ENDOGENOUS REGULATORY PEPTIDES

Chemistry, biology and medical significance

Editor J. Menyhárt

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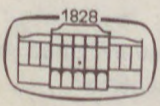
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Editor:

J. Menyhárt
United Research Organization
of the Hungarian Academy of Sciences
and Semmelweis University Medical School,
Budapest, Hungary



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PREFACE

Until about the early sixties our knowledge of endogenous regulatory peptides (ERPs) was virtually restricted to a few peptide hormones produced by the classic (i.e. glandular) endocrine organs and gastrointestinal tract of mammals. The position today is very different. Over the past 20 years or so, there has been a spectacular increase in the number of ERPs identified and in the range of biological/pathological functions in which they have been found to participate.

ERPs occur virtually in all organisms comprising the animal kingdom, irrespective of their position at the phylogenetic scale. As a result, ERPs emerged as a huge class of molecules of universal significance which, in spite of the monotonous building principle manifested in their primary structure, carry the most diverse biological information.

Thus, it is easy to conceive why the research of biologically active peptides is one of the most rapidly developing areas. Novel techniques such as the cloning and sequencing of cDNAs encoding precursors of various peptides, peptide receptors and of non-peptide messenger substances revolutionized this field, enabling investigators to identify precursors sometimes consisting of hundreds of amino acids and often giving rise to several novel biologically active sequences, in a matter of months. Let us recall that in the case of one of the oldest known neuropeptides, substance P (an undecapeptide), forty years had to elapse between the first detection (Von

Euler and Gaddum 1931) and the identification of primary structure (Chang and Leeman 1971).

Considering the rate of development, one might get dispirited to engage in a hopeless race, i.e. to accept the challenge of compiling a comprehensive and up-to-date material on endogenous biologically active peptides. We do believe, however, that our book may be useful to the scientific community in more than one ways.

A large number of monographs and symposia proceedings providing detailed information about one or other class of ERPs has now been published. However, this book represents the first attempt aimed at covering the whole field of "peptidology" by bringing together the basic information about ERPs at present known to have function(s) with biological and/or medical significance, while leaving details to the extended list of cited references.

When selecting a compound for inclusion in this book, the decisive factor has been its biological function of regulatory nature irrespective of its molecular size. As a consequence, ERPs with a size order characteristic of oligopeptides, polypeptides and proteins have been included alike, except enzyme proteins. While the 11 classes of ERPs selected for inclusion points to the editor's intention to provide a possibly comprehensive survey of the field as a whole, those classes of ERPs which have been recognised for decades (e.g. classical peptide hormones) are discussed in less detail than those discovered more recently such as neuropeptides, immunopeptides, peptide growth factors and non-mammalian peptides. It is the editor's hope that this book may render everyone a good service who wishes to inquire about the latest development in the field of ERPs in general, or who wants to get acquainted with one or another class of these molecules of universal biological significance.

I wish to express my gratitude to all who have contributed to the accomplishment of this book, either professionally or technically. I am indebted to Professor J. Szentagothai, the former president of the Hungarian Academy of Sciences and Professor B. Halasz, president of the Medical Section of the Hun-

garian Academy of Sciences for the moral support on writing this book. Professors L. Graf and I. Schon gave valuable help by their comments and criticism. I owe a debt of gratitude to Dr. A.Z. Ronai of the Eötvös Lorand University of Budapest for devoting many hours to updating a substantial proportion of the text. Special thank is due to Dr. Zsuzsanna Kirilly, who, by running an excellent library at the Department of Comparative Physiology, L. Eötvös University, facilitated the collection of materials. The contribution of Drs Z. Marcsek and G. Simon as programming mathematicians can hardly be overestimated. I must also express my deepest thanks to Mrs G. Bodolay, a coworker of mine for more than three decades, who, in cooperation with Miss Judit Molnar, excelled in helping to review and rearrange the text of the manuscript.

It was a pleasure to work with the staff of Akadémiai Kiadó, and especially with Mrs A. Berky, Mrs K. Csóka, Mrs Gy. Tanay and Miss Krisztina Takács; they produced this book with their usual care and high standards. Finally, I thank may wife, who has patiently endured my writing and editing this book remaining an everlasting source of encouragement.

The Editor

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CHAPTER 1

OUTLINES OF THE CHEMISTRY AND BIOCHEMISTRY OF ENDOGENOUS PEPTIDES

K. NIKOLICS, G. SETALO and J. MENYHART

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1.1. DEFINITION OF ENDOGENOUS PEPTIDES

Peptides, i.e., oligomers and polymers of amino acids joined by peptide bonds, are present at all levels of life and display a very wide variety of functions. Peptides serve as structural elements of organisms and regulators or mediators of a great many biological processes. Peptides are produced by bacteria, fungi, plants and animals. Viral polypeptides coat the nucleic acid core of viruses. Bacterial peptides serve as cell wall constituents, ionophores, and some of their representatives can be used as antibiotics. Many of the fungal toxins, insect venom toxins, snake venom toxins are peptides. More important, however, are peptides which exert regulatory functions and those which mediate various physiological mechanisms. Such peptides are also present at all levels of living organisms. Peptide hormones regulate the growth, circulatory and metabolic processes, sexual cycle, lactation, pigmentation, response to stress stimuli of vertebrates among other processes. Peptides of the immune system act in a concerted manner to protect the integrity of higher vertebrates against foreign substances and organisms.

Today, we are witnessing an abundance of discoveries demonstrating the significant regulatory functions of various peptides. Peptides are becoming to be acknowledged as general regulators of biological functions at intracellular, intercellular and organismic levels.

Apart from a relatively short overview of nonmammalian peptides the primary aim and scope of this book is focused on the role and significance of peptides in biological processes of higher vertebrates, especially mammals. In addition, special attention is given to the human aspects and medical significance of regulatory peptides. Therefore, the term endogenous peptides or alternatively endopeptides will be applied for compounds produced, processed and utilized by higher vertebrates with special reference to humans. Consequently, the terms exogenous peptides or exopeptides will be descriptive for compounds originating from other sources. These may still have biological activities in the vertebrate organism, suggesting that either the exopeptide itself, or a related endogenous substance may have some bearings on the physiological and/or pathological processes in the vertebrate organism. Without further definition, the above terms will be used throughout the book. Philosophically, any peptide is endogenous for the organism it is produced by. The classification of peptides into endogenous and exogenous classes is certainly an artificial yet practical one, and reflects the aim of this treatise. For example, it is important to distinguish, especially for the human body, between endogenous and exogenous "immune peptides". Endogenous immune peptides act in a coordinated way in regulating the function of lymphocytes and leukocytes and other immune cells and they do not provoke antibody production (with the exception of some pathologic conditions). Exogenous immune peptides are recognized as foreign and thus stimulate antibody production or alarm leukocyte chemotaxis. These aspects will be discussed in greater detail later. This chapter will review general principles of the physical and chemical properties of peptides and also deal with some basic biochemical aspects, namely biosynthesis, and metabolism of endogenous peptides. Endogenous peptides show a very wide organ distribution in mammalian organisms. Peptides have been isolated from and identified in the pituitary gland, pineal gland, gonads, gastrointestinal tract, hypothalamus, cerebrospinal fluid, different areas of the brain, blood and other tissues. There is a substantial difference between the pro-

cessing and metabolism of endogenous and exogenous peptides. Endogenous peptides are transported by internal body fluids and they are degraded by rather specific enzymes. Exogenous peptides, with the exception of viral or bacterial infection, are mostly consumed as food constituents, and they are degraded and taken up by proteases and peptidases of the gastrointestinal tract. The processing and uptake of exogenous peptides is of growing interest today and our whole view of nutrition will certainly be greatly influenced by recent findings in this area.

1.2. PHYSICAL AND CHEMICAL PROPERTIES OF ENDOGENOUS PEPTIDES

Endogenous peptides which play various significant regulatory functions in mammals are extremely different in size and chemical characteristics. Peptides, with a few exceptions that have branched chains, are linear-chain oligomers and polymers of amino acids coupled by the characteristic CO-NH peptide bond as first described and defined by Fischer (1906). Among the smallest representatives of endopeptides are kyotorphin: Tyr-Arg (Takagi et al. 1979) consisting only of two amino acids, thyrotropin releasing hormone: pGlu-His-Pro-NH_2 (Boler et al. 1969, Burgus et al. 1969) with three, and tuftsin: Thr-Lys-Pro-Arg (Najjar 1974) with four amino acids, respectively. The upper limit is more difficult to determine, the largest representatives may contain several hundred amino acids.

The IUPAC-IUB Commission on Biochemical Nomenclature proposed a general usage of abbreviations and terms for peptides e.g., in J. Biol. Chem. 242: 6489-6497 (1970) and 250: 3215-3216 (1975). According to these proposals and earlier tradition, Greek prefixes are used to describe the number of amino acids in a peptide. Thus, we have di-, tri-, tetrapeptides, etc. However, to simplify this nomenclature Bodanszky (1977) proposed the use of arabic numerals as prefixes above 10 amino acids, for example, 17-peptide.

Peptides containing between 2 and approximately 10-20 amino acids are generally called oligopeptides. The term polypeptide applies for structures containing a minimum of 10 amino acids. There is basically no upper limit for the term polypeptide and thus there is no clear distinction from which point the term protein should be used. However, proteins can have complex structures constructed of more polypeptide chains, they can contain lipids, carbohydrates, etc.

In such cases, we speak about the polypeptide constituent(s) of the protein. On the other hand, peptides may also contain carbohydrate residues forming glycopeptides or complex certain metal ions making metallopeptides, etc. The classification of peptides on the basis of size also serves practical purposes. Very frequently applied separation methods of peptides (gel permeation chromatography, ultrafiltration, dialysis, SDS-polyacrylamide gel electrophoresis, etc.) are based on the distinction between molecules of different ranges of size. Since one amino acid residue contributes approximately 110 daltons to the total molecular weight of the peptide, peptide chain length can be estimated from molecular weight determinations.

The physicochemical, chemical and consequently the biochemical and physiological properties of peptides are determined by their constituent amino acids. Thus, there are peptides having acidic, neutral and basic isoelectric points depending on the number of basic and acidic side chains and free termini of the peptides. In addition to the net electrical charge, other physicochemical properties of peptides also depend on the properties of the main peptide chain and side chain functional groups. All peptides absorb UV light in the 205-215 nm range, which is characteristic of the CO-NH peptide bond, but only peptides containing tryptophan, tyrosine and phenylalanine show absorbance in the 275-280 nm region specific for aromatic rings. Fluorescence can only be observed with peptides containing tryptophan. The light absorption and fluorescence properties of peptides, similarly to electrical properties, are additive, however, they are also dependent on the conformational position of the absorbing chromophores.

In contrast, optical rotatory dispersion and circular dichroism are predominantly dependent on the conformation of the peptide and less influenced by the increments contributed by the individual amino acid residues. In fact, both methods can be used for the estimation of helicity in the peptide conformation (Moffitt and Yang 1956, Carver et al. 1966, Greenfield and Fasman 1969, Bodanszky et al. 1969). In turn, peptide conformation of a known sequence can be predicted with reasonably good accuracy based on statistically analyzed data of proteins with known X-ray conformations (Chou and Fasman 1974a,b).

The three-dimensional structure of peptides is much more flexible than that of proteins. Peptide conformations, therefore, can only be estimated from data obtained from various spectroscopic measurements (optical rotatory dispersion, circular dichroism, nuclear magnetic resonance, electron spin resonance, etc.). For a more detailed review of spectroscopic methods suitable for investigation of peptide conformation and other characteristics see Brown (1980) and Edelhoch and Chen (1980).

From a great many aspects peptides display characteristics on an extremely broad spectrum. This variability and abundance of structures showing a variety of chemical and physicochemical properties have certainly contributed to the natural selection of peptides to become a general class of regulatory substances that possess a great variety of biochemical and physiological functions. In addition, peptides can be formed in all types of cells by general mechanisms and also degraded by general proteolytic enzymes as will be discussed in the next section.

1.3. BIOCHEMISTRY OF ENDOGENOUS PEPTIDES

The formation, degradation and mechanism of action of endogenous peptides is an extremely complex interplay of events having general, as well as particular individual characteristics which differs not only from peptide to peptide but also from one species to another or even from one tissue or region

to another within the same species. In this chapter we shall review the general characteristics of biosynthetic and degradative pathways involved in the homeostasis of endogenous peptides. The mechanism of action of endopeptides which involves recognition of the peptides by tissue-specific receptors and transduction of the information carried by the peptide via second messengers is by itself a distinct field and will be treated separately in Chapter 2.

1.3.1. FORMATION OF ENDOGENOUS PEPTIDES

The formation of endopeptides involves biosynthetic and processing mechanisms. Endopeptides of regulatory importance are initially synthesized as larger polypeptides by ribosomal protein biosynthesis and processed proteolytically to yield their active forms. We only mention herewith that certain peptides, as demonstrated in the case of peptides of bacterial origin, are formed by nonribosomal enzyme-directed synthesis (Lipmann 1971). For example, gramicidins and tyrocidins are synthesized by multienzyme complexes which function similarly to the fatty acid synthetase system. According to the proposed scheme, the peptide chain is synthesized by consecutive cycles of pantothenate-assisted peptide condensations which are mediated by thioester activation (Kleinkauf et al. 1971, Bauer et al. 1972). However, in the light of recent findings, this type of peptide synthesis is not characteristic of mammalian endogenous peptides. Endogenous peptides of different tissue origin have been demonstrated to be synthesized as higher molecular weight precursors by ribosomal polypeptide synthesis.

Protein biosynthesis occurs on ribosomes as an extremely complex, yet very rapid chain of reactions tightly coordinated in a unique manner. Ribosomes in eukaryotic cells are approximately 4×10^6 dalton multicomponent particles, essentially fully automated machineries for synthesizing polypeptides. This mechanism is the most complex of all biosynthetic mechanisms requiring the participation and cooperation of over 300 different macromolecules. The whole process is extremely

rapid: for example, a chain length of 100 amino acids is synthesized within approximately 5 seconds. For the synthesis, amino acids are first activated by transfer to spescific tRNAs at the expense of ATP energy. The aminoacyl-tRNA formed has a high-energy bond which assures high group-transfer potential required for rapid coupling reaction. Ribosomal protein synthesis proceeds through a series of steps including (1) formation of an mRNA-ribosome initiator-tRNA complex, (2) binding of aminoacyl-tRNA, (3) peptide bond formation, (4) translocation and (5) termination. The complex mechanisms have been revealed in detail and can be found in expert reviews (as e.g., by Weissbach and Pestka 1977). Polypeptides synthesized by the ribosomal apparatus undergo post-translation processing which is of basic impact on the formation of endogenous peptides (for a more detailed review see Docherty and Steiner 1982, Douglass et al. 1984, Loh et al. 1984).

The generation of endogenous peptide products possessing different biological activities greatly depends on the processing of the nascent polypeptide chain and recent insights in this area have been of major influence on our present view of the formation of endopeptides. The nascent proteins or polypeptides are most widely termed preproteins or prepolypeptides after preproparathyroid hormone (Kemper et al. 1974) and IgG light chain precursors (Milstein et al. 1972). Immediate precursors of the active, known forms of endogenous peptides are usually termed proproteins or propolypeptides, after proinsulin (Steiner and Oyer 1967). This is, however, not a general rule, since processing mechanisms, although having common characteristics, are not necessarily identical as we shall see on certain examples.

Immunoglobulin light chain precursors (Milstein et al. 1972) and preproparathyroid hormone (Kemper et al. 1974) were the first to be recognized as rapidly processed precursors extended on their amino terminals by 15-30 hydrophobic amino acids long "pre" or "signal" sequences. Since then, a great number of further endopeptides have been shown to be processed from precursors possessing such pre or signal sequences (reviewed by Kreil 1981). According to the "signal hypothesis" of

Blobel and Dobberstein (1975a), the function of these structurally different, but physicochemically similar sequences is the "guidance" of the nascent polypeptide to interact with the membranes of the endoplasmic reticulum to promote the formation of membrane-bound polyribosomal complexes leading to peptide translocation. At present, however, the mechanism of this interaction and the following peptide translocation into the cisternae of the endoplasmic reticulum have not been clarified. Cleavage of the pre- or signal sequence occurs very rapidly, either cotranslationally or shortly after translation (Blobel and Dobberstein 1975b). The existence of a "signal peptidase" has already been confirmed, but it has not been purified or isolated (Jackson and Blobel 1977, Zwizinski et al. 1981).

After removal of the signal sequences, polypeptides undergo further processing reactions. These involve further cleavage of certain sequences, glycosylation, acetylation, amidation, etc. These further processing steps are considerably slower than the cleavage of the signal peptide sequence and last approximately 15 minutes to a few hours depending on different cell types and reaction types (Steiner 1977, Habener and Potts 1978, Eipper and Mains 1980). The cleavage of pro-sequences and other post-translational reactions occur at the stage of secretory processing in the Golgi apparatus and the secretory granule (Jamieson and Palade 1977). It is remarkable that in most propolypeptides, pairs of basic amino acids separate the peptide sequences to be cleaved and secreted, suggesting the involvement of trypsin-like enzymes in this cleavage step (Kemmler et al. 1971, Hamilton et al. 1974, Nakanishi et al. 1979, Noda et al. 1982). In the light of current studies indicating that many of the paired basic residue-specific enzymes have properties quite unlike trypsin, Loh and his coworkers (1984) recommended that these enzymes should be referred to as proprotein converting enzymes rather than as "trypsin-like" ones. In certain cases carboxypeptidase-like enzymes are also involved, as for example with insulin, proACTH/LPH and enkephalin (Kemmler et al. 1973, Nakanishi et al. 1979, Hook et al. 1982).

The C-terminal amino acid of a number of endopeptides is found in the amidated form due to the action of a specific peptidase. As a result of this C-terminal amidating enzyme an X-Gly bond is cleaved and converted to the C-terminally amidated form of the peptide, X-NH (Bradbury et al. 1982).

Since the discovery of proinsulin (Steiner and Oyer 1967), propolypeptides have been isolated or their presence demonstrated in the case of a number of polypeptides including albumin (Brennan and Carrell 1978), glucagon (Patzelt et al. 1979), somatostatin (Hobart et al. 1980, Lechan et al. 1983), gastrin (Noyes et al. 1979), cholecystokinin (Rehfeld 1978, Deschenes et al. 1984, Beinfeld 1985), LH (Liu et al. 1979), FSH (Reichert and Ramsey 1977), LHRH (Seeburg and Adelman 1984), TRH (Jackson et al. 1985), vasopressin (Land et al. 1982), substance P (Nawa et al. 1983), and many others. The case is less unequivocal for growth hormone (Stachura and Frohman 1975 vs. Spielman and Bancroft 1977, Lewis 1984) and TSH (Klug and Adelman 1977 vs. Chin et al. 1978); no propolypeptide forms could be detected in the case of prolactin (Maurer and McKean 1978 and Lewis 1984) and the two-subunit glycoprotein hormone hCG (Daniels-McQueen et al. 1978), however, complex processing steps involving glycosylation and subunit association occur in the latter case.

At present, the physiological role of the propolypeptide sequences and propolypeptide forms cannot be understood in most cases. While the C peptide in proinsulin would serve as a positioning bridge to facilitate the proper disulfide bond formation between the A and B chains, it is unclear why proalbumin and proPTH contain their N-terminal extensions. Also the role of the N-terminal region in the proACTH/LPH precursor needs further studies.

The most exciting findings of polypeptide biosynthesis and processing came with the discovery of the common precursor of corticotropin (ACTH) and lipotropin (LPH) by Mains et al. (1977) and Roberts and Herbert (1977). Studies on this 30,000 (30K) molecular weight precursor molecule have revealed a unique and complex mechanism by which the anterior and intermediate pituitary and the brain synthesize and secrete a whole

family of active endogenous peptides: ACTH, LPH, α-melanotropin (MSH), corticotropin-like intermediate lobe peptide (CLIP), β-endorphin, γ-LPH, β-MSH and in addition an N-terminal glycosylated 112 amino acid-polypeptide (reviewed by Eipper and Mains 1980). It is still difficult to see what evolutionary and functional background would explain this example of cellular economy. The situation is even more complex, since different processing mechanisms of the same precursor molecule have been found in the anterior and the intermediate lobe of the pituitary gland (Mains and Eipper 1979). In the anterior pituitary ACTH, β-LPH and some endorphins are the major secretory products, while in the pars intermedia α-MSH, CLIP, β-endorphin, β-MSH and some other smaller peptides. This example clearly shows the basic role of post-translational proteolysis in the formation of various active endopeptides. It is interesting to note that the 30K precursor contains three identical sequences contained in the melanocyte stimulating peptide segments of the polypeptide. Another example of such economy of functional significance is provided by the gonadotropin-releasing hormone precursor which appears to generate two peptides which play important roles in mammalian reproduction (Nikolics et al. 1985 and Phillips et al. 1985). Another fascinating biosynthetic and processing mechanism has been revealed with the discovery of the preproenkephalin precursor (Gubler et al. 1982, Noda et al. 1982). This 30K polypeptide contains 6 copies of Met-enkephalin and one copy of Leu-enkephalin. Two of the Met-enkephalin segments are extended at their C-termini by 2 and 3 amino acids, respectively. In this case, direct duplication of ancestral DNA segments could have formed this unique structure. The physiological role of this multiplied precursor is still questionable.

Further exciting questions are raised by the discovery of somatostatin precursors. Somatostatin is a 14 amino acid peptide present in the central nervous system and the gastrointestinal tract and was originally isolated and identified from ovine hypothalami (Brazeau et al. 1973). Most propolypeptides are either completely inactive or significantly less active than their secreted final products. Therefore it was surpris-

ing that two prohormonal forms of somatostatin: a 25- and a 28-peptide were both equipotent with somatostatin-14 (Esch et al. 1980, Pradayrol et al. 1980, Schally et al. 1980).

The examples of proACTH/LPH, proenkephalin and prosomatostatin show different biosynthetic and processing mechanisms as shown in the following scheme:

(1) proACTH/LPH precursor	several structurally and functionally different peptides
(2) proenkephalin precursor	several structurally and functionally similar peptides
(3) prosomatostatin precursor	several structurally different, functionally identical peptides

These different mechanisms demonstrate the extreme regulatory potential lying in biosynthetic/processing mechanisms which an organism can utilize. Since enkephalins are also contained in other precursor molecules, we can probably add new mechanisms to the above scheme in the near future.

The biosynthetic and processing mechanisms also offer a potential for feedback regulation by various factors which can act at different levels. In the case of parathyroid hormone (PTH), increased levels of Ca^{2+} ions stimulate the intracellular degradation of PTH before secretion (Habener et al. 1975, Mayer and Hurst 1978). Growth hormone levels are regulated by glucocorticoids and thyroid hormones, these seem to increase the corresponding mRNA amount. Similarly, thyrotropin releasing hormone (TRH) and prostaglandins increase the amount of prolactin mRNA (Martial et al. 1977, Tushinski et al. 1977, Evans et al. 1978). Certain endopeptides are not formed by intracellular processing but their precursors are secreted polypeptides and they are formed by proteolytic cleavage extracellularly. Several body fluids, especially serum, are the sites where some already known, and probably a number of still unknown, endopeptides are generated. For example, angiotensins

and kinins are produced by consecutive cleavage steps from high molecular weight precursors in the circulation (Skeggs et al. 1957, Braun-Menendez and Page 1958, Schroder and Lubke 1966). Similarly tuftsin, a naturally occurring antitumor tetrapeptide, is cleaved from the heavy chain of γ-globulin (positions 289-292) by extracellular proteases (Najjar et al. 1981).

Serum contains active proteases with various substrate specificity, therefore it is highly probable that a great number of yet unknown undetected, or unidentified peptides with different biological activities are present in the systemic circulation which are formed by proteolytic enzymes.

1.3.2. DEGRADATION OF ENDOGENOUS PEPTIDES

The separate discussion of the degradation of endogenous peptides is highly artificial. The biosynthesis, secretion, receptor binding in target cells and degradation of most regulatory endopeptides forms a tightly regulated complex mechanism, which should be viewed at as a whole. As already mentioned, a feedback signal such as an elevated Ca^{2+} level, can activate proteases degrading parathyroid hormone already prior to its secretion (Habener et al. 1975, Mayer and Hurst 1978). Similarly, insulin degradation before secretion is stimulated by elevated concentrations of glucose (Halban and Wollheim 1980) or mannoheptulose (Halban et al. 1980). Such examples demonstrate that degradation can indeed be an integral part of a complex mechanism of endopeptide homeostasis.

The idea that degradation of peptide hormones in an appropriate means for the regulation of peptide levels was put forward by Knights et al. (1973). Two basic models were proposed: one where the degrading enzyme was independent of the hormonal system acting merely as an inactivating, antagonizing mechanism and one where the degrading enzyme activity would be regulated directly or via mediators by the hormone. Endogenous peptides with various regulatory roles usually act as rapid stimuli on physiological processes. Therefore, efficient de-

grading mechanisms must exist for the elimination of endogenous peptides as a prerequisite of efficient regulation. The first type of degrading mechanism proposed by Knights et al. (1973) would be characteristic of the general proteases and peptidases present in the blood and kidney (Marks 1977, 1978), and urinary excretion also serves as a nonspecific means of elimination for several smaller peptides (e.g., Carone et al. 1980).

The second model is, in fact, more sophisticated and requires a more detailed discussion. Degrading enzymes can act at the sites of production, transport and target tissue of endopeptides. (Schwartz 1983, Burbach et al. 1984, Kenny et al. 1984, Schwartz et al. 1984). Therefore, physiologically relevant degradation can only be verified by experiments in which the endopeptides are followed by adequate methods. Unfortunately, a great number of experiments are carried out with simplified systems which do not reflect physiological conditions. Crude tissue homogenates are mostly used for degradation studies and since the cytoplasm and lysosomes are rich in various proteolytic enzymes, it is not surprising that such preparations degrade peptides. But it is rarely proven that endopeptides under physiological circumstances ever get into contact with those enzymes (for reviews see Marks 1977, Griffiths and Kelly 1979, Chertow 1981, Nikolics et al. 1982).

Within the cells of production, secretory granules containing the peptides to be secreted can fuse with lysosomes (Farquhar 1969). The phenomenon, known as crinophagy, can be the pathway of intracellular peptide degradation. In several cases, secretory granules were also found to possess receptors for endopeptides which would mediate the signals for the induction of crinophagy (Sussman et al. 1982).

In the target cells, peptides are bound to specific cell surface receptors. Recently, in the case of a number of different endopeptides acting on diverse target cells, including insulin, glucagon, nerve growth factor, epidermal growth factor, α_2-macroglobulin, low density lipoprotein, enkephalin, gonadoliberin and others, internalization (endocytosis) of the peptide-receptor complex has been demonstrated to follow re-

ceptor binding and microaggregation of the peptide-receptor complex (reviewed by King and Cuatrecasas 1981, Middlebrook and Kohn 1981, Gorden et al. 1982). Internalized endocytotic vesicles can also fuse with lysosomes, thus leading to a similar degradation mechanism as in the case of crinophagy (Chertow 1981, Gorden et al. 1982). The physiological significance of this process, however, is yet questionable, since both crinophagy and fusion of lysosomes with endocytotic vesicles are slow processes requiring several hours to days following exposure of cells to either regulatory agents or peptides (Farquhar 1969, Masur and Holtzmann 1969, Muller et al. 1980a,b). In several cases endopeptides were found to be degraded by enzymes bound to the plasma membrane of target cells (Terris and Steiner 1975, Dial et al. 1977, Clayton et al. 1979, Baumann and Kuhl 1980, Schwartz 1983, Burbach et al. 1984, Schwartz et al. 1984). This degradation would occur prior to endocytosis of the endopeptides and add an extra point of homeostatic control. In fact, in a number of cases, degradation of various endopeptides by target cell plasma membranes interfered with binding experiments and caused artefacts. This phenomenon, however, requires further clarification. In conclusion, degradation of endogenous peptides either by producing cells or target cells can only be evaluated when a more conclusive picture of the whole mechanism is reached. Studies with tissue homogenates can be useful but data obtained from such experiments must be correlated with experiments which relate the in vitro results to physiological mechanisms.

As an overall, generalized scheme, biosynthetic, nascent polypeptides undergo consecutive degradation reactions and among the intermediate products of this chain of events certain forms are more stable and exert definite biological activities. Certainly, the timing and location of these processes are of basic importance. It is an intriguing question why certain peptide forms are more stable during this process than others. Although no general answer can be given to this question today, increasing evidence suggests that those peptides which are metabolically more stable have a more "compact" and ordered conformation which is more difficult for

peptidases to attack (Graf and Hollosi 1982, Lintner et al. 1982).

1.4. QUALITATIVE AND QUANTITATIVE ANALYSIS OF ENDOGENOUS PEPTIDES

Endogenous peptides are produced in different organs of various mammalian species. The pituitary, pineal, thyroid glands, several areas of the central nervous system, the gastrointestinal tract, the gonads, the adrenals and other tissues are known to contain varying amounts of endogenous peptides. Studies on such peptides require identification, localization and quantification of the amounts of intracellular and secreted endopeptides. Both analytical and localization methods must be carried out with extreme caution to avoid artefacts.

The analysis of endopeptides in tissue extracts or different body fluids is very difficult due to their extremely low actual concentrations. Most endopeptides are present typically below µg/g wet weight tissue levels. Biological assays used for the identification of endogenous peptides are usually considerably more sensitive than methods of chemical analysis, however, they can be influenced by several factors and result in artefacts. Additional problems arise from tissue preparation methods. Tissue homogenates contain a mixture of proteolytic enzymes which can degrade the peptides to be measured (discussed in the previous section). Also, autolysis in autopsy material can lead to the same problem. Peptides generated by autolytic degradation have been isolated and identified with, e.g., growth hormone releasing and corticotropin releasing properties (Schally et al. 1969, 1978). Recently applied microwave irradiation has the advantage to heat-denature proteases that can lead to artefacts (Stein 1981).

Analytical methods applied for endogenous peptides have been extensively improved during the past decade. The sensitivity of chemical methods was left far behind by immunological methods during the sixties after the introduction of ra-

dioimmunoassay (RIA) techniques (Yalow and Berson 1960). RIA has become a routinely applied method with very high sensitivity and generally good selectivity. More recently, however, chemical analytical methods have largely been improved in both selectivity (resolving power) and sensitivity by specific reactions and detection methods. Through these improvements, chemical methods are approaching the sensitivity of immunological detection. In this section, we shall briefly review some of the more recent advances in this field.

1.4.1. ANALYSIS OF ENDOGENOUS PEPTIDES BY CHEMICAL METHODS

Extremely remarkable developments have been achieved in chromatographic techniques during the past decade. High-performance liquid chromatography (HPLC) has become a reliable analytical and preparative method with a high resolving power. Adequate instrumentation has made the use of small (3-10 m diameter), porous, uniformly sized, mechanically stable particles as supports for adsorption, partition, ion exchange and permeation chromatography possible (for a review of HPLC principles see Snyder and Kirkland 1974). Densely packed HPLC columns can withstand very high pressures, therefore high flow rates can be applied which result in rapid analyses. Peak broadening is minimal and well-controlled conditions yield highly reproducible chromatographic separations. Typically, reverse phase chromatography on various organo-silane supports is applied for the separation of peptides, but ion exchange, adsorption and permeation are also suitable for definite purposes. HPLC has been successfully applied for the separation of peptides in complex mixtures, amino acid analysis and peptide sequencing (reviewed in Hearn et al. 1983, and Horvath 1983).

Other separation techniques have also been further developed including gel electrophoresis, electrofocusing, isotachophoresis and others (see Gross and Meienhofer 1981). Among detection methods applied for the different separation methods, fluorescent detection is the most powerful: amino

acids and peptides can be detected at picomole levels. Fluorescamine (Weigele et al. 1972), 2-methoxy-2,4-diphenyl-3(2H)furanone (MDPF, Weigele et al. 1973), o-phthalaldehyde (Roth 1971) and fluoresceine isothiocyanate (Muramoto et al. 1978) and some others have been used as derivatizing reagents giving fluorescent products with primary amines. Both postcolumn (Bohlen et al. 1975, Stein and Moschera 1981) and precolumn derivatization (Gruber et al. 1976, Wideman et al. 1978) are suitable for analytical purposes when column effluents are not collected for further processing. In addition, chromatographic techniques can be combined with appropriate bioassays and radioimmunoassays yielding complex information about the materials under investigation. For example, the neurohypophyseal peptides oxytocin and vasopressin were quantitated in individual rat pituitaries by the HPLC-fluorescent detection method (Gruber et al. 1976). The peptide peaks were identified by retention times and these data were confirmed by internal standards, as well as amino acid analysis and bioassay of collected peaks. Similar methods were applied for the measurement of proACTH/LPH and peptides derived from this precursor (Rubinstein et al. 1978).

In special examples, radioactive detection can yield even higher sensitivity than fluorescent detection. Radioactive amino acids with very high specific radioactivity were incorporated into the biosynthetic proACTH/LPH precursor which permitted the complex analysis of the biosynthetic and processing mechanisms of tumour cells and normal cell cultures secreting LPH, ACTH and other peptides derived from these (Mains and Eipper 1978). In these experiments the simple, yet high resolution of sodium dodecyl sulfate-polyacrylamide gel electrophoresis served for peptide separations.

1.4.2. ANALYSIS OF ENDOGENOUS PEPTIDES BY IMMUNOLOGICAL METHODS

Immunological methods utilizing specific antibodies against endopeptides are extremely useful in measuring or localizing endopeptides in complex mixtures or matrices. Specific immunoglobulins or antibodies can recognize even minute structural differences and due to the very high association constants of the peptide-antibody reactions, these can be used to detect very low amount of endopeptides, usually within or below their physiological concentration ranges. Antisera have mostly been produced by a variety of immunization schemes in different animal species, mostly rabbit, sheep and goat. Generally, peptide antigens were injected intradermally at different sites or intramuscularly with or without Freund's adjuvant (Ross et al. 1971, Jolles and Paraf 1973, Lynch and Shirley 1975). Small peptides are very poor immunogens by themselves or do not exhibit immunogenic properties at all, however, they are haptens and coupled to a carrier molecule like serum albumin, they induce antibody production. Antisera produced by such methodology can be applied for radioimmunological determination, tissue localization and affinity chromatography of peptides and also other purposes such as immunoprecipitation of peptides from complex mixtures.

More sophisticated production of specific immunoglobulins or antibodies is offered by the use of myeloma-lymphocyte cell hybrids or hybridomas (Kohler and Milstein 1975). This method, known as monoclonal antibody production, offers homogenous immunoglobulins synthesized by single cell lines or clones which can be well characterized for their recognition site specificity (for reviews on monoclonal antibody techniques see Kennett et al. 1980 and Fellows and Eisenbarth 1981). Radioimmunoassay (RIA, Yalow and Berson 1960, Yalow 1978) based on the competitive binding of a radioactively labeled antigen and nonlabeled antigen by specific antibodies allows the quantitation of endopeptides at extremely low concentrations: (Fig.1.1). Radioactive labeling of endopeptide "tracers" is achieved mostly by ^{125}I-incorporation into tyrosine, histidine

residues or amino groups by specific reagents (Greenwood et al. 1963, Marchalonis 1969, Bolton and Hunter 1973). Today incubation procedures, separation of bound and free antigens and evaluation of data are well established and RIA has become a routinely applied analytical method which has tremendously contributed to the spectacular developments in the field of peptides (Yalow 1978). The procedures and applications of RIA have been reviewed in detail by several authors (see e.g., Kirkham and Hunter 1971, Odell and Daughaday 1971, Jaffe and Behrman 1978). The use of monoclonal antibodies for RIA has improved the sensitivity and selectivity of the method as compared to "classically" produced antisera (Eisenbarth and Jackson 1982).

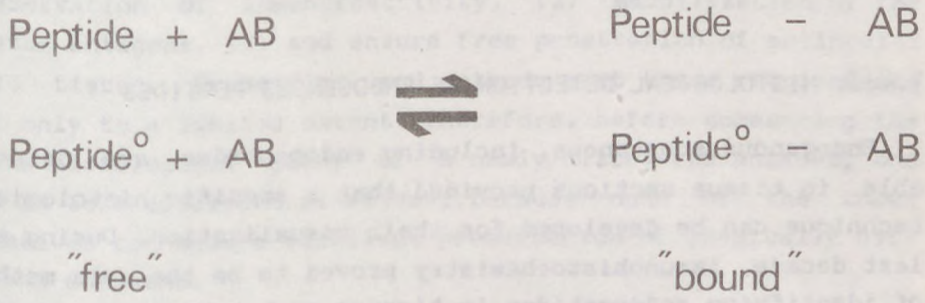


Fig. 1.1. Competitive antigen-antibody reactions on which radioimmunoassay is based. Labeled and unlabeled antigen compete for binding sites of the antibody to form labeled and unlabeled complexes the ratio of which can be measured and calibrated. Labeled antigen (peptide) is indicated by circles

Alternative competitive binding assays have also been developed. The immuno-radiometric assay (Woodhead et al. 1974) employs radiolabeling of the antibody-antigen complex which has been found to have advantages in certain cases. Enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) avoid the use of radioactive materials, however, at present offer lower sensitivity than RIA (Pal 1978).

Target cell plasma membranes have been utilized for radioreceptor assays to be discussed in Chapter 2.

Specific antibodies against peptide sequences can be used for double antibody immunoprecipitation of the peptides from complex mixtures such as tissue extracts or cell culture

media. This approach was found to be very useful for the analysis of the processing mechanism of the pro-ACTH/LPH precursor protein (Mains and Eipper 1976, 1978). Antibodies can also be suitably combined with other methods for the tissue localization of endopeptides (see next section). The immune recognition of foreign molecules by immunoglobulins is a highly specific and sensitive reaction. Today, extensive use is made of this reaction in studying endopeptides. Chemical analysis of endopeptides at present does not reach the level of sensitivity offered by immunological methods, however, the two methods used in combination or complemented with bioassay data can yield very complex information about endopeptide production and degradation as well as other aspects of investigation.

1.4.3. HISTOLOGICAL DETECTION OF ENDOGENOUS PEPTIDES

Endogenous substances, including endopeptides, are detectable in tissue sections provided that a specific histological technique can be developed for their visualization. During the last decade, immunohistochemistry proved to be the sole method of identifying endopeptides in tissues with sufficient reliability, i.e., with satisfactory accuracy, precision, sensitivity, specificity (Borth 1952), and efficiency (Petrusz et al. 1975). In essence, immunohistochemical staining methods are antigen-antibody reactions adapted to the peculiar requirements of histological methodology. Specific antibodies bearing a label and bound to their antigenic determinants in a tissue can be made visible, hence will indicate the location of the substance to be detected. If the specific antibodies themselves bear the label, the applied technique is called direct, while in indirect techniques, the label is bound to the detectable antigen by the mediation of shorter or longer chains of antibodies immunologically coupled to each other in the course of consecutive incubations. Regarding their basic principles, two groups of immunohistological methods have been invented: techniques utilizing (a) labeled, or (b) unlabeled antibodies. Each of these basic principles has a number of dif-

ferent technical applications, some of which have only a historical value, while others are still in use. Practical details of hese methods have been published in a recent monograph of Sternberger (1979).

1.4.3.1. FIXATION, EMBEDDING, SECTIONING

It is a generally accepted view that adequate fixation is the prerequisite of the immunohistological location of antigens. In addition to preserve the morphological details of the tissue as close to the living state as possible, fixation in immunohistology has to provide the following conditions: (1) preservation of immunoreactivity, (2) immobilization of the tissue antigens, (3) and ensure free penetration of antibodies into tissue. Presently used methods meet these requirements but only to a limited extent. Therefore, before commencing the immunohistological study of a newly discovered antigen, one has to test different fixatives, because none of the known fixatives can assure excellent preservation of chemically different antigens.

Buffered formaldehyde solution (4%, pH 7.5) is probably the most commonly used fixative (Pearse 1968). Recently, Berod et al. (1981) demonstrated excellent fixation of brain tissue using formaldehyde solutions at variable pH values. Short perfusion with formaldehyde at pH 6.5 provides fast, homogeneous distribution of the fixative, however, with a slow rate of fixation. Changing the pH of the perfusate to a higher value (pH 11), increases the rapidity of peptide crosslinkage in a tissue, while immunoreactivity of the detected antigen is retained.

Glutaraldehyde is the most effective bifunctional aldehyde to preserve fine morphological details in tissues. By condensation reactions with proteins, it rapidly leads to inter- and intra-molecular crosslinks, hence provides fast fixation. Nevertheless, when used in perfusion fixation, it hampers deep penetration of the fixative due to rapid perivascular fixation, causing conformational changes of protein molecules. It

also decreases immunoreactivity. However, when used in low concentrations (0.1-2.5%), glutaraldehyde fixation was found to satisfy even immunoelectron microscopic requirements for the detection of both cell surface antigens (Van Ewijk et al. 1980), and endopeptides stored in secretion granules (Morel et al. 1980).

Formaldehyde and glutaraldehyde are frequently used together. Whether these aldehydes are used individually, or in combination, perfusion fixation is the method to be followed in order to remove blood cells causing nonspecific staining when peroxidase is used in the immunohistological technique (Ordronneau and Petrusz 1980).

Fixation with formaline sublimate, or with Bouin-Hollande sublimate, as well as with the so-called Zamboni's fixative (Zamboni and De Martino 1967) are common practices used for the light microscopic detection of endopeptides. It was found that some endopeptides retain their immunoreactivity even after OsO_4 fixation (Baskin et al. 1979). Recently, fixation with acrolein has been introduced for the immunocytochemical detection of brain peptides (King et al. 1983).

As a consequence of histotechnical processing, immunoreactivity of tissue antigens becomes more or less damaged. Therefore, cryo-microtomy or cryo-ultramicrotomy (Morel et al. 1980, Tokuyasu 1980) of fixed tissue samples cause the least impairment of immunoreactivity. Immunofluorescence techniques generally use cryosections. Recent immunohistological techniques prefer the use of vibrating microtome (vibrotome) sections for the peroxidase-antiperoxidase complex (PAP) staining procedure. Since the PAP method is one of the most sensitive immunohistological techniques, it can be used with excellent results for sections cut from embedded materials. Paraffin, polyethylene glycol (Mazurkiewicz and Nakane 1972), or epoxy resins can be used with equal success. In the case of epoxy sections, which are commonly processed for electron microscopy, the embedding material must be etched (Moriarty and Halmi 1972), or removed (Baskin et al. 1979). Staining itself can either precede, or follow embedding. In the case of preembedding staining, the 20-100 µm thick vibrotome or frozen

sections must be treated with alcohol or a detergent (for example 0.15% Triton X-100) prior to incubation with the primary antibodies in order to facilitate penetration of the IgG molecules into the tissue (Tougard et al. 1980).

1.4.3.2. METHODS USING LABELED ANTIBODIES

Immunohistological methods of this type are similar in the sense that they apply labels chemically or physically bound to the antibodies. The label can either be a fluorochrome, ferritin, colloidal metal, an enzyme or radioactive tracer. The respective methods are known as immuno-fluorescence, -ferritin, -colloid, -enzyme methods, or immunoradioautography.

I m m u n o f l u o r e s c e n c e methods. The idea to detect antigens with fluorochrome-labeled antibodies was first conceived by Coons, who also brought the idea to practical success (Coons et al. 1941, 1942). The most frequently used fluorochromes are fluorescein isothiocyanate (FITC), and rhodamine isothiocyanate (RITC), resulting in yellow-green and red emitted light, respectively. They are coupled to free amino groups of the antibodies by stable covalent bonds at an alkaline pH. Following incubation of the sections with the purified, fluorochrome-labeled antibodies, specific immuno-fluorescence is detected with fluorescence microscopy.

I m m u n o f e r r i t i n and i m m u n o c o l l o i d techniques. These are immunohistological methods using antibodies tagged with electron opaque substances, such as ferritin or colloidal gold. To label IgG with ferritin, the latter is first reacted with a bifunctional reagent. When reactants are admixed in appropriate proportions, one reacting arm of the bifunctional reagent reacts with ferritin, while the other remains free. In the second step, this free arm will bind the IgG added to the system. Unconjugated IgG molecules must be separated because they would compete for antigenic determinants during the staining procedure. Time limitation of the second step of conjugation is an important parameter in order

to prevent polymerisation of immunoglobulins. Colloidal gold particles in water can adsorb IgG molecules on their surface leaving the binding sites of IgG active. The bonds between gold particles and IgG molecules are loose, and intracytoplasmic protein molecules, possessing stronger positive charges than IgG, may replace IgG leading to nonspecific gold-labeling of the tissue.

I m m u n o e n z y m e methods. Horseradish peroxidase (HPO) has been introduced as a marker in immunohistochemistry by Nakane and Pierce (1966). Bifunctional reagents were first used for the conjugation of HPO with IgG, but soon the periodate conjugation technique substituted this method (Nakane and Kawaoi 1974). At first the carbohydrate moiety of HPO is oxidized to aldehydes, then these are allowed to bind to the amino groups of IgG at alkaline pH. To prevent self-coupling of the oxidized peroxidase molecules, their own amino groups must be previously blocked with fluorodinitrobenzene. The conjugate is finally stabilized with sodium borohydrate. The enzyme is then detected with histochemical methods. HPO forms a primary complex with its specific substrate H_2O_2, while the hemeprostetic component of the enzyme becomes oxidized. In the absence of an electron donor, the reaction would cease at this stage; however, when an electron donor is available, the oxidized enzyme forms a secondary complex with it. By the rapid dissociation of this complex, the enzyme becomes reduced and reactivated, ready to react with new molecules of the substrate. Meanwhile the liberated electron donor becomes oxidized. This form of the electron donor is a coloured product, and makes the detection of the antigen-antibody reaction possible with transmission microscopy. 3,3'-diaminobenzidine (DAB) proved to be the best electron donor, because its oxidized form has a contrasting brown colour, it is stable and almost insoluble in water preventing nonspecific translocation of the reaction product in the tissue. A further advantage of DAB as electron donior is that its oxidized form becomes electron opaque following osmication (Graham and Karnovsky 1966), hence it can be also used for the detection of tissue antigens at an ultrastructural level.

I m m u n o r a d i o a u t o g r a p h y. The antibodies used in this technique are labeled with a radioactive tracer. Location of the specifically bound labeled antibodies is detected with radioautography.

A f f i n i t y h i s t o c h e m i s t r y. Affinity histochemistry is the combination of immunologic and affinity binding (Heggeness and Ash 1977). The specific, or the bridging antibodies are labeled with biotin. During the last incubation, avidin labeled with FITC, or an enzyme, will bind to specifically bound biotin and can subsequently be detected as mentioned above (Bayer et al. 1976, Guesdon et al. 1979, Boorsma 1983). The advantage of affinity histochemistry lays in the extemely strong binding of avidin to biotin, which makes possible the use of very high dilutions of the antibodies, and extensive washings between sequential incubations. Both of these circumstances provide better chances to prevent nonspecific binding of the reactants used in immunohistochemistry.

1.4.3.3. METHODS USING UNLABELED ANTIBODIES

The common and most important disadvantages of the immunohistological methods using labeled antibodies are the partial destruction of the immunoactivity of the labeled antibodies due to the chemical manipulations, and the uncontrollable binding of the immunocomplexes formed in the course of conjugation to the tissue leading to disturbing background staining. To eliminate these drawbacks, chemical or physical labeling of the antibodies leading to altered steric configuration of the antibody reaction sites should be replaced by pure immunologic manipulations.

The unlabeled a n t i b o d y b r i d g e e n z y m e method. In 1969 Mason et al. and Sternberger and Cuculis independently discovered that antibodies can be tagged with an enzyme using only specific immunologic bindings. In a second incubation, following the incubation of the tissue section with the specific antibodies, antibodies against the IgG of the

species providing the first antibodies can be bound to the first antibodies already present in the tissue. These antibodies have to be used in excess to let only one of their two antibody reaction sites react during this incubation. With their second antibody reaction site (being identical to the one already bound), these "bridging" antibodies can bind antibodies from the species in which the first antibody was raised, but this time bearing antibody reaction sites specific against the enzyme label, for example HPO. In the next incubation, the enzyme, i.e., HPO is bound to this antibody, and finally the enzyme is histochemically detected.

Because of the elimination of all possible factors leading to antibody destruction, the unlabeled antibody bridge enzyme method was thought to be the ideal immunohistological method. However, besides being time-comsuming because of the five consecutive steps, due to the peculiar characteristic of the anti-HPO antibodies, this method proved to be superior to the methods using labeled antibodies in only a few laboratories possessing anti-HPO of exceptionally high quality (Petrusz et al. 1980). Using whole antiserum to HPO, IgG molecules without antibody reaction sites to HPO will compete for the free binding sites of the "bridge antibodies", by this means weakening the effectivity of the staining method. The use of purified HPO antibodies would be the solution. The best ways to purify antibodies are the dissociation of the specific antibodies from a solid phase immunoabsorbent at low pH, or the addition of excess antigen to the immune precipitate of this antigen and its antibodies. In the case of anti-HPO, these methods proved to be unsuccessful, because the binding forces between HPO and anti-HPO are unusually strong. Purified anti-HPO IgG therefore contains only the antibodies of the lowest affinity. As a consequence of this, even 75 per cent of the HPO bound in the final incubation may be lost through subsequent extensive washing.

The p e r o x i d a s e - a n t i p e r o x i d a s e complex (PAP) method of Sternberger. The problem to purify HPO-antibodies with high affinity led to the invention of the immunohistological method most widely used nowadays. Stern-

berger and his coworkers (1970) achieved continuous dissociation of anti-HPO from the immunoabsorbent using a low pH, and the immediate binding of the freed antibodies by the addition of excess HPO. The HPO-antiHPO complex formed this way remained soluble and, following neutralization, stable enough to be used for months. The proportion of HPO-anti-HPO in the complex was found to be 3:2, and in the complex itself, three molecules of HPO and two anti-HPO IgG molecules formed a characteristic pentagonal ring easily detectable under the electron microscope. Incubation with the specific antibodies is followed by incubation with a bridge antibody capable of sticking to the antigenic determinants of the specific antibody with one of its antibody reaction sites, and of binding with its second antibody reaction site in the following incubation to the IgG from the same species in which the first antibody was raised. Since in the PAP complex the components used in the 3rd and 4th steps of the unlabeled antibody bridge enzyme method are already coupled to each other, immunohistological staining with the PAP complex is shorter in time. It was found that the PAP method is more sensitive than the unlabeled antibody bridge enzyme method, or any of the immunohistological methods utilizing labeled antibodies. In the absence of immunocomplexes inevitably formed during chemical or physical labeling, PAP staining is practically free of background staining. This makes the oxidized DAB end-product of the PAP staining method suitable for physical intensification, which further increases the sensitivity of the method by about two orders of magnitude (see section 1.4.3.5).

1.4.3.4. SIMULTANEOUS LOCALIZATION OF MULTIPLE TISSUE ANTIGENS

Localization of multiple antigens in tissues is possible either by using different markers for the specific antibodies, or by utilizing the different colours of various capturing agents in the immunohistological methods using enzyme markers (Nakane 1968). Combination of immunofluorescence and immunoperoxidase methods on the same histological section provides

another possibility to detect different antigens simultaneously (Lechago et al. 1979). Very recently, excellent results have been achieved in demonstrating two antigens in the same section utilizing the difference in colour and density of the DAB end-product of the traditional PAP-DAB method, and that of the silver intensified PAP-DAB (Liposits et al. 1983, 1984).

It is advised to elute antibodies bound to the tissue during the location of the first antigen, because their reactive sites may interfere with the immunoreactions that follow. Dissociation of the antibody complexes from tissue can be achieved by extensive washing in buffers of a low pH (Nakane 1968), or by the combination of low pH and electrophoresis (Vandesande et al. 1977). However, excellent contrasting colour has been achieved by others without eluting the antibodies used for the detection of the first antigen (Joseph and Sternberger 1979).

1.4.3.5. INTENSIFICATION OF THE DAB REACTION

Intensification of the DAB reaction product was first practiced by Graham and Karnovsky (1966) using OsO_4, and recently by Adams (1981), using either cobalt alone, or cobalt and nickel salts together. Besides increasing the sensitivity of the immunohistological methods, they also produced an electron dense deposit suitable for electron microscopic immunohistochemistry.

In 1982, Gallyas et al. published a new method suitable for intensifying the visibility of the end-product of the oxidative polymerization of DAB. Using this method, dark, electron dense deposits show up at sites of the tissue section exhibiting otherwise no discernible signs of the reaction product. The procedure is based on the ability of the oxidized DAB to catalyse the reaction between silver ions and a reducing agent present in the physical developer of Gallyas (1971), producing metallic silver grains. Following the DAB reaction, immunohistologically stained sections are treated with thioglycolic acid to suppress the catalytic activity of the tissue, thus

preventing nonspecific silver deposition. After thorough washing, reacted sections are treated with the physical developer under microscopic control until the desired degree of intensification has been achieved. Intensification is stopped in acetic acid. Following dehydration and clearing, intensified sections are ready for mounting (Gorcs et al. 1983), or can be processed for electron microscopy.

1.4.3.6. SPECIFICITY TESTS FOR IMMUNOHISTOCHEMISTRY

Antibodies used in immunohistochemistry recognize only antigenic determinants of substances to be localized, i.e., smaller or larger regions of that antigen, and not the whole molecule. This has to be kept in mind whenever the question of specificity is discussed.

In order to qualify the specificity of an immunohistochemical staining method, one has to answer two questions (Petrusz et al. 1980): (1) Is the method used specific?, (2) Are the antibodies used specific?

M e t h o d s p e c i f i c i t y means that staining exclusively results from the immunochemical reaction between the primary antibodies and their complementary antigenic determinants present in the tissue. To prove this, the performance of the following tests is recommended: (a) Simultaneous staining of consecutive sections with increasing dilutions of the primary antiserum must lead to different staining intensity. Staining which is independent of the degree of dilution of the primary antiserum represents method non-specificity. With this test, the optimal working dilution of the primary antiserum can also be determined. It may happen that staining with higher concentrations of the primary antiserum results in weaker staining than with higher dilutions of the same antiserum (Vandesande 1979). This can be the case when primary antibodies bind to their antigenic determinants in the tissue so close to each other (because of high concentration of the antigen) that bridging antibodies can find specific binding sites for both of their reaction sites. This way no reaction

sites remain on the "bridge" for the antibodies of the PAP-complex. Increasing the dilution of the primary antiserum, more and more bridging antibodies will find only one primary antibody molecule within their reaction sphere, sticked to the tisue, this way saving their second reaction site for the PAP complex; (b) Omitting each reagent, one at a time, from the staining protocol, the factor leading to method non-specificity can be traced; (c) Nonimmune binding of the bridging antibodies to the tissue also leads to method non-specificity. This can be eliminated by preincubating the sections with a 1-2 per cent dilution of a normal serum derived from the species serving the bridging antibodies. (d) Absorption of the primary antiserum with appropriate tissue powder (usually derived from a tissue of the species under investigation, which does not contain antigenic determinants for the primary antibodies) can significantly reduce background staining caused by nonimmune binding of the primary antiserum.

It is a difficult task to prove antibody specificity. Immunization with any antigen will always lead to the generation of a large number of antibody populations bearing different antibody reaction sites for many of the antigenic determinants of that antigen. Some of these antibodies show various degrees of complementary fit to a single determinant site. The closer the complementary fit of an antibody to its antigenic determinant, the higher its affinity and specificity. Further complications arise from the fact that different antigens may share similar, or identical determinant sites. During immunization, this can lead to the generation of crossreacting antibodies. In other words, in any given antiserum, antibody populations are present that can react, although with different affinity, with substances of the tissue other than the antigen under investigation. An antibody can be qualified specific if it is exempt from such crossreacting antibodies. This can be achieved by (Petrusz et al. 1976, 1977): (a) Adsorption on a solid phase immunoadsorbent, to which the crossreacting antigens are stuck. In the case of precipitating antibodies, the addition of the crossreacting antigen in suitable amounts to the antiserum leads to the precipitation of the disturbing im-

munoreactants, and this way serum non-specificity can be eliminated; (b) Staining with increasing dilutions of the primary antiserum facilitates recognition and "elimination" of crossreacting antibodies; (c) Absorption tests with the antigen used for immunization is a basic control in immunohistology, but does not guarantee the absence of crossreacting antibodies from the antiserum; (d) Absorption tests with fragments of the antigen molecule (especially in the case of peptides) help to recognize antigenic determinants against which an antiserum contains antibody populations. This way other antigens with known chemical structure can be purposefully tested for possible crossreactions, and if necessary, the antiserum can be absorbed with this antigen; (e) Radioimmunoassay may complete our knowledge about an antibody, but cannot prove specificity of that serum when used for immunohistochemistry. Different antibody populations have been found to act in RIA and in immunohistochemistry (Swaab et al. 1977).

The use of monoclonal antibodies in immunohistochemistry has significantly reduced the hazard of nonspecific staining. However, due to possible common antigenic determinants shared by different antigens, interpretation of the results of an immunohistological study requires caution even if monoclonal antibodies have been used (Mason et al. 1983).

1.5. ISOLATION AND STRUCTURE ELUCIDATION OF ENDOGENOUS PEPTIDES

In view of the generally low concentration of endopeptides present in tissue material, the extraction and isolation of a given endopeptide requires carefully designed, well-conceived coordinated programs. Typically, such stepwise purification programs are directed toward the isolation of the material responsible for a certain biological activity in pure form. Thus, an isolation program is a combination of physicochemical separation methods with appropriate detection of biological (or immunological) activity in each step.

As already defined in the introduction, endopeptides are regulatory substances in different tissues of higher organisms. Such compounds have been isolated from whole brain, the hypothalamus, cerebrospinal fluid, the pituitary, various areas of the gastrointestinal tract, the thymus, the gonads, blood and several other tissue preparations.

1.5.1. EXTRACTION AND PURIFICATION OF ENDOGENOUS PEPTIDES

The amount of starting tissue material is basically dependent on the sensitivity of the final analytical method used for structure determination and the actual tissue concentration of the peptide to be isolated, provided the isolation procedure recovers the peptide in high yield. At the beginning of the isolation program, we do not want to isolate a certain "peptide" but a biological factor with yet unknown structure. However, as more and more regulatory substances turn out to be peptides, there is high probability that the isolation and purification of regulatory substances from mammalian tissue will result in a peptide structure.

Earlier isolation programs required large amounts of fresh tissue because detection techniques (biological and immunological assays) and structural methods were not as sensitive as today. In "classical" examples of endopeptide purifications several kilograms of fresh tissue had to be processed and consequently large amounts of processing chemicals were required which made such efforts exceedingly expensive and heroic.

The common, generally applied steps described in various isolation programs for hypothalamic, pituitary and gastrointestinal peptides are briefly discussed here. Their use and combination are highly dependent on the nature of the biologically interesting factors. The tissue material is first defatted with organic solvents either freshly or following lyophilization of the tissue. The pulverized dry tissue material is then extracted with an aqueous solution (buffer, dilute acid or base) and this extract is lyophylized. Provided the biologically active material is contained in the aqueous extract,

this can further be purified with specific methods. For the separation of hydrophilic compounds gel permeation chromatography or gel filtration on dextran (Sephadex) or polyacrylamide (BioGel) proved to be a very convenient and therefore frequently applied first step (Fischer 1980). This allows fractionation by size and provides a first estimate of the molecular weight of the biologically active factor.

The biochemist carrying out an isolation program has had an ever growing arsenal for the separation of complex biological mixtures at his disposal. Countercurrent distribution (CCD, Craig and Craig 1956) dominated as a very powerful method for the isolation of many endogenous peptides for a long period (e.g., Schally et al. 1971). The advantages of the method have largely contributed to its revival in the form of droplet countercurrent distribution (Tanimura et al. 1970, Brenner et al. 1979) which also offers high resolution and high recoveries due to the absence of carrier materials. Partition chromatography developed by Yamashiro (1964) is also based on the same principle, however, a dextran bed is used as column support. This technique has proved to be very effective in separating even structurally highly related peptides (Yamashiro 1980). Ion exchange chromatography on cellulose or dextran type supports (Peterson 1970) and polystyrene type supports (Schroeder 1972b) has been very widely applied for the purification of various peptides. Different electrophoretic methods have also been used besides analytical work for preparative scale purifications as well (Gordon 1975, Righetti and Drysdale 1976). In addition, immobilized binding proteins covalently attached to carrier matrices provide an additional, highly specific purification potential in the form of affinity chromatography (Lowe 1979).

The above separation steps were monitored by the measurement of UV absorption of the fractions or protein determination with specific reagents from aliquots of the fractions. Most important, fractionation was based on the measurement of the desired biological activity following each step of the purification scheme. Biological assays, however, were not as sensitive and reliable as today and thus required considerable

amounts of active materials. The development of radioimmunoassay has greatly contributed to the improved sensitivity of bioassays used today (Yalow 1978).

Contemporary purification projects are based on sophisticated methodology on all levels. Separation methods have been largely improved in the past decade. Detection methods, both chemical and biological or immunological, have reached extreme levels of sensitivity. Structure analytical methods using microtechniques allow sequencing of peptides on nanomolar or even picomolar levels. Isolation work is becoming more realistic than before as a result of these developments.

For obtaining appropriate tissue material, animals are usually killed by microwave irradiation (Stein 1981) to prevent autolytic damage of the peptide to be isolated. Tissue obtained this way is defatted and then extracted with aqueous solutions. Enzyme inhibitors are usually added to the extraction buffer at this stage also to avoid proteolytic peptide degradation. Purification of the active material is today typically achieved by a first gel filtration step followed by preparative HPLC separation (Vale et al. 1981, Guillemin et al. 1982, Rivier et al. 1982). This way pure substances can be prepared within two or three chromatographic steps compared to 5-10, or even more using classical schemes.

HPLC has become an invaluable tool in peptide chemistry in both analytical and preparative work due to its yet unmatched performance in separation power. Reversed phase HPLC is the mostly applied version for peptides (reviewed by Hearn et al. 1983, Horvath 1983).

As already discussed in section 1.4.1, fluorescent detection of peptides after derivatization with o-phthalaldehyde, fluorescamine or other reagents is highly superior in sensitivity against direct UV detection or other peptide or protein reagents. Thus fluorescent detection of aliquots can be effectively applied for preparative work also (Stein 1981).

The development of RIA, and its convenience, has had a remarkable effect on isolation of endopeptides. In addition to RIA, other specific binding assays, as, for example, receptor binding assay can form the basis of peptide isolation, which

has been successfully applied for the isolation of enkephalins using opiate receptor binding (Hughes et al. 1975).

Peptides subjected to structure determination should be homogeneous and contain no other peptide contaminants. For the determination of homogeneity, methods discussed in section 1.4.1 are generally applied. HPLC has also been found to be very efficient in final purification and verifying homogeneity.

1.5.2. SEQUENCING OF ENDOGENOUS PEPTIDES

Since peptides are oligomers or polymers of amino acid building blocks joined by peptide bonds, determination of peptide structures principally means reading the sequence of constituent amino acids. The development of modern sequencing techniques started with the pioneering work of Edman (1950) who proposed phenylisothiocyanate (PITC) for the stepwise degradation of peptides from the N-terminal amino acid residue. This principle and its basic chemistry formed the basis of all further sequence studies on peptides and proteins. The first successful complete structure elucidation of a polypeptide (insulin) was accomplished by Sanger and Tuppy (1951) as well as Sanger and Thompson (1953). Several examples followed and these laid the foundations for today's sequencing strategies. Further significant progress came with the development of automated amino acid analysis (Spackman et al. 1958) which largely increased the speed and accuracy of structural work on peptides.

The repeated sequence of reactions used in the Edman degradation offered the possibility of automation which was successfully solved by the design of a special apparatus incorporating a reaction vessel where the peptide or protein was kept in a liquid phase film during sequencing (Edman and Begg 1967). Automatic sequencers became commercially available which greatly promoted structure determinations of various peptides and proteins. The common strategy used to analyze amino acid sequences of peptides is briefly summarized here.

Extensive reviews and handbooks are referred to for further details (Needleman 1975, Konigsberg and Steinman 1977, Birr 1980, Allen 1981, Liu et al. 1981).

First the disulfide bonds, if present, are split by reduction and alkylation prevents their reformation. Then the amino acid composition (molar ratios of amino acids) of the whole peptide is determined after hydrolysis using an automatic amino acid analyzer. N- and C-terminal amino acid residues are determined next. N-terminal amino acids can be identified by PITC, (Edman 1950), dansyl chloride (Gray 1972) or 4-dimethylaminoazobenzene-4'-isothiocyanate (DABITC, Chang et al. 1978) reagents which yield easily detectable derivatives with the free amino terminus. C-terminal amino acids can be identified by hydrazinolysis (Schroeder 1972a) or carboxypeptidase cleavage (Ambler 1972).

Provided the peptide has a free amino terminus, an aliquot of the whole peptide is then subjected to direct sequence determination. The automatic sequencer performing sequential Edman degradations (reviewed by Edman and Henschen 1975) can determine N-terminal sequences of 30-70 residues on large polypeptides and 20-40 residues on small peptides. Typically, the sensitivity of the liquid phase sequencer lies in the micromole range. Due to several technical improvements, this sensitivity has increased to approximately 10-100 nanomoles (Wittmann-Liebold 1981). A new type of sequencer incorporating HPLC separation and determination of PTH amino acids can analyze peptide sequences from as little as 50-100 picomoles of starting material (Hunkapiller and Hood 1980, 1981).

Since several technical problems can arise during liquid phase sequencing, an alternative approach was developed by covalently linking the peptide to an insoluble support (Laursen 1966). The "solid phase sequencing" methods using different supports and attachment reactions have found wide applicability (Laursen 1975, Previero and Colletti-Previero 1977), however, they do not exceed the sensitivity of advanced liquid phase micro methods. Solid phase sequencing also offers automation and such sequencers are commercially available.

After the N-terminal sequence of the original molecule has been determined, the peptide is broken into fragments by internal cleavage at specific points. For this purpose specific enzymes: endopeptidases, trypsin, chymotrypsin, thermolysin, pepsin and some others can be used as well as specific chemical reagents, e.g., cyanogen bromide (reviewed by Allen 1981). This cleavage yields smaller fragments which are separated and their amino acid compositions and N-terminal sequences are determined similarly as for the whole molecule. At least two independent cleavage procedures must be carried out. These provide "overlap peptides" whose ordering results in the complete sequence of the whole molecule. Finally, the location of the disulfide bonds has to be determined, which is generally done by partial sequence analysis of fragments obtained by internal cleavage of the intact, unreduced peptide.

Additional structural elements of peptides require further analytical investigations. Such structures can be blocked at N- and C- termini, by γ-carboxy-glutamic acid, phosphorylated and methylated amino acids, or carbohydrate residues attached to the peptide backbone and several other unusual elements.

Using the strategy outlined above with recent methodology, the size of polypeptides which can be structurally elucidated has greatly increased, and recently the complete sequence of β-galactosidase from E. c o l i consisting of 1,021 amino acids could be determined (Fowler and Zabin 1977).

It should be noted that the simplicity and speed of recent DNA-sequencing (Maxam and Gilbert 1977, Sanger et al. 1977) offers an alternative approach to protein and peptide sequence determination. Cloned cDNA can be derived from purified mRNA whose sequence can be rapidly determined by the new methods. Nevertheless, DNA sequences also contain extensions which are not expressed and it is difficult to exactly find the start and end of the expressed fragment. Therefore at least partial sequence determination of N- and C-terminal amino acid sequences must be carried out to locate the frame of the DNA sequence from which the protein structure is deduced. In addition, intervening sequences in eukaryotic DNA makes the deduction of peptide sequences difficult (Abelson 1979). Further-

more, posttranslational modifications can only be determined by direct peptide analysis. Therefore, the analysis of peptides by the sequencing methods described above certainly remains unavoidable.

At present, although not fully elaborated and complete, mass spectrometry also offers the possibility of peptide sequence determination. This method, with further technical and methodical improvements, is likely to become an extremely valuable tool for peptide sequencing (e.g., Biemann 1981).

1.6. SYNTHESIS OF ENDOGENOUS PEPTIDES

1.6.1. CHEMICAL SYNTHESIS OF ENDOGENOUS PEPTIDES

The contribution of chemical peptide synthesis to the investigation of biologically active peptides is very complex. Chemical synthesis of a natural peptide serves as proof for the correctness of the structure determined. Chemical synthesis is also the major tool for the preparation of modified species of a given molecule. Single or multiple modifications reveal a great deal of the contribution of the individual amino acid side chains to the biological effect of the peptide. In addition, peptides of biological interest can be prepared in large quantities by chemical synthesis. This can even be scaled up for the production of very large amounts for medical and veterinary applications. Despite successful efforts in genetic technology, chemical synthesis probably remains dominant for the preparation of smaller size peptides and modified derivatives.

The aim of this section is to very briefly summarize the present state of chemical peptide synthesis along with its possibilities and limitations. The scope and size of the paper permit only a brief account and only a reference is made to the exhaustive monographs in the field (Schroder and Lubke 1965, 1966, Merrifield 1969, Meienhofer 1973, Wunsch 1974, Bodanszky et al. 1976, Gross and Meienhofer, 1979, 1980, 1981 and The Chemical Society Special Periodical Reports 1969).

The present methodology of chemical peptide synthesis is a unique combination of organic chemistry, physical chemistry and biochemistry. The techniques permit a reasonably rapid and safe preparation of peptides up to about 50 amino acids and even beyond, however, if we compare this with the speed and fidelity of peptide and protein biosynthetic mechanisms it becomes apparent that further developments are still required. Chemical peptide synthesis, in general, is based on the following steps and techniques: temporary protection of certain functional groups, activation of carboxyl groups for rapid peptide bond formation, removal of protecting groups and purification of the synthetic peptide. It is obvious that side reactions are avoided as much as possible. Since these cannot be totally suppressed, sophisticated separation methods are required for the purification of synthetic peptides. The specific combination of these methods is often referred to as the strategy and tactics of peptide synthesis. We shall review generally applied protecting groups, coupling reactions, possible synthetic routes and demonstrate the present possibilities of chemical peptide synthesis with a few prominent examples.

1.6.1.1. PROTECTING GROUPS

Directed coupling reactions require the temporary protection of the functional groups in certain amino acid residues of the peptide. Peptide chemists have developed a large arsenal of protecting groups for the α-amino, α-carboxyl and different side chain functions of amino acids. From these, only common types will be discussed, others with more special applications can be found in the monographs cited.

For a m i n o g r o u p s mostly urethane type protective groups have been applied. Among these the most frequently used ones have been the benzyloxycarbonyl, Z (Bergmann and Zervas 1932), the t-butyloxycarbonyl, Boc (McKay and Albertson 1957 and Anderson and McGregor 1957), the 2-(p-biphenylyl)propyl (2)oxycarbonyl, Bpoc (Sieber and Iselin

1968), the α ,α dimethyl-3,5-dimethyloxybenzyloxycarbonyl, Ddz (Birr et al. 1972) and the 9-fluorenylmethyloxycarbonyl, Fmoc (Carpino and Han 1972) groups. The more recent protective groups can be removed under very mild conditions which do not cause severe damage to the peptide chain. During synthesis, different protective groups are used for the α-amino and ω-amino functions to ensure selectivity, i.e., unidirectional peptide chain elongation.

C a r b o x y l g r o u p s can be conveniently protected as alkyl and aralkyl esters. Both the introduction and removal of the ester groups is usually straightforward, thus only special applications include other types, e.g., substituted hydrazides as carboxyl protection.

For the protection of (other than amino or carboxyl) side chain functional groups a great variety of protective groups are available, of which only cysteine protection is mentioned here. In addition to the peptide chain, disulfide bridges form the most important covalent bond in peptides and the directed formation of disulfide bonds in peptides and proteins has always been a difficult challenge. Sulfhydryl groups of cysteine can be effectively protected by trityl (Amiard et al. 1956), p-methoxybenzyl (Akabori et al. 1964) or acetamidomethyl (Veber et al. 1968) groups. Improved conditions for the removal of these protective groups have greatly contributed to the successful application of these in more recent syntheses.

1.6.1.2. COUPLING REACTIONS

Coupling reactions between amino acids, i.e., formation of a peptide bond requires the activation of the participating carboxyl group (Fig.1.2).

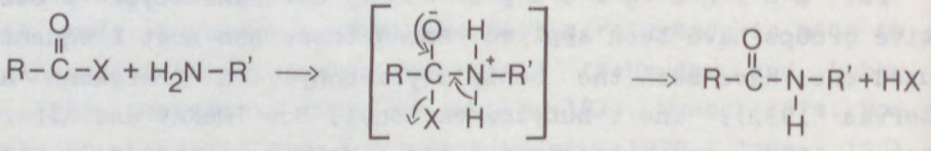


Fig. 1.2. Mechanism of peptide bond formation

Activation can be achieved in many ways. The four major methods of peptide bond formation are based on the use of active esters, azides and mixed anhydrides of amino acids as well as carbodiimide as an additive to facilitate the coupling reaction.

A c t i v e e s t e r s of N-protected amino acids have been very popular for peptide bond formation since the preparation of the first useful representatives of these types of reagents (Bodanszky 1955). The most widely applied active esters have been aryl (among these p- and o-nitrophenyl and 2,4,5-trichlorophenyl) esters and hydroxylamine derivatives (among these O-acyl derivatives of N-hydroxysuccinimide and N-hydroxybenzotriazole) (reviewed by Bodanszky 1979).

Due to their advantages a z i d e s of N-protected amino acids and peptides are still being used for peptide bond formation. The azide method provides a low racemization potential, high coupling yields even with large segments and good conversion of amino acid or peptide esters to azides via hydrazides. Because of these advantages, azide coupling has been most frequently applied for the synthesis of large polypeptides and proteins employing segment condensations. Expert reviews of the azide method can be found in Schroder and Lubke (1965), Klausner and Bodanszky (1974) and Meienhofer (1979).

The use of m i x e d a n h y d r i d e s of N-protected amino acids was developed independently by Boissonnas (1951), Vaughan (1951) and Wieland and Bernhard (1951). The method, which almost exclusively uses alkylchloroformates for the formation of mixed anhydrides, has gained wide application because of its easy manipulation, low cost and availability of reagents. The method, however, was found to lead to a relatively high degree of racemization because of the strong carbonyl activation. For a long period it was not used for segment condensations. However, with the introduction of precise reaction conditions (Wieland et al. 1971) it can now safely be used for segment condensations. A revival of the method has been the development of the repetitive excess mixed anhydride (REMA) procedure as a simple and rapid peptide synthetic method (Tilak 1970) which will be discussed later.

The carbodiimide method for peptide bond formation was first applied by Sheehan and Hess (1955) and due to its successful application in both classical and solid phase peptide syntheses, this reaction has reached a high level of popularity. Due to the simplicity and relatively high speed of the reaction, dicyclohexylcarbodiimide has become the most widely applied reagent both directly for peptide bond formation, and for the preparation of active esters of amino acids. A great improvement of the carbodiimide method was the introduction of 1-hydroxybenzotriazole for accelerating the rate of the coupling reaction and suppressing racemization (Konig and Geiger 1970). The method is generally used for the coupling of N-protected amino acids to a growing peptide chain, but it can also be used for the coupling of large segments.

Related to the two latter methods, symmetrical anhydrides formed from N-protected amino acids by dicyclohexylcarbodiimide have also been used for peptide bond formation. The use of symmetrical anhydrides is becoming more popular especially in solid phase peptide synthesis (Hagenmaier and Frank 1972, Yamashiro and Li 1974). During the synthesis of corticotropin, the overall yield of 3% obtained with the conventional DCC coupling could be increased to 30% by using symmetrical anhydrides (Lemaire et al. 1977).

In conclusion, the different coupling methods offer unique features which have to be carefully studied and considered for the synthesis of individual peptides. For example, the azide method, which had previously been considered to be racemization-free, showed up to 40% racemization during a segment condensation reaction (Sieber et al. 1970), calling for the need of strict tests for side rections, especially racemization. In spite of such examples, the azide coupling reaction can still be considered to be the safest from the point of yielding high chiral purity. Nevertheless, critical experiments must precede the selection of coupling reactions and the strategy of constructing a certain peptide.

1.6.1.3. PEPTIDE SYNTHESIS

The construction of peptides from amino acids is a complex sequence of chemical reactions. Peptide synthesis can be carried out in a homogeneous solution (classical solution synthesis) and on the surface of an insoluble carrier (solid phase synthesis, SPPS). Another general distinction between synthetic routes is the fashion in which the peptide chain is assembled. In this respect we can distinguish between stepwise chain elongation and segment condensation.

S t e p w i s e c o u p l i n g of amino acids to the growing peptide chain can be applied in solution and in solid phase synthesis. In fact, the idea of stepwise chain elongation had a major impact on the development of SPPS and this is still the major route applied in SPPS. Stepwise repeated reactions offer the possibility of automation and today's "automatic" peptide synthesizers are all based on stepwise SPPS. Also, the so-called "rapid" techniques carried out in solution employ stepwise strategies. The REMA method (Tilak 1970, Van Zon and Beyerman 1973) uses mixed anhydrides of N-protected amino acids in slight excess over the amino component, which, after the coupling reaction, can be easily removed. The products can be purified usually by crystallization or extraction. Two rapid methods are based on the use of activated esters. One uses o-nitrophenyl esters (Bodanszky et al. 1973), while the other pentafluorophenyl esters (Kisfaludy et al. 1974) of N-protected amino acids in excess, which after the coupling is removed by various washing steps. The usefulness of all three rapid methods was demonstrated on syntheses of different peptides up to 27 amino acids, however, they have not reached general acceptance.

S e g m e n t c o n d e n s a t i o n is a frequently applied strategy for the synthesis of large peptides or proteins, especially in solution; but there are examples of segment condensation in SPPS. Segment condensation offers the major advantage that the end product of the coupling reaction is generally considerably different from the starting peptides

and thus a very efficient purification can be achieved as compared to stepwise chain elongation.

In certain cases semisynthesis of peptides is a possible alternative for the preparation of complex polypeptides or proteins (Offord 1980). Peptides of natural origin are converted or modified to desired structures by chemical reactions. An example of successful semisynthesis is the conversion of porcine insulin to the human sequence.

As briefly outlined, the basic methodolgy of peptide synthesis has become a reliable means of preparing peptides with known natural sequences, modified derivatives thereof and large quantities of pharmacologically potent peptides. We shall have a look at the present frontiers of peptide synthesis on selected representative examples.

The directed formation of disulfide bonds has always caused serious synthetic problems for the peptide chemist. One elegant solution for such a problem has been the total synthesis of human insulin (Sieber et al. 1974, 1977) using an ingenious combination of sulfhydryl, amino and carboxyl protecting groups and specific cleaving conditions for their independent removal. Three disulfide bridges were formed independently of each other during the synthesis of the two-chain polypeptide containing 51 amino acid residues. The two chains were constructed by segment condensation using azide and dicyclohexyl carbodiimide/1-hydroxybenzotriazol coupling reactions.

One of today's most remarkable achievements in peptide synthesis has been the total synthesis of crystalline ribonuclease A (Fujii and Yajima 1981, Yajima and Fujii 1981). The single chain protein consists of 124 amino acids and it was built up from 30 smaller segments using mainly azide coupling reactions. The disulfide bonds were formed after the construction of the whole peptide chain. In contrast to smaller peptides, where the conformation of the molecule is generally very flexible, ribonuclease has a preferred three-dimensional structure, which determines the correct formation of the disulfide bonds. The final product, very carefully purified, was characterized by a variety of biochemical and physicochemical methods which proved the enzyme to be even more potent

than the natural RNase. In 1969 well-characterized, fully active RNase A could not be prepared either by classical solution (Hirschmann et al. 1969) or solid phase synthesis (Gutte and Merrifield 1969).

Solid phase peptide synthesis, however, using improved methodology (symmetrical anhydrides for obtaining very high coupling yields and repeated swelling and shrinking of the peptide-resin), can result in fully active compounds as it was demonstrated with β-lipotropin (Yamashiro and Li 1978). A general problem in solid phase synthesis is the formation of failure sequences as a result of incomplete coupling yields. Precise analytical tests can be very helpful in revealing the presence of failure sequences. Marki et al. (1981) reported the formation of des-Pro^2-gastrin releasing peptide, a 27-peptide prepared by routine SPPS methods. The formation of this product could only be revealed by peptide sequencing. A more controlled synthesis, however, led to the preparation of homogeneous gastrin releasing peptide. Such examples call for the necessity of critical analytical tests of synthetic products, which, taking advantage of today's sophisticated techniques, is a realistic requirement.

Criteria of purity toward synthetic peptides have become extremely strict due to the improved methods used for the analysis of peptides. Both separation techniques and detection methods have been highly improved and as a result, synthetic peptide preparations can be efficiently analyzed. A most useful analytical method seems to be reversed phase HPLC as discussed in detail by Hearn et al. (1983) and Horvath (1983). This technique has been found to be powerful enough to separate even stereoisomers with identical peptide sequences. Preparative scale HPLC successfully supplements today's peptide synthetic methods to meet high standards of purity.

1.6.2. BIOLOGICAL SYNTHESIS OF ENDOGENOUS PEPTIDES: CLONING AND EXPRESSION OF GENES ENCODING SPECIFIC PEPTIDES AND PROTEINS

The still developing techniques of DNA recombination represent a new and, in some respect, a more efficient route of production of endogenous peptides (EPs) than conventional laboratory synthesis. With the aid of these techniques, production of EPs whose molecular size would otherwise prohibit their synthesis by the conventional methods, can also be accomplished. The number of EPs produced thus far by recombinant DNA techniques has remarkably increased in recent years, and still continues to grow. In addition, we are now witnessing the birth of a new branch of industrial activities which is based on, and makes use of the techniques of genetic engineering, as well as fermentation.

Due to the increasing theoretical and practical importance of genetic engineering in EPs research a brief account on the main features of DNA recombination is warranted.

1.6.2.1. THE TOOLS AND TECHNIQUES OF DNA RECOMBINATION

DNA recombination techniques were developed with the aim to transfer genetic information from one cell to another, allowing the production of a selected gene product, most often an interesting peptide or protein. In practice, this requires the introduction of exogenous (foreign) DNA fragments coding for the sequence of the selected gene product into bacterial cells where they subsequently are expressed and replicated with the final result of synthesizing substantial quantities of desired gene product. To achieve this goal, one has to be in possession of the appropriate biological tools and techniques. These include: methods by which a DNA segment coding for a selected protein can be obtained by specific cleavage of the DNA molecules; procedures by which a DNA segment can be introduced into bacteria in a replicating form; specific probes for the identification of the selected gene product; and the means by which expression of genes can be achieved in bacteria.

1.6.2.1.1. SOURCES OF SPECIFIC DNA SEQUENCES

The DNA sequences encoding specific proteins may be obtained from three sources: (i) chromosomal or genomic DNA, which contains essentially all the genetic information of a given organism, (ii) mRNA obtained from tissues that synthesize the protein of interest, and (iii) chemical synthesis, if the amino acid sequence of the protein is known.

The term recombinant molecule or recombinant DNA (recDNA) is applied for hybrid molecules that are constructed by joining a selected DNA sequence to the DNA of an appropriated vector (see later). Generally, recombinant molecules containing fragments of genomic DNA are the most easily constructed, and they should contain all of the DNA sequences present in the organism. In contrast, recombinant molecules carrying DNA sequences derived from mRNA will represent only those genes transcribed in the tissue from which the mRNA was obtained. Complete chemical synthesis of specific genes is at present applicable to only small genes. Short synthetic oligonucleotides corresponding to partial sequences in the protein of interest, however, may be used as probes to isolate the entire genes from a collection of genomic or mRNA sequences by techniques known as molecular hybridization and cloning.

1.6.2.1.2. CLONING OF GENOMIC DNA

For the isolation of specific genes from genomic DNA, it is necessary to (i) cut large DNA molecules into fragments using restriction endonucleases, and (ii) isolate and insert these fragments into the DNA of a vector, or join small pieces of DNA, if required.

(a) Restriction endonucleases

Restriction endonucleases are enzymes having the capability of cleaving optional DNA sequences at strictly determined sites, a property called the sequence specificity of such enzymes. These enzymes have been found in nearly every microorganism examined and are known to catalyze double-strand breaks in DNA, to yield restriction fragments. Since 1970, when the first restriction nuclease was discovered (Kelly and Smith 1970), the number of known enzymes has increased to more than 250, and the number of different sequence specificities determined so far is also outnumbering 100 (Roberts 1982). Because a restriction enzyme cleavage pattern is specific for a given DNA and enzyme, a restriction fragment, when isolated on a preparative scale, represents a homogeneous population of DNA molecules. The multitude of restriction endonucleases, and the large variety of their sequence specificities now allow the specific and reproducible cleavage of double stranded DNA molecules at a large variety of locations, and thereby the production of specific DNA fragments or genes.

(b) Joining pieces of DNA

Joining pieces of DNA including insertion of DNA fragments into foreign DNAs, can also be achieved by enzymatic methods. Currently, two kinds of enzymes are used most frequently for this purpose.

Pieces of DNA can be joined by one of the several DNA ligases. The enzyme used most frequently is the ATP dependent DNA ligase obtained from bacteriophage T4-infected E. c o l i. Due to its ability to link covalently double-stranded DNA segments possessing complementary single-stranded ends ("sticky ends"), or ends without a single-stranded protrusion ("blunt ends"), DNA ligases proved to be excellent tools for joining DNA fragments of different origin (Seeburg et al. 1977).

Pieces of DNA can also be joined by annealing homopolymer tails. The use of terminal deoxynucleotidyl transferase of calf thymus origin allows the attachment of a homodeoxy nu-

cleotide protrusion to the 3'-ends of any pieces of DNA. Since single-stranded 3'-ends ensure the most efficient terminal addition, DNA fragments with 5'-ends extensions are usually pretreated in a controlled reaction with phage λ exonuclease to remove a few nucleotides from the 5'-ends of each strand (Lobban and Kaiser 1973).

On the other hand, two stretches of DNA having complementary homopolymer protrusions can be noncovalently attached to each other by simple base-pairing. Following introduction into a living cell, the noncovalent bonds will be quickly transformed to covalent ones by repairing enzyme activities.

By using any of the two enzymes mentioned above, long linear polymeric chains and circular molecules are equally formed. However, the relative quantity of the two products can be easily influenced by relatively simple laboratory techniques (Dugaiczyk et al. 1975).

(c) Vectors

Exogenous DNA fragments introduced into bacteria ordinarily do not replicate spontaneously. In order to make it replicative, the foreign DNA segment must be linked to a DNA molecule capable of intracellular replication within its bacterial host by virtue of sequences known as origins of replication , which are recognized by the host cell's DNA replication enzymes. The DNA molecules which have the specific capability of self replication are termed vectors. Presently four types of vectors are used most frequently for introducing foreign DNA segments and propagating them in E. c o l i.

P l a s m i d s are extrachromosomal genetic elements of bacterial origin. They consist of double stranded, closed circular DNA molecules with a size ranging between about 1 and more than 200 kilobases (kb). Under laboratory conditions, plasmids can be transferred to new host cells by the process of transformation originally described by Avery and associates (1944). The process of transformation requires the previous uptake of plasmid DNA by the new host cells. To promote this process, a certain proportion of candidate host cells should

be made temporarily permeable to plasmid DNA. Permeability of the bacterial cell wall can be enhanced, e.g., by pretreatment of the bacteria with $CaCl_2$ Plasmid DNA should confer certain phenotypic properties (e.g., resistance to antibiotics; degradation of complex organic compounds; production of antibiotics, colicins, endotoxins, restriction and modification enzymes, etc.) upon the recipient cells, which make the selection of transformed host cells relatively easy.

The enzymes involved in the replication of plasmid DNA are identical to those participating in the duplication of the bacterial chromosome. A good vector is required to ensure the production of a high copy number in the recipient cell by its own replication. In contrast to plasmids under stringent control, the generation of a relatively high co, number (10-200 per cell) in the host can be only achieved with plasmids under relaxed control, meaning that the replication of plasmid DNA is not coupled to that of the host (for review see Novick et al. 1976). The copy number of a relaxed plasmid can be substantially increased by blocking the protein synthesis of the host, e.g., by chloramphenicol treatment (Clewell 1972). Under the condition of blocked protein synthesis, replication of the relaxed plasmids continues, whereas that of the chromosomal DNA, or of plasmids under stringent control ceases in the host cells.

To be a good vector, a plasmid should possess a number of specific properties: it should be relatively small in size; it should replicate in a relaxed fashion; it should possess a selectable marker(s) allowing identification of the transformants; it should have the ability to be maintained in the bacterial population; it should possess a recognition site for a restriction enzyme(s) in a region which is not involved in its replication. The foreign DNA segment is inserted into this restriction site which should be located preferably within the genes coding for the selectable marker(s), and thus the insertion of a foreign DNA will result in the inactivation of this gene. For more details on plasmid vectors and their required properties, the reader is instructed to read the review by Bernard and Helinski (1980).

Bacteriophage λ (phage λ) is a double stranded, linear DNA virus with a size of approximately 50 kb, which has a 12-nucleotide long complementary end ("sticky end" or "cohesive end"). Since the discovery of phage λ as a cloning vehicle, a large variety of vectors of this type has been constructed (for review see Williams and Blattner 1980). Although, the successful use of phage λ as a vector is hardly possible without a basic understanding of its molecular biology, the space available here prohibits even a superficial discussion of the topic. Thus, the interested reader should consult the pertinent literature for more detail.

Cosmids are vectors specifically designed for introducing and cloning large fragments of eukaryotic DNA, which could not be accomplished by λ type vectors due to their limited size capacity. The properties essential for a cosmid vector can be summarized as follows: (i) a small size (about 46 kb in length) allowing the accommodation of eukaryotic DNA fragments with a maximal length of 45 kb; (ii) a DNA fragment carrying the ligated "sticky end" site of phage λ ; (iii) a drug resistance marker and replication ability of plasmid origin; (iv) one or more specific restriction sites for coupling exogenous DNA fragments. As the cosmids presently available can only be used under a limited number of specialized conditions, plasmids and λ type vectors will probably continue to remain the vectors of choice in an overwhelming majority of cases due to their greater versatility and demonstrated effectiveness.

Single - stranded phages: The best single-stranded bacteriophage vectors are those originating from M13, a bacteriophage having a closed circular DNA genome with a size of about 25 kb. After its penetration into the host, the single stranded phage DNA is converted into a double-stranded replicative form which, following its isolation from the cells, can be used as a double stranded DNA cloning vector. After the replicative forms have reached a concentration of 100-200 per cell, the M13 phages start to produce only one of the two DNA strands which, after being incorporated into mature phage particles, are continually ex-

truded from the infected cells. The single-stranded DNA released in the phage particles is homologous to only one of the complementary strands of the cloned DNA, and thus can be used as template for DNA sequencing by the dideoxy method described by Sanger et al. (1977). M13 vectors can be used for sequencing of up to 350 bases from a single clone, or for the generation of single-stranded DNA probes used to select RNA, or as substrates for in vitro mutagenesis. Sequencing of longer stretches of DNA can be carried out by sequencing overlapping cloned DNA fragments. With the only exception of a 507 nucleotide region, termed the intergenic sequence, the entire M13 genome is essential for its replication. The intergenic sequence, however, is able to accept foreign DNA inserts without affecting phage viability, including replication.

(d) Rendering a foreign DNA fragment replicative in bacterial cells

As it was mentioned before, most foreign DNA fragments coding for some interesting proteins do not replicate spontaneously in bacterial cells. To make them replicative, they should be linked to a DNA molecule capable of intracellular replication, i.e., a vector. In theory, cloning and replication of a foreign DNA segment in appropriate bacteria, usually E. c o l i , is a very straightforward process, and can routinely be performed by anyone with training in sterile techniques. A common procedure includes the following steps (Fig.1.3).

Let us suppose that a double-stranded DNA segment has to be inserted into a plasmid DNA, and that the genome of the plasmid contains not only the replicating gene, but also a gene which provides the plasmid with resistance to tetracycline, a common antibiotic. As a first step, the circular plasmid DNA should be transformed into a linear form. This can be achieved by using one of the restriction nucleases, which cleaves the plasmid DNA at a region involved neither in replication nor in the antibiotic resistance of the plasmid. As a next step, polydeoxyguanidyl [poly(dG)] tails have to be at-

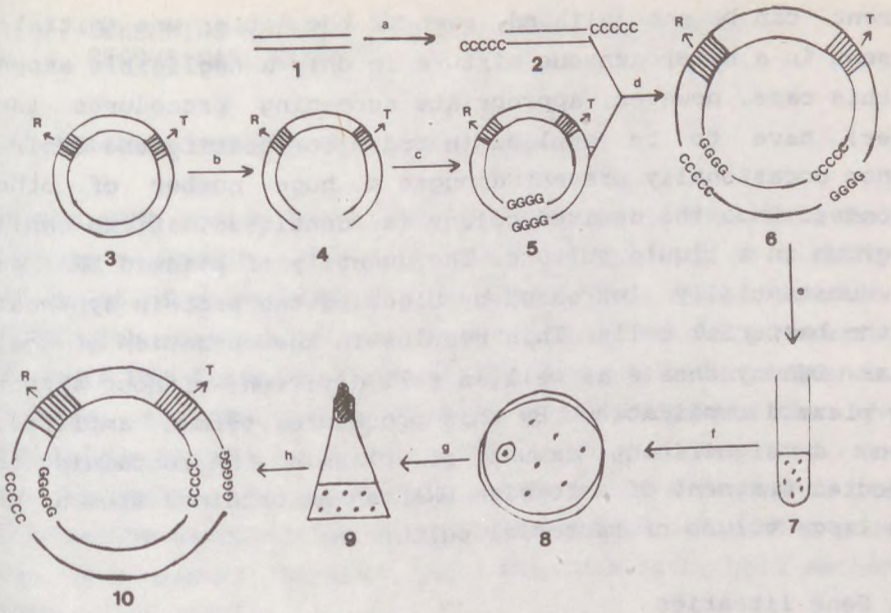


Fig. 1.3. Cloning and replication of a foreign DNA segment in E. c o l i with the aid of a plasmid vector. 1) foreign DNA segment; 2) foreign DNA elongated by poly(dC) tails; 3) circular plasmid; 4) linear plasmid; 5) linear plasmid with attached poly(dG) tails; 6) foreign DNA segment annealed to the plasmid vector; 7) calcium treated E. c o l i; 8) E. c o l i plated on dish containing tetracycline (TETR) to obtain TETR-resistant colonies; 9) single colony grown in liquid culture; 10) plasmid DNA containing foreign DNA insert; a) terminal transferase + dCTP; b) restriction nuclease; c) terminal transferase + dGTP; d) annealing; e) transformation; f) transfer of $CaCl_2$-treated E. c o l i to plating dish: g) transfer of a single colony to liquid culture; h) plasmid-DNA extraction

tached to the 3'-termini of the cleaved plasmid DNA using the enzyme terminal deoxynucleotidyl transferase. Simultaneously, polydeoxycytidyl [poly(dC)] tails are attached to the foreign DNA segment using terminal deoxynucleotidyl transferase again, and the DNA segment is annealed to the vector. The recombinant plasmid is now applied to $CaCl_2$ pretreated E. c o l i cells, and the latter are spread on an agar layer in a Petri dish containing tetracycline to prevent non-transformed E. c o l i from growing. The number of E. c o l i cells applied should be chosen so that each surviving (transformed) cell forms a separate colony, i.e., a clone. By such a cloning procedure, the isolation as well as the replication of a foreign DNA

segment can be accomplished, even if the latter was initially present in a heterogeneous mixture in only a negligible amount. In this case, however, appropriate screening procedures (see later) have to be applied in order to identify the desired clones occasionally present amongst a huge number of other colonies. Once the desired colony is identified, it can easily be grown in a liquid culture. The quantity of plasmid DNA can be substantially increased by blocking the protein synthesis of the bacterial cells. This results in the cessation of cellular DNA synthesis as well as cell division, without affecting plasmid replication. By this procedure, termed amplification, a significant amount of plasmid DNA containing the selected fragment of a foreign DNA can be obtained from a not too large volume of bacterial culture.

(e) Gene libraries

Vectors vary in size of inserted DNA that they can accomodate. Those with the largest capacities (cosmids) can accept up to 40,000 base pairs (40 kilobase pairs = 40 kb) of DNA. If the human haploid genome (approximately 3×10^6 kb of DNA) is broken randomly into fragments of 40 kb, it would take at least 7×10^4 independent clones to encompass all its sequences. Such a set of clones is referred to as a gene library or gene bank. In practice, a larger library is required to ensure that any single sequence has a high probability of being represented. To ensure a 99% probability that a single sequence of interest is represented in a human genomic library composed of 40 kb fragments, it is necessary to construct approximately 4x10 clones. Such expanded libraries are relatively easy to construct. The major difficulty lies in the identification of a particular clone of interest within any large library. To overcome this difficulty, various screening procedures have been developed.

1.6.2.2. SCREENING METHODS FOR THE IDENTIFICATION OF RECOMBINANT CLONES

(a) Restriction and DNA sequence analysis

If the mRNA coding for a selected protein is available in a mixture in a quantity which allows the preparation of a sufficient amount of complementary DNA (cDNA), then the digestion of cDNA with restriction nucleases may lead to the generation of discrete DNA fragments containing the desired sequence. Frequently, the considerable purity of the fragments thus obtained allows their direct and immediate restriction and sequence analysis. In possession of the correct fragment, its cloning can be accomplished without employing a screening procedure. Rat growth hormone has been cloned by this method (Seeburg et al. 1977).

(b) Hybridization and assay of the hybrid

Hybridization to mRNA, and subsequent assay of the hybrid in one of the translational systems is a method used when the amount of a given mRNA in a mixture is very low. In such cases, the presence of the sought mRNA can be detected by its activity in a cell-free translational system (e.g., reticulocyte lysate), or in a cell-bound system. Oocytes of the frog *Xenopus laevis* proved to be an excellent cell-bound translational system. Namely, during oogenesis in *Xenopus*, the major enzymes, organelles (e.g., ribosomes) and precursors (e.g., tRNAs) are present in a vast amount in the immature eggs (oocytes) as compared to the somatic cells of the frog. These maternal materials are used physiologically during the early development of the frog. However, these materials also preserve biological activity in vitro, thereby providing a sensitive test system for the translation of exogenous mRNA. In contrast to prokaryotic mRNAs, all eukaryotic mRNAs thus far tested were found to be able to direct the synthesis of the appropriate protein following their injection into living oocytes (for review see Lane and Knowland 1975).

In addition, the oocyte system not only translates mRNAs into appropriate proteins, but also modifies or matures the latter correctly. If the injected mRNAs code for secretory proteins, the newly synthesized protein product is also secreted from the oocytes (Colman et al. 1981). Thus, injected oocytes provide a complete system for the study of all events associated with protein synthesis and secretion. It is noteworthy that oocytes translate injected mRNAs much more efficiently than cell-free systems do.

The desired protein produced by any of the two translational systems mentioned above can be subsequently assayed by gel electrophoresis, immunoprecipitation, and/or bioassay. If a cloned DNA segment, or a mixture of segments containing sequences complementary to the mRNA studied is immobilized on a filter paper or on a column, the mRNA can be selectively bound to the immobilized DNA by hybridization, and, after elution, it will be able to direct the synthesis of the correct protein. DNA coding for interferon has been identified by such a method (Nagata el al. 1980).

A modified version of the systems described above is based on the observation that the synthesis of a protein is inhibited if the mRNA coding for this protein is converted to an RNA-DNA hybrid upon the addition of a cloned DNA fragment containing homologous sequences. The method, termed hybrid-arrested translation was introduced by Paterson et al. (1977).

(c) In situ hybridization of bacterial colonies

In situ hybridization of bacterial colonies or bacteriophage plaques is a powerful screening method described by Grunstein and Hogness (1975), which is preferably used when screening of a small number of colonies (100-200) dispersed over several agar plates is to be accomplished. The procedure is based on direct in situ hybridization of a radioactive nucleic acid probe to a denatured DNA from lysed bacterial colonies, or bacteriophage plaques. The transformed colonies dispersed over several agar plates are simultaneously consolidated onto a master agar plate and onto a nitrocellulose

filter laid on the surface of a second agar plate. After a certain period of growth, the colonies on the filter are lysed with alkali, which also denatures DNA. After neutralization, the denatured plasmid DNA is fixed (immobilized) to the filter by baking in vacuo, and the filters are incubated with a radioactive hybridization probe, either pure mRNA or pure denatured DNA fragment of a desired sequence. After thorough washing, the filters are monitored by autoradiography. The latter will reveal the colonies containing sequences able to base-pair with the radioactive probe.

In plaque hybridization (Benton and Davis 1977), the filter is applied to the surface of a plate containing bacteriophage plaques, so that there is a direct contact between the plaques and the filter. Molecules of unpacked bacteriophage DNA present in the plaque bind to the filter, and are hybridized as described above.

Application of the methods outlined above are limited to cases in which a homologous nucleic acid is available as a hybridization probe. Accordingly, cloned cDNA probes are frequently used to screen for the homologous chromosomal gene in populations of cellular DNA clones.

Although the use of synthetic oligonucleotides as hybridization probes presents some difficulties, hybridization of a synthetic oligonucleotide has been successfully used to determine the sequence of gastrin mRNA (Noyes et al. 1979). Instead of autoradiography, a solid phase radioimmunoassay, based on a colony blotting technique similar to that described above, can also be used to screen for bacterial clones which are synthesizing a desired protein under the direction of an exogenous gene (Brome and Gilbert 1978).

(d) Blot hybridization analysis

The combined use of gel electrophoresis and restriction endonuclease analysis allows a detailed and accurate physical mapping of a DNA molecule or chromosome. However, in order to construct a precise genetic map indicating the size and location of genetic coding region on this molecule, more informa-

tion is required. This information can be obtained by combining agarose gel electrophoresis with hybridization analysis by means of the blotting technique originally described by Southern (1975).

The fact that complementary single strands of DNA and RNA can anneal or hybridize to generate a homoduplex of DNA-DNA or a heteroduplex of DNA-RNA has been thoroughly documented. The finding that this hybridization can take place even when one of the two complementary strands is immobilized on a nitrocellulose filter serves for the conceptual basis of the Southern blot technique. In this method, restriction DNA fragments are denatured in the gel and blotted onto a sheet of nitrocellulose by elution or electrophoretic transfer in a manner that does not disturb their original pattern. After the single-stranded DNA is permanently bound to the nitrocellulose, the sheet is incubated in a solution containing radioactively labeled probe, i.e., complementary DNA or RNA. Once the homologous sequences have had time to anneal, the nitrocellulose is washed free of unhybridized probe and placed in contact with an X-ray film. The resulting autoradiograph will indicate which restriction fragments bear homology to the nucleotide sequence on the probe. The autoradiographic pattern can be compared to the ethidium bromide staining pattern to determine which restriction fragments correspond to coding region in the DNA.

It should be mentioned that the concept of blotting itself has been extended to include RNA and protein. These blotting procedures, now popularly termed Northern and Western blotting, respectively, differ from the Southern techniques in two principal ways. Northern blots involve the blotting of RNA onto diazotized paper where the RNA becomes permanently bound by covalent crosslinking. With Western blotting, proteins are separated electrophoretically, blotted and bound to nitrocellulose filters, and then challenged with radioactively labeled (^{125}I) antibody to one particular protein. Both procedures are excellently suited for establishing the presence and approximate size of a particular species of RNA or protein.

1.6.2.3. METHODS BY WHICH A DNA SEGMENT CODING FOR A DESIRED PROTEIN CAN BE OBTAINED

Specific segments of DNA coding for an interesting protein can be obtained by their direct isolation from native cellular or viral DNA, and by the template dependent or template independent synthesis of appropriate DNA segments.

(a) Isolation of specific DNA segments

As to the expression of specific DNA segments (genes) isolated from appropriate living sources, significant differences exist between the genes isolated from higher eukaryotes (e.g., mammals) and those obtained from prokaryotes or from lower eukaryotes (e.g., yeast). Namely, the coding sequences or exons of the genes in higher eukaryotes are usually interrupted by noncoding intervening sequences or introns, and the bacterial machinery is unable to express such genes. (Since, in the eukaryotic genome, the exons and introns are equally transcribed, the primary transcript will consist of both exon- and intron-derived RNA sequences, and only a subsequent event, termed processing ensures formation of the authentic mRNA molecule by eliminating intron-derived sequences and joining exon-derived sequences.) Moreover, the fact that a single gene in a higher eukaryotic cell may represent an infinitesimally small proportion of all DNA sequences constituting the genome presents further difficulties due to the need for a powerful screening procedure and a considerable effort to isolate such genes. On the other hand, prokaryotic genes in general, or the genes coding for nuclear proteins in lower eukaryotes do not have intervening sequences, and so they can be expressed relatively easily in bacteria. In addition, several genes of the eukaryotic DNA viruses, for example those isolated from simian virus 40 (SV40) and hepatitis B viruses, which contain sequences coding for viral proteins proved to be exceptions to the general rule, and could be expressed in E. c o l i due to the lack of introns in the DNA segment to be expressed (Burell et al. 1979, Pasek et al. 1979, Roberts et al. 1979).

(b) Template dependent enzymic synthesis of complementary DNA

The most commonly used procedure of this type utilizes eukaryotic mRNA as a template (for review see Williams 1981). As a first step, synthesis of the first DNA strand is performed with the aid of the enzyme reverse transcriptase (RNA-dependent DNA polymerase) resulting in a RNA-DNA hybrid. The reaction needs a priming by oligodeoxythymidylic acid which anneals to the 3'-terminal poly(A) of the mRNA. The RNA template is subsequently removed by alkaline degradation, and now the freshly synthesized single-stranded DNA serves as a template for the synthesis of the second strand of DNA. This step is catalysed by E. c o l i DNA polymerase I. The latter uses the 3'-end of the single-stranded DNA template as a primer in a self-primed reaction, eliminating the need for the addition of a primer oligonucleotide. Finally, the loop connecting the first and the second DNA strands is to be opened with the aid of the single-strand-specific nuclease S1, and the double-stranded DNA segment is then usually cloned by the homopolymer tailing method as outlined earlier in this paragraph. The DNA segment thus cloned is commonly referred to as complementary DNA (cDNA). The procedure is outlined schematically in Fig.1.4.

It is important to note that cDNA segments of eukaryotic origin, by being derived from mRNA, do not contain intervening sequences. Thus, they can be expressed in bacteria without considerable difficulties, provided they are preceded by appropriate starting signals for transcription and translation.

Various mRNAs are copied into the appropriate DNA with different efficiencies. Conditions that are optimal for copying one species of mRNA may prove not to be so good for another. In general, when one deals with heterogeneous populations of mRNA, conditions safeguarding the greatest overall yield of cDNA are recommended. Optimization of the conditions for producing appropriate cDNA transcripts have been published in a number of papers (Efstratiadis et al. 1976, Buell et al. 1978, Retzel et al. 1980).

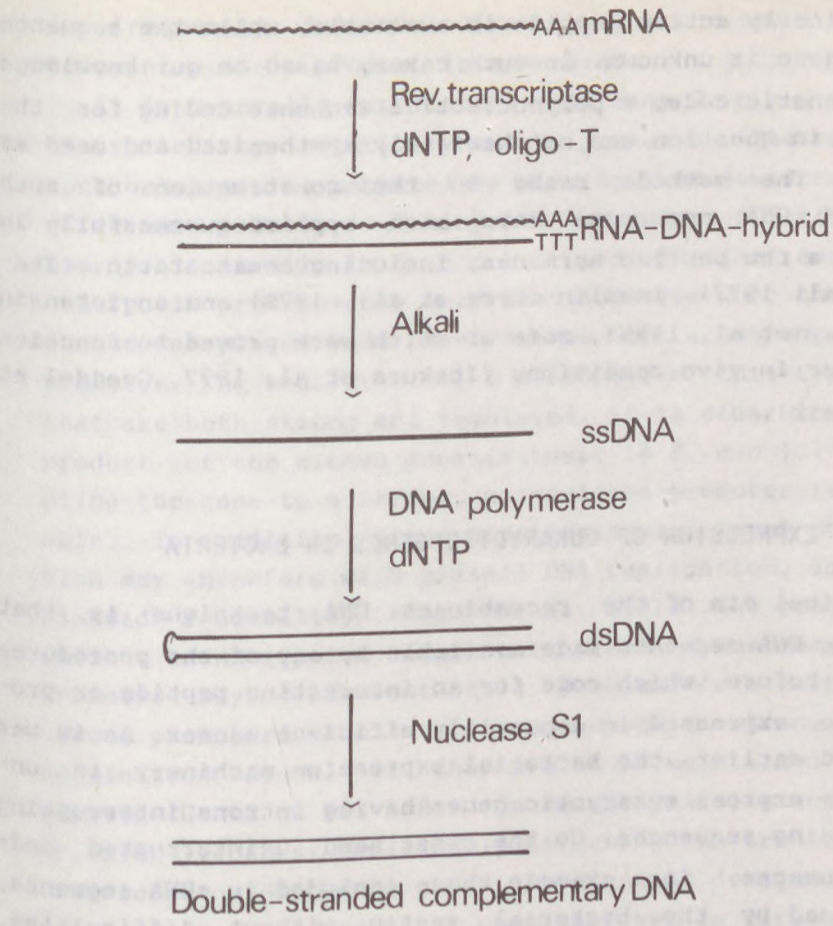


Fig. 1.4. Preparation of double-stranded DNA complementary to a messenger RNA. See text for explanation. mRNA=messenger RNA; dNTP=deoxy-nucleoside triphosphate; oligo-T=oligo-thymidylic acid; ss=single-stranded; ds=double-stranded

(c) Template independent synthesis of specific DNA segments

These methods utilize the techniques presently available for the laboratory synthesis of DNA coding sequences. The synthetic procedures play an important role in several fields of genetic engineering. Until recently, the most excellent results have been achieved in the synthesis of short coding sequences. It may often occur that the amino acid sequence of

a biologically active peptide is available, while the sequence of its gene is unknown. In such cases, based on our knowledge on the genetic code, a polynucleotide sequence coding for the peptide in question can be chemically synthesized and used as a probe. The methods based on the construction of such "derived" DNA sequences have been applied successfully in cases of a few peptide hormones, including somatostatin (Itakura et al. 1977), insulin (Crea et al., 1978) and angiotensin II (Koster et al. 1975), some of which were proved to function also under in vivo conditions (Itakura et al. 1977, Goeddel et al. 1979a,b).

1.6.2.4. EXPRESSION OF EUKARYOTIC GENES IN BACTERIA

The final aim of the recombinant DNA technique is that specific DNA segments made available by any of the procedures outlined before, which code for an interesting peptide or protein, be expressed in a possibly efficient manner. As it was mentioned earlier, the bacterial expression machinery is unable to express eukaryotic genes having introns interrupting their coding sequences. On the other hand, uninterrupted coding sequences, for example those included in cDNA segments, can be read by the bacterial system without difficulties. However, even in such cases, the provision of appropriate initiation signals of bacterial origin is necessary for successful transcription and translation. In order to provide the system with such signals, eukaryotic genes have to be attached to expression vectors, DNA sequences containing promoters and translation initiation signals originating from bacterial genes. Promoters are DNA sequences that direct RNA polymerase to bind to DNA, and to initiate RNA synthesis. Strong promoters cause the initiation of mRNAs at high frequency, whereas weak promoters direct the synthesis of rarer transcripts. The only true test of the efficiency of a promoter is to measure the frequency with which the synthesis of the appropriate mRNA is initiated. Because this value is difficult to obtain from in vivo studies, the efficiency of a promoter

is frequently deduced indirectly from the level at which the relevant protein product is expressed.

It is generally experienced that many E. c o l i genes are controlled by relatively weak promoters. The expression of such genes can be enhanced by placing them downstream from an efficient promoter. Eukaryotic promoters function extremely poorly, if at all, in E. c o l i, and efficient expression of eukaryotic proteins has only been achieved when the coding sequence was placed under the control of a strong E. c o l i promoter. The best E. c o l i promoters of this type are those that are both strong and regulated. It is clear that, if the product of the cloned gene is toxic to E. c o l i , then coupling the gene to a strong, unregulated promoter is not desirable. In addition, a constitutively high level of transcription may interfere with plasmid DNA replication, and lead to plasmid instability (Remaut et al. 1981). The presence of efficient transcription terminators placed downstream from the promoter may circumvent this problem. As a matter of fact, strong promoters require the presence of a strong downstream termination signal if they are to be maintained stably on a plasmid (Gentz et al. 1981). In recent years, a number of expression vectors efficiently directing the transcription and translocation of cloned genes have been designed. Although several strong promoters were employed in various laboratories, no single method has been used to compare their efficiencies. Thus, the choice among promoters remains somewhat arbitrary at present.

Utilization of the bacterial gene for β-lactamase provides the basis of a relatively simple procedure, by which eukaryotic genes can be attached to DNA segments containing promoters and translational initiation signals of bacterial origin. This gene is responsible for the resistance to the antibiotic ampicillin, and is also a part of several vectors, including the most frequently used plasmids. There is a cleavage site for a restriction endonuclease (Pst I) within the gene which can easily be used for the insertion of an exogenous gene. The latter is usually done by the homopolymer tailing method outlined in Fig. 1.1. Although the β-lactamase gene is inactivat-

ed as a result of insertion of the foreign gene, continuation of plasmid selection remains ensured due to the presence of another gene segment responsible for the resistance to tetracycline, another antibiotic. Foreign DNA segments inserted into the β-lactamase gene will be copiously transcribed from the β-lactamase promoter. As a consequence, and if the insertion occurred in the correct orientation, the transcripts thus generated will consist of mRNA hybrids containing the mRNA sequences coding for the foreign protein and the β-lactamase mRNA sequences. The hybrid mRNA will actively synthesize protein in vivo. Construction of the protein products will depend on several factors. If ribosomes initiate exclusively at the β-lactamase initiation site, hybrid protein products will be generated, and correct translation of the foreign gene will occur only on transcripts in which the foreign mRNAs are linked to β-lactamase mRNAs in the appropriate reading frame. As the homopolymer tailing method results in a statistical distribution of reading frames, and the probability of the correct orientation of the foreign gene is 50%, not more than about 20% of the plasmids constructed by this method can be expected to express the foreign gene. The product is expected to be a fused protein consisting of the foreign protein sequence linked at its N-terminal to the N-terminal part of β-lactamase via an oligo-glycine bridge. Isolation and characterization of such products were, in fact, reported, e.g., in the case of the proinsulin gene (Villa-Komaroff et al. 1978). However, protein products lacking β-lactamase sequences have also been described, indicating that the initiation of protein synthesis may have taken place at initiation sites located on the eukaryotic mRNA sequence (Chang et al. 1978, Pasek et al. 1979). Essentially the same principle was employed in experiments which resulted in the expression of both somatostatin and insulin. In these cases, however, the foreign inserts were fused to the β-galactosidase gene, a part of the lactose operon. Both peptide hormones were successfully removed from the fused protein by chemical cleavage (Itakura et al. 1977, Goeddel et al. 1979b). The lactose promoter was also used for the expression of human growth hormone (Goeddel et al. 1979a) and the SV40 t-antigen (Roberts et al. 1979). In

these cases, however, the formation of a fused protein was avoided by linking the initiator triplet of the foreign coding sequence directly to the region controlling the initiation of protein synthesis for β-galactosidase, thereby creating a hybrid ribosomal initiation site.

If the native protein is unstable in E. c o l i, the expression of the gene product as part of a fusion protein may be desirable, especially if the native protein can be chemically cleaved from the purified fusion protein, as it was the case with both somatostatin (Itakura et al. 1977) and insulin (Goeddel et al. 1979b). The same applies for cases when the protein is to be used to elicit the production of antibodies (Kleid et al. 1981). Alternatively, some proteins may be stabilized by their large-scale synthesis in E. c o l i (Shimatake and Rosenberg 1981).

Expression vectors allowing the fusion of foreign genes to DNA encoding a signal sequence may be useful for transporting proteins out of the cytoplasm, especially if the signal peptide is cleaved during protein exportation. Export of the proteins may also be helpful in subsequent purification, and may also allow their isolation from cytoplasmic proteases.

To achieve high levels of expression in E. c o l i, it is necessary to use, not only strong promoters ensuring an abundant generation of mRNA, but also ribosome-binding site to ensure that the mRNA is efficiently translated. In E. c o l i, the ribosome-binding site includes an initiation codon and the so-called Shine-Dalgarno, or SD sequence, which is complementary to the 3'-end of E. c o l i 16S rRNA (Shine and Dalgarno 1975). Binding of the ribosome to mRNA is suspected to be promoted by base-pairing between the SD sequence in the mRNA and the sequence at the 3' end of the 16S rRNA.

In most cases, however, the expression levels of eukaryotic genes in E. c o l i is considerably lower than expected. Although some of the factors which influence the expression levels have been determined, conditions necessary for optimization of expression have not been generalized, and thus each new protein poses a different problem at present. These problems now are the subject of intensive investigation.

1.6.2.5. SITE DIRECTED MUTAGENESIS AS A TOOL FOR CHANGING THE PRIMARY STRUCTURE OF A PROTEIN

The artificial variation of the amino acid sequence of peptides and proteins is widely used in studies concerned with the investigation of structure-activity relationships. Such variations may occasionally yield a variant having more favourable (e.g., therapeutic) properties than the original molecule. In cases of proteins the genes of which are available by genetic engineering techniques, such variation in sequence can be achieved, at least in principle, by artificial introduction of specific mutation into the coding DNA sequence. This can be done, for example by enzymic introduction of a mutagenic analogue of deoxyribonucleoside triphosphate (e.g., N^4-hydroxy-cytosine-deoxyriboside triphosphate) at specific sites on the DNA. By this method, a number of variants of cloned rabbit β-globin cDNA have successfully been produced. It has been demonstrated that specific mutation can also be achieved by using synthetic oligonucleotides. (Hutschison et al. 1978, Kossel et al. 1978, Razin et al. 1978).

It is now clear that presently, the production of EPs and proteins by recombinant DNA techniques in bacteria have become a realistic alternative to the conventional techniques of isolation from biological sources, or chemical synthesis. It is hardly questionable that, in a short time, a considerable number of products will become available by this route, many of which (e.g., hormones, interferons, vaccines, antibodies, enzymes) may turn out to be not only of scientific, but also of therapeutic or commercial interest. However, due to the numerous difficulties that should be overcome before genetic engineering techniques can be routinely used, the methods of isolation as well as chemical synthesis and semisynthesis will certainly continue to be used for the production of interesting peptides, as well as of peptides containing unusual amino acids.

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CHAPTER 2

THE RECEPTORS: APPLICATION TO PEPTIDE LIGANDS

A.Z. RONAI

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2.1. THE RECEPTOR MODEL: CHANGING CONCEPTS

The receptors are constituents of a sophisticated machinery subserving intercellular communication as well as receiving chemical messages from the outer environment. The signal carrying substances (first messengers, e.g., hormones, neuroregulators, growth factors, etc.), as they reach the cells which have specialized receivers for the message, induce a complex cellular response in the target cell.

The term receptor, as used by a biologist, may cover the entire assembly of "addressed" cell constituents involved in primary signal transduction. A biochemist would much rather restrict the receptor designation to the ligand binding unit, which, in several instances, is distinct from, and even may not be permanently coupled to, the signal transducing unit (see below). This stems historically from technical reasons: when a biologist/pharmacologist of the past attempted to characterize receptor-mediated processes he/she did so by using drug-induced whole organ/organism or tissue responses as an indicator. Technical progress made possible to study the various phases of receptor-linked events separately: thus, the simple term "receptor-mediated cell response" was split into "ligand binding", "signal transducing/modulating" and "effector" steps (Ariens et al. 1979). When it became possible to measure the binding process separately, a distinction had to be made between "receptors" and "acceptors" (Ariens 1984). The term "acceptor" applies to binding sites that have not been shown to be linked to a response: when a response-generating

mechanism is detected the receptor status of such a site should be considered. The classical model considered the receptor as a rather stationary complex. A closer insight into the signal transducing mechanisms altered drastically this attitude. The discovery of receptor-regulated cAMP-generating machinery (Sutherland and Rall 1960, Sutherland et al. 1965, De Lean et al. 1980, Levitzki 1982) stimulated the recognition of other, receptor-controlled second messenger-producing systems (for review see Lichtstein and Rodbard 1987). A further cornerstone was the finding and characterization of guanine nucleotide-binding regulatory proteins (for review see Graziano and Gilman 1987). The number of receptors recognized as constituents of an ion-channel forming macromolecular complex, is also growing. Furthermore, there is a class of transductive systems that depends on the internalization of the primary signalling substance, i.e., although signal transduction in such systems may also involve changes at the memebrane surface, the process remains incomplete until the primary messenger and/or its receptor reach targets within the cell itself (Carpenter and Cohen 1976, Davies et al. 1980, King and Cuatrecasas 1981, Schlessinger 1981, Cuatrecasas 1982, Walaas and Horn 1982). Further evidence suggests that the cytoskeleton may modulate cell surface properties and thereby receptor function, and is perhaps even the primary target for some signalling processes (Fowler and Branton 1977, Golan and Veatch 1980, Edelman 1982, Steck 1982). Thus, the emerging evidence suggests that the transductive process may be more complex than previously thought.

The theoretical and practical significance of two further phenomena, receptor adaptation and receptor heterogeneity, has received distinguished attention in the recent years. It has been recognized that the receptor machinery is not a static assembly of functionally connected units but its function is subject to regulation. This regulatory process may be induced by its own ligands (homologous regulation) or by other factors (heterologous regulation) (Su et al. 1976, for review see Hollenberg 1981). Thus the encounter of the receptor with the ligand would result not only in the primary processes triggering

cellular response, but would also initiate events producing short- or long-term alterations in the binding and/or transductive properties of the receptor itself. (Strittmatter et al. 1979, Chuang et al. 1980, Daly et al. 1980, for review see Hoffman and Lefkowitz 1980, Creese and Sibley 1981, Hollenberg 1981).

The concept that receptor types exist, is now widespread (Snyder and Goodman 1980, Molinoff et al. 1981). It appears to be a general rule that there are multiple types of receptors for each of the many biological active endogenous substances (hormones, neuroregulators, etc.) in the organism. Much work has to be done until the relevant properties of each receptor type will be found, which are conserved from one tissue/or function to the next, and will make the receptor subclassification meaningful.

The receptor-related knowledge was and is still intended to be surveyed mostly in context of neurotransmitter/modulator and hormone receptors for several reasons. First of all because information is most abundant in this field. Furthermore, the basic principles on neurotransmitter/modulator and hormone receptors are mostly valid also for other receptors. Last but not least, this predilection was favored by the authors' expertise and interest.

2.2. THE FUNCTIONAL ANATOMY OF RECEPTORS: CELL MEMBRANE

According to the fluid mosaic model (Singer and Nicholson 1972) the structural determinants in the plasma membrane are composed of asymmetric phospholipid bilayers. The membrane proteins are also asymmetrically disposed. They can be embedded within the lipid bilayer, at times penetrating the membrane completely. These proteins are firmly bound to membranes and may be surrounded by shell of immobilized lipid known as boundary lipid or lipid annulus (Singer and Nicholson 1972, Singer 1974). Thus, they are called integral, intrinsic or endomembrane proteins (Singer 1974). The second type of proteins is located at the surface of membranes; they are weakly bound

by electrostatic forces or by intermediate divalent cations. They interact with the integral proteins rather than with the lipid bilayer (Singer and Nicholson 1972, Singer 1974). These proteins are called peripheral, extrinsic or exomembrane proteins.

The cytoskeleton (the term refers to groups of self-assembling filamentous protein arrays), besides providing dynamic intracellular compartmentation and determining cell shape (Lasek 1981), serves not only as a mechanical support for the plasma membrane but may interact with integral membrane components as well (Bennett and Stenbuck 1979) and can influence membrane function in many other ways (Steck 1982).

The recognition sites for the majority of biologically active signalling substances (first messengers), are located in the plasma membrane (Singer 1974, De Robertis 1976, Tanford 1978, Laduron and Ilien 1982). The elements for the complete receptor function (i.e., for all steps of primary signal transduction) may be present in the membrane, while in other cases the process remains incomplete until the messenger and/or its binding substance reach targets within the cell itself. The known examples of the latter class of transductive systems that depend on the actual internalization of primary signalling substance are the insulin-, epidermal growth factor-, and nerve growth factor-receptor systems (Carpenter and Cohen 1976, King and Cuatrecasas 1981, Cuatrecasas 1982). To discuss the properties of intracellular receptors such as the ones for the steroid hormones is beyond the scope of this overview.

The consensus is that the recognition sites of receptors consist mainly of large oligomeric proteins (Hollenberg 1981). It is also widely accepted that most of the membrane proteins (70-80%), thus, the receptor proteins, are easily recognized as intrinsic, integral or transmembrane proteins (Singer 1974, Tanford 1978, Laduron and Ilien 1982). A good example of transmembrane topography is the nicotinic cholinergic receptor from T o r p e d o (Wennogle and Changeux 1980, Taylor and Sine 1982).

The contribution of nonprotein moieties to ligand recognition (Loh and Law 1980) and signal transduction (Rimon et al. 1978, Axelrod 1982, Helmreich and Rodbell 1982, Michell 1982) is also acknowledged. Thus, membrane lipids, tightly receptor-associated via noncovalent mechanisms may serve as recognition sites (Loh and Law 1980). Furthermore, the lipids that surround the receptor molecules (the lipid annulus of the receptor) may modulate the receptor's three-dimensional structure and thereby regulate the ligand affinity for the receptor (Loh and Law 1980). Membrane phospholipids, partly via transmethylation mechanisms (Hirata et al. 1979, Axelrod 1982) may participate directly in signal transduction (Michell 1982). Finally, the lipid domain in which the receptor is situated regulates the lateral mobility of the receptor by determining membrane fluidity; this regulatory mechanism gains special significance in the transduction of signal in receptor-cyclase systems (Rimon et al. 1978, Helmreich and Rodbell 1982).

2.3. RECEPTOR FUNCTIONS

Before discussing receptor functions some terms and principles which are instrumental in understanding receptor-related phenomena should be clarified. Some of these terms have already been used deliberately in previous sections of this chapter; others are to be mentioned later.

Ligands are all the chemical substances that are recognized by and thus are capable of interacting specifically with a receptor. The ligands may be of natural or synthetic origin; the natural ligands which are generated within the organism that contains the receptor in question are designated as endogenous ones. The ligand which binds specifically to the receptor and contains the information that enables it to trigger a chain of events leading to the biological response is designated as an agonist. The ligand which has the capability of binding only to the same site as the agonist, but cannot initiate a biological response is a competitive antagonist. It can prevent or reverse the action of an agonist by competing for the very

same binding site; otherwise, in the absence of an agonist, the binding of a pure competitive antagonist to the receptor is biologically "silent". The so-called "purity" of the antagonist character, which may be present in nonendogenous ligands, is always a relative one, and may not be preserved as such from one test system to the other; thus, when speaking of an antagonist, the biological system and the conditions whereby this property was characterized, must be strictly defined. It is the author's opinion, which may be not shared by others, that assuming the existence of endogenous competitive receptor antagonists makes no sense biologically at least not under nonpathological conditions. The occasional reports on endogenous antagonists of certain receptors such as the opioid (Margules, 1979, 1984, Kaneko et al. 1985) or benzodiazepine (Costa 1982) receptors should be considered with caution.

Recently, a new category of receptor ligands, namely the inverse agonists have been introduced in the context of benzodiazepine receptors (Haefely 1984). It implicates that on the very same receptor, events leading to opposite biological responses can be triggered by agonists and inverse agonists, respectively.

Besides the site directly involved in triggering the biological response, the receptor may contain other specific binding site(s). Ligand binding to these sites can regulate allosterically the binding and/or triggering events at "the" receptor. These allosteric sites are seldom referred to as receptors; a noteworthy exception is the case of benzodiazepine receptors, which, in fact, are allosterically related to the GABA receptor-Cl^- ion channel complex (Haefely 1984).

Usually, the receptors are named after their characteristic endogenous ligand (e.g., dopamine, acetylcholine; Green 1987). If these endogenous ligands are not identified yet, the most characteristic natural or synthetic ligand class may lend the name (e.g., opiate or benzodiazepine receptors). If, however, the endogenous ligands become known, their name originally given after an opium alkaloid should be altered accordingly (i.e., opioid rather than opiate is the proper designation; Goldstein and Kosterlitz 1983).

2.3.1. LIGAND BINDING

Ligand binding involves the interaction of a chemical susbstance with a highly specialized domain on the target cell. The interaction is the result of the discriminative recognition of the information contained within a part of the ligand molecule (termed as haptophore or address; Ariens et al. 1979, Schwyzer 1980a,b) by chemically matching entities of the specialized target cell domain. These entities can be referred to as the receptor.

Recognition is offered primarily by a sterically proper arrangement of polar groups in the ligand involving point to point interaction with the receptor (Ariens et al. 1979). The region of ligand molecule providing hydrophobic interaction may also contribute to the recognition; the hydrophobic interactions are factors of primary importance in the high-affinity binding of antagonists (Ariens et al. 1979). The interacting moieties of receptors were mapped mostly by studying the structure-activity relationships of natural and synthetic ligands, as it was the case for the ACTH (Schwyzer 1980 a,b) and opioid receptors (Beckett and Casy 1954, Beckett et al. 1956, Feinberg et al. 1976, Galt 1977), adrenoceptors (Ariens et al. 1979, Unger et al. 1980), acetylcholine, histamine and serotonin receptors (Ariens et al. 1979). In fact, the rational design of synthetic ligands involves a speculative fitting of ligand structure to the putative interacting counterparts at the receptor (e.g., Bajusz et al. 1976).

2.3.2. FUNCTIONAL CONSEQUENCES OF LIGAND BINDING

2.3.2.1. PRIMARY CONSEQUENCE OF (AGONIST) LIGAND BINDING: SIGNAL TRANSMISSION

The binding of an agonist ligand to the receptor initiates a chain of events leading to the cellular response (N.B. this can be taken as a definition of agonist type ligand). The information that triggers this process must be coded by the ligand structure; the structural moieties involved in receptor activation represent the "actophore" (Ariens et al. 1979) or "message" (Schwyzer 1980a,b) domain of ligand molecule. The agonist-induced first stimulus might be the direct alteration of the permeability of an ion channel (Taylor and Sine 1982), or a change in the activity of an enzyme (reviewed by Hollenberg 1981, 1982, Cuatrecasas 1982, Walaas and Horn 1982). The receptor-linked enzymes may generate so-called "second messengers" upon agonist ("first messenger") binding (for review see Lichtenstein and Rodbard 1987). Finally, if the agonist must reach an intracellular target to initiate the next step, the membrane receptor serves as a sort of carrier; one possible intracellular event is the production of a second messenger in this case, too (Cuatrecasas 1982, Walaas and Horn 1982). It should be mentioned that the very same first messenger, acting at various receptor types, may initiate the cellular response by different mechanisms (Roth and Chuang 1987).

2.3.2.1.1. SIGNAL TRANSMISSION BY DIRECT CONTROL OF ION CHANNEL PERMEABILITY

The best known examples are the nicotinic cholinergic receptor, where the assembly of tightly connected subunits of receptor macromolecule forms the perimeter of the ion channel subserving sodium entry (Taylor and Sine 1982) and the $BABA_A$ receptor, which is known to control chloride ion channel permeability (Obata et al. 1967, Gold and Martin 1984, McBurney

1984); constituents of the latter receptor complex might be assembled similarly to the pattern verified for the nicotinic cholinergic receptor (Haefely 1984).

It is unlikely that too many peptide receptors, if any, would be found in such a direct connection with an ion channel as the ones mentioned above. It is not surprising since very few of the nonpeptide neurotransmitter receptors, namely, the ones capable of transmitting fast, sharp signals (nicotinic cholinergic, $GABA_A$, glycine and excitatory amino acid receptors) are known to be coupled directly to ion channels.

However, quite a few peptide receptors were shown to influence ion conductance mechanisms though less directly, but, as far as it is assumed to be the case at present, without the intervention of second messenger generating systems (see next paragraph).

A functional coupling between opioid receptors (μ, κ and δ types) and calcium conductance reduction (Illes 1982, Dougall and Leff 1987, Shimahara and Icard-Liepkins 1987) as well as potassium conductance increase, (μ and δ receptor types; Williams et al. 1982, North et al. 1987) has been shown to exist. μ and δ opioid receptors appear to belong to a family of receptors that are coupled to potassium channels via guanine-nucleotide binding regulatory protein (G_κ) but, apparently, without the intervention of a soluble second messenger (North et al. 1987, see also next paragraph). It should be mentioned that other tentative explanations have also been offered to explain the action of opioid agonists on K^+-conductance (Shimahara and Icard-Liepkins 1987). Somatostatin receptor-mediated stimulation of K^+-channels was also shown to be conveyed by G_κ protein (Yatani et al. 1987) but without the participation of known second messenger-generating systems.

The so-called σ receptor which has been "expelled" from the opioid receptor family, appears to be allosterically related to one of the cation-channel controlling excitatory amino acid receptors (of NMDA type, Anis et al. 1983, for review see Winger 1987). This receptor, designated now as σ/PCP receptor is likely to possess an endogenous, probably peptide, ligand (Quirion et al. 1984, Zukin et al. 1987).

2.3.2.1.2. SIGNAL TRANSMISSION BY MECHANISMS COUPLED TO SECOND MESSENGER GENERATING SYSTEMS

Upon the discovery of cyclic AMP (Sutherland and Rall 1957) and cyclic GMP (Ashman et al. 1963) several experimental results have been used to conclude that cyclic nucleotides may function as intracellular mediators of hormone action (Sutherland and Rall 1960). Thus, Sutherland and his coworkers (1965) put forth the generalized concept of second messengers in hormone action.

According to the original proposal, the interaction of non-penetrating humoral regulating substances (first messengers) with their receptors would bring into action effector systems in the plasma membrane resulting in the elaboration of intracellular mediators (second messengers). Very few (if any) modifications should be made in the wording of the concept to cover the most recent developments; it should be added perhaps that internalized hormone/receptor complexes may also act via second messengers (Cuatrecasas 1982, Walaas and Horn 1982).

As opposed to the several hundred first messengers of diverse structure and their respective receptors, the number of known second messengers - cyclic nucleotides, members of phosphoinositide pathway, arachidonic acid and its metabolites and, perhaps, intracellular calcium - is surprisingly modest (for review see Lichtstein and Rodbard 1987, Axelrod et al. 1988). Ca^{2+} is much more like a "third" rather than a "second" messenger candidate (Putney 1987); arachidonic acid as well as some of the arachidonate metabolites can act also as first messengers (Lichtstein and Rodbard 1987, Axelrod et al. 1988).

The main features of receptor-regulated second messenger-generating systems have been delineated first in context with the cyclic AMP-producing machinery (De Lean et al. 1980, Helmreich and Rodbell 1982, Levitzki 1982, Lefkowitz et al. 1984). In general, the receptors are coupled to the various effectors (adenylate cyclase, cGMP, phosphodiesterase, phospholipase C, phospholipase A_2 etc.) manufacturing the actual second messengers by the intervention of different types of guanine nucleotide binding regulatory proteins (G proteins;

Graziano and Gilman 1987, Lichtstein and Rodbard 1987, Axelrod et al. 1988). These G proteins, designated originally as "N unit" or "G/F protein" in context with the adenylate cyclase system (Sternweis et al. 1981) appear to play a key role in signal transduction. The list of peptide- and nonpeptide receptors which are coupled to second messenger-generating mechanism is extensive (Jakobs and Schultz 1980, Pert and Taylor 1980, Schwyzer 1980a,b, Costa 1982, Helmreich and Rodbell 1982, Levitzki 1982, Iversen 1984, Graziano and Gilman 1987, Putney 1987, Axelrod et al. 1988).

The second messengers often activate protein kinases which lead to the phosphorylation of one or more specific enzymes or other proteins (Huganir and Greengard 1987, Lichtstein and Rodbard 1987).

2.3.2.2. CONTROL OF RECEPTOR RESPONSIVENESS: HOMOLOGOUS AND HETEROLOGOUS RECEPTOR REGULATION

The responsiveness of the cell to signalling molecules may be regulated at several levels; of these, the control can take place at the level of receptors. The phenomenon has long been known; if the ultimate result was an enhanced cellular response, hypersensitivity or supersensitivity, in the opposite direction hyposensitivity, desensitization, tachyphylaxis or tolerance was the word used to denote it. As it turned out in many instances, this phenomenon constituted the functional basis of some pathological processes (Olsen et al. 1980, Lefkowitz et al. 1984), therapeutic and/or unwanted drug effects (Creese and Sibley 1981) and also certain physiological regulatory mechanisms like the circadian and seasonal variations in cell responses (Kafka et al. 1981). The nomenclature used recently is not quite unitary. Some investigators (e.g., Olsen et al. 1980) use the term "up regulation" in the collective sense, i.e., for all the possible functional variations when the change in receptor state (number of binding sites in unit of tissue sample, ligand affinity, coupling to the effector and effector function) is such as to lead to enhanced cel-

lular response; if the opposite happens, the term "down regulation" serves to denote the process. Others (e.g., Lefkowitz et al. 1984) appear to restrict the "up" or "down" regulation term for cases when the change involves the actual number of binding sites. The changes in the receptor function may be induced by the ligands of respective receptors or by other cellular effectors including ligands of other receptors. According to the categorization applied by Su et al. (1976) to the phenomenon of desensitization, homologous regulation should denote the former, whereas heterologous regulation the latter process (rev. by Hollenberg 1981, 1985a,b,c).

2.3.2.2.1. HOMOLOGOUS RECEPTOR REGULATION

H o m o l o g o u s "u p" r e g u l a t i o n. Binding of β-adrenoceptor agonists to their receptors in rat reticulocyte ghosts may unmask more β-receptors in the membrane probably via methylation and translocation of membrane phospholipids (Strittmatter et al. 1979, Axelrod 1982). This process amplifies the initial binding event and could presumably lead to further cyclase activation. As another example, prolactin appears also to cause an "up" regulation of its own receptors (cf. Hollenberg 1981).

H o m o l o g o u s "d o w n" r e g u l a t i o n. It has long been known that long- or even short-term exposure to high concentrations of the agonist usually induces a desensitization in many receptor systems (Daly et al. 1980, Hoffman and Lefkowitz 1980, Hollenberg 1981, 1985a, Axelrod 1982). In cyclase-coupled receptor systems where the phenomenon was analyzed most extensively, two types of desensitization were differentiated.

In the case of short-term desensitization, as it has been demonstrated e.g., for β-adrenergic and muscarinic cholinergic receptors, there is a rapid fall in the agonist-induced cellular response without a similar change in the overall binding capacity of receptors for antagonist ligands (Harden et al. 1979, El-Fakhany and Richelson 1980a,b, Hoffman and Lefkowitz

1980). Thus, the receptor is uncoupled from more distal components of signal transduction system (Harden et al. 1979) The uncoupled receptors will not bind agonists with high affinity (Kent et al. 1980).

Long-term desensitization is accompanied by an actual loss of receptor binding capacity, which could be demonstrated for both agonist and antagonist binding (Hoffman and Lefkowitz 1980, Kent et al. 1980, Shifrin and Klein 1980, Hollenberg 1981), naturally the cellular responses to agonists are also reduced. Receptor internalization was proposed as the most probable mechanism to account for the loss of binding sites (Chuang et al. 1980).

2.3.2.2.2. HETEROLOGOUS RECEPTOR REGULATION

Up to now several examples of heterologous regulatory interaction have been given (for review see Hoffman and Lefkowitz 1980, Hollenberg 1981, 1985a). Both heterologous "up" and "down" regulations were shown to occur upon the interaction of epidermal growth factor, platelet-derived growth factor and fibroblast growth factor receptor (Wrann et al. 1980). Muscarinic cholinergic receptor stimulation can increase the affinity of α_1-adrenoceptor for its antagonist ligand in rat cardiac tissue (Yamada et al. 1980). Insulin is capable of enhancing the binding of insulin-like growth factors, multiplication stimulating activity and basic somatomedin by rat adipocytes (Hollenberg 1981). Thyroid hormones appear to participate in the regulation of adrenoceptors (Hoffman and Lefkowitz, 1980). A well-known example of heterologous receptor regulation is the complex reciprocal relationship between the binding of gamma-aminobutyric acid and the benzodiazepines (and putative endogenous ligands) in isolated membrane preparations (Guidotti et al. 1978, Braestrup and Nielsen 1980, Muller 1981).

2.4. THE CODING OF INFORMATION BY THE LIGAND: SPECIAL FEATURES OF PEPTIDE MOLECULES

The receptor-ligand interaction involves the encounter of an "addressed" cell and a molecule with properly organized structural constituents (ligand) to provide specific recognition by properly organized counterparts at the target cell and, in the case of agonist ligands, to initiate a chain of molecular events which generate the "initial stimulus", leading to the biological response.

Thus, the ligand must contain the elements which enable the target cell receptor to recognize the molecule as its own ligand; these elements are designated as the "address"or "haptophore" (Ariens et al. 1979, Schwyzer 1980a,b). In addition, agonist ligands must possess structural constituents ("message" or "actophore" part) which make it possible that the content of information be translated by the target cell (Ariens et al. 1979, Schwyzer 1980a,b).

The peptide ligands are linearly organized and flexible molecules; the peculiarities of their receptor interactions could be attributed mostly to these properties (De Lean et al. 1979, Schwyzer 1980a,b). Fundamentally, ligand binding to the receptor may take place in a "lock and key fit" manner or may follow the so-called "zipper" pattern (Loew and Burt 1978, De Lean et al. 1979). The former is characteristic of more or less rigid ligand molecules, whereas the latter of flexible ones. The spatial orientation of interacting moieties in a peptide is determined by the actual conformation of the molecule. The preferred conformations could be predicted with good probability from the amino acid sequence (Chou and Fasmann 1978); for the shorter peptides the low energy conformers can be determined by energy calculations (De Coen et al. 1977, Loew and Burt 1978, Wijnne 1980). There are several methods at hand (CD spectroscopy, NMR, etc.) to obtain data on the ordered structure of peptides. However, the actual conformation of a peptide ligand at the binding site is strongly influenced by the environment provided by the receptor itself: consequently it is not necessarily the "low-energy", predicted or

experimentally determined "probable" conformations are assumed by the ligand at the receptor (Loew and Burt 1978). Several studies and calculations have been performed to determine the preferred conformation of peptide ligands such as β-lipotropin, β-endorphin (Graf et al. 1977, Yang et al. 1977, Wu et al. 1981), enkephalins (Garbay-Jaureguiberry et al. 1976, De Coen et al. 1977, Loew and Burt 1978, Wijnne 1980), or substance P (Mehlis et al. 1980, Cotrait and Hospital 1982). A very convincing demonstration of the statements made above on the peculiarities of peptide ligand receptor interaction was given for the opioid peptides (Bajusz 1978, Loew and Burt 1978, Wijnne 1980), where the geometry of receptors has been mapped with the aid of nonpeptide opiates (Beckett and Casy 1954, Beckett et al. 1956, Porthogese 1965, Loew and Berkowitz 1975, Feinberg et al. 1976, Horn and Rodgers 1976, Galt 1977). In addition, in the case of opioid peptides the structural orderedness in itself, though it may not be a primary determinant, appears to contribute to the selectivity of ligands towards different receptor types (Ronai et al. 1979, Soos et al. 1980).

Owing to the rapid development of synthetic peptide chemistry, the properly designed synthetic analogs in each biologically active peptide family greatly contributed to our understanding about the organization of information in a peptide molecule (for review see Schwyzer 1980b). According to a rather attractive theory (Schwyzer 1980a,b), the different functional aspects of receptor-related information (i.e., "address", "message" and "potentiator", see the definitions below) are coded either by adjacent or partially overlapping amino acid sequences (sychnologic organization) or the information-carrying elements are scattered throughout the peptide molecule (rhegnylogic organization).

As a definition, the address conveys receptor-specific affinity but not activity, the message triggers the receptor as well as displaying affinity, and the potentiator enhances the triggering effect of the message without having triggering capability on its own. Schwyzer (1980b) listed many polypeptide hormones and neuroregulators like ACTH, α-MSH, gastrin, gluca-

gon, VIP, substance P, parathyroid hormone, enkephalins and endorphins among the sychnologically organized ligands, and mentioned insulin as an example of rhegnylogically organized ones.

In the case of rhegnylogical ligands the scattered elements of information must be gathered at the surface of the molecule to form a well-defined active site; this arrangement must be provided by a rather stable tertiary structure. As to the sychnologic peptides, one and the same ligand may contain more than one address and several distinct message sequences, as it was demonstrated for ACTH and α-MSH (Schwyzer 1980a,b). For the substance P molecule, it has been demonstrated that different portions of the peptide are responsible for the smooth muscle activating and histamine releasing (Blumberg and Teichberg 1980, Mazurek et al. 1981) capability. The studies on the structure-activity relationships made possible the functional mapping of ligand molecules in several peptide families such as ACTH, α-MSH (De Wied et al. 1975, Eberle and Schwyzer 1976, Elliott et al. 1976, Schwyzer 1980a,b) opioid peptides, i.e., endorphins (Li 1978, Smyth and Zakarian 1979) enkephalins (Beddell et al. 1977, Frederickson 1977, Morley 1980, 1983) β-casomorphin (Henschen et al. 1979), dynorphins (Huidobro-Toro et al. 1981, Wuster et al. 1981b, Corbett et al. 1982), proenkephalin B-related peptides (Wuster et al. 1981a, Oka et al. 1982), substance P (Blumberg and Teichberg 1980, Mazurek et al. 1981), angiotensin (Fitzsimmons 1980) gastrin, cholecystokinin (Dockray and Gregory 1980), and parathyroid hormone (for review see Sheppard 1977, 1978, 1979, see also Schwyzer 1980b).

Nevertheless, there are indications, that the distinction between sychnologic and rhegnylogic organization might not be so clearcut. A striking example is the β-endorphin molecule where the message function was attributed to the N-terminal tetrapeptide sequence (Smyth and Zakarian 1979) or even to a shorter N-terminal portion (Schwyzer 1980b). It is obvious, however, that the C-terminal tetrapeptide region (Geisow et al. 1977, Wuster et al. 1979) also plays a vital role in message and/or address function. It was speculated that at the

receptor site the molecule of untriakontapeptide assumes such a folded structure which brings the N- and C-terminal domains into spatial proximity via a head-to-tail interaction (Graf et al. 1977).

2.5. MULTIPLE RECEPTOR TYPES

The term receptor multiplicity/heterogeneity means that for the very same endogenous ligand molecule (or family of ligands, see below) more than one receptor with truely distinct properties are found in the organism. Thus, the distinction should not be based on data reflecting e.g., the different functional states of the very same receptor type and, on the other hand, the experimental conditions must be chosen such as not to obscure really existing heterogeneity.

Nowadays the fact that multiple types of the same receptor do exist is rather widely accepted due to the plethora of pharmacological and biochemical evidences (Martin et al. 1976, Lord et al. 1977, Kebabian and Calne 1979, Seeman 1980, 1982, Snyder and Goodman 1980, Cools 1982, Creese 1983, Fagg 1985, Quirion 1985, Dutta 1987, Goldstein 1987, Regoli 1987). The task now is to separate clearly facts from artifacts (Goldstein 1987), to establish an informative and unitary nomenclature (for recommendations see Goldstein and Kosterlitz 1983, Green 1987) to find a common denominator to explain the molecular and developmental basis of multiplicity and to better understand the physiological consequences of receptor heterogeneity.

The mental process of assuming the existence of multiple receptor types and designing synthetic analogs for them is a self-stimulating circuit. The possession of potent and selective ligands (the selective being the operative word) facilitates to establish receptor heterogeneity (Chang 1984, Dion et al. 1987, Goldstein 1987). Whereas, in general, several agonists with good selectivity are needed to convincingly demonstrate receptor multiplicity in properly chosen battery of test systems (Chang 1984, Dion et al. 1987), even a single,

not type-selective (see below) competitive antagonist might provide an opportunity to make a clear differentiation (Ronai et al. 1977a).

It is a special pleasure to devote a brief paragraph to peptide receptor antagonists since the general lack of such antagonists has been an old complaint of both pharmacologists and biochemists. Because the possession of specific, competitive and selective antagonists is one of the most powerful tools in characterizing receptors, the growing number of these antagonists is promising. Most of them are synthetic peptide analogs but there are some antagonists of non-peptide structure; the extent of specificity and selectivity is varying. It appears a common feature of receptor antagonist synthetic peptides that the substitutions/alterations lend a considerable conformational rigidity to the natural peptide ligand(s) of the resulting molecules as compared to the natural peptide ligand(s) of the same receptor (Fahrenholz et al. 1984, Hruby 1987).

To better understand Table 2.1. some comments should be made on the nomenclature to be followed. As it was recommended in connection with the opioid receptors by Goldstein and Kosterlitz (1983), the receptor category/class should be named after the natural/endogenous ligands (e.g., kinin/bradykinin, tachykinin/neurokinin, bombesin etc.). To denote the main trends of heterogeneity, the type should be designated next ($\mu, \kappa, \delta, \varepsilon$, and historically, σ for the opioid receptors) and, if there is any, the subtype should be given by index numbers (μ_1, μ_2, etc. for the opioid receptors). Until the receptor nomenclature becomes unified, it might be a source of confusion that within certain receptor categories the types are designated also by index numbers (e.g., B_1 and B_2 for bradykinin receptors, NT_1, and NT_2 for neurotensin receptors, etc.). The antagonists listed in Table 2.1 will be characterized in terms of competitive (C) or noncompetitive (NC) mode of action, specificity (S, selectivity for a given receptor category/class) and type selectivity (TS). Definite statements about the character of an antagonist will be avoided in cases where

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ambiguity may be suspected; this holds particularly for the type selectivity of tachykinin/neurokinin receptor antagonists. In this receptor class, several uncertainties in defining clearly the receptor types remain to be settled (Dutta 1987, Regoli 1987).

Before giving an example of peptide receptor heterogeneity by discussing opioid receptor multiplicity in details, I should like to point out a general distinctive feature between multiple peptide and nonpeptide receptor systems. Whereas in the case of nonpeptide receptors, in general, the very same endogenous substance is likely to serve as the endogenous ligand for all of its receptor types, for the peptides it might be a trend rather than an exception that the receptor types have distinct "preferred" endogenous ligands (for review see Chang 1984, Quirion 1985, Hollt 1986, Dutta 1987, Goldstein 1987, see also Table 2.2). The implications of this phenomenon are not clearly understood as yet (Herkenham 1987).

The opioid receptors are located mainly on neural elements; the receptor types have a distinct regional distribution in the brain with considerable interspecies variation (Herkenham 1987, see also Chapter 5). At the periphery, there are organs which may contain mainly or almost exclusively one or the other receptor type (for review see Chang 1984).

Attempt has been made to find a matching between a receptor type and a single effector mechanism (e.g., inhibition of adenylate cyclase, Pert and Taylor 1980). This approach, though it was attractive, did not seem to lead to the correct solution, as it failed to do so also in the case of other receptors (Roth and Chuang 1987). Thus, both μ (Pert and Taylor 1980) and δ (Wahlstrom et al. 1977) receptor types have been shown to be coupled to adenylate cyclase in an inhibitory fashion; on the other hand, both μ and δ receptor types have been demonstrated to be coupled to potassium channels in a way quite unrelated to cAMP-generating enzyme (North et al. 1987). A more likely proposal has been put forward by Barnard and Demoliou-Mason (1983); they made an attempt to explain opioid receptor heterogeneity by assuming different combinations of various subunit structures.

Table 2.1. Antagonists of some peptide receptors

Receptor category/class	Antagonist structure (name)code	Character of antagonism	Reference
Opioid	naloxone, naltrexone	C,S,TS$^{\pm}$ (μpreference)	Lord et al. 1977 Rónai et al. 1977
	D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-OI (SMS 201-995)[b]	C,S$\pm$ (minor somatostatin-like activity TS (μ)	Maurer et al. 1982a,b
	D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2[b,c]	C,S$\pm$ (minor somatostatin-like activity) TS (μ)	Shook et al. 1987
	M_r 2266[d]	C,S,TS$\pm$ (μ=$\kappa$$\gg$$\delta$)	Lord et al. 1977
	N,N-diallyl-Tyr-Gly-Gly-ψ-(CH_2S)-Phe-Leu (ICI-154,129)	C,S,TS (δ)	Morley 1983
	N,N-diallyl-Tyr-Aib-Aib-Phe-Leu (ICI-174,864)[e]	C,S,TS (δ)	Cotton et al. 1984
Tachykinin/ Neurokinin	(D-Pro^4,D-Trp^{7,9,10}, Phe^{11})SP(4-11)[f]	C,S,TS$\pm$ (?)	Regoli et al. 1985, rev. Dutta 1987
	(D-Pro^4,Lys^6,D-Trp^{7,9,10} Phe^{11})SP(4-11)[f]	C,NS (antagonizes also bombesin) TS$\pm$	Regoli et al. 1985 rev. Dutta 1987
	(D-Arg^1,D-Trp^{7,9}, Leu^{11})SP[f] (spantide)	C,S,TS$\pm$(?)	Folkers et al. 1984, Hunter and Maggio 1984
	Spantide analogs	C,S	Folkers et al. 1984,
	($N^{\alpha c}$-Z-Arg^1,N^{ε}-Z-Lys^3--D-Trp^{7,8},D-Met^{11})-SP[f]	C,S,TS$\pm$(?)	Dutta et al. 1986
Kinin/bradykinin (BK)	(Leu^8)des-Arg^9-BK	C,S,TS (B_1)	Regoli et al. 1978, 1986.
	(Thi^{5,8},D-Phe^7)BK[g]	,S,NTS (B_2 and B_1)	Regoli et al. 1986
	(D-Arg.Hyp^3,Thi^{5,8}, D-Phe^7)BK[h] (B-3824)	C,S,TS,(B_2)	Schachter et al. 1987
	(Lys,Lys,Hyp^3,Thi^{5,8} D-Phe^7)BK (B-4310)	C,S,TS($B_2\pm$?)[i]	Griesbacher and Lembeck 1987
Cholecystokinin (CCK)	Proglumid	C,S,TS (CCK_1)	Gardner and Jensen 1984, Mac Vicar et al. 1987
	CR 1409[j]	C,S,TS (CCK_1)	Makovec et al. 1986
	L-364,718[k]	C,S,TS (CCK_1)	Chang and Lotti 1986
	CCK(27-33)	C,S,TS$\pm$ (CCK_1)	Gardner and Jensen 1984 Mac Vicar et al. 1987

Table 2.1. cont.

Bombesin (BB)	(D-Pro^{4},Lys^{6},D-Trp^{7,9,10}, Phe^{11}) SP(4-11)	NC, NS	Regoli et al. 1985, Regoli 1987
	(Leu^{13}-ψ/CH_2NH/-Leu^{14})BB	C,S	Coy et al., ref. in Cowan 1988
Neurotensin (NT)	(D-Trp^{11})NT	C,S,TS(NT_1)	Regoli 1987
Oxytocin (OT)	(d /CH_2/$_5$-Tyr /CH_3/^{2}, Thr^{4},Tyr-NH_2)OVT^{1,m}	C, S	Elands et al. 1987
Vasopressin (VP)	(d /CH_2/$_5$-Tyr /CH_3/-^{2}-Arg^{8})VP^{1} (SK & F 100223)	C,NS(antagonizes also OT) TS(V_1)	Kruszynski et al. 1980
	(d /CH_2/$_5$-D-Ile^{2}, Val^{4}, Arg^{8})VP^{1}	C,TS(V_2)	Manning et al. 1982
Luteinizing hormone releasing hormone (LH RH)	(N-Ac-pCl-D-Phe^{1}, pCl-D-Phe^{2},D-Trp^{3} D-Arg^{6},D-Ala^{10})-LHRH		Taken from Regoli 1987
	(N-Ac-Pro^{1},pF-D-Phe^{2}, nal/2/^{3,6})-LHRH^{n}		

a) For the abbreviations see the text
b) Cyclic somatostatin analogs
c) Pen=penicillamine
d) (-/2-/3-furylmethyl)-5,9-diethyl-2'-hydroxy-6,7-benzomorphan
e) Aib=α-amino-isobutyric acid
f) SP=substance P
g) Thi=β-(2-thienyl)-L-alanine
h) Hyp=L-4-hydroxyproline
i) Antagonizes BK also in systems where the receptor types have not been established as yet
j) Proglumid-analog, D,L-(3,4-dichloro-benzoyl-amino)-5-(dipentyl-amino)-5-oxo-pentanoic acid
k) 3 s(-/N-/2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-1H-indole-2-carboxamide
l) d(CH_2)$_5$=1-(β-mercapto-β, β-cyclopentamethylenepropionic acid)
m) OVT=Vasotocin
n) nal(2)=3-(2naphtyl)-D-Ala

Table 2.2. Type selective/prototype synthetic and endogenous ligands of opioid receptors [a,b]

Receptor type	Synthetic	Endogenous	Reference
	Sufentanyl		Wüster et al. 1981b
	(D-Ala^2,/N-CH_3/-Phe^4, Met/O/-Ol^5)enkephalin (FK-33824)		Kosterlitz et al. 1980
	(D-Ala^2,/N-CH_3/-Phe^4, Gly-Ol^5)enkephalin (DAGO)		Kosterlitz and Paterson 1981
μ	Tyr-Pro-Phe-Pro-NH_2 (Morphiceptin)		Chang et al. 1981
	(/N-CH_3/-Phe^3,D-Pro^4, Gly^5)Morphiceptin (PLO17)		Chang et al. 1983
	Tyr-D-Met (O), Gly,(N-CH_3) -Phe-ol (Syndyphalin, SD-25)		Kiso et al. 1981, Quirion et al. 1982
		peptide E /μ>κ>δ/[c]	Garzon et al. 1983
		BAM-22 P /μ>κ>δ/[c]	Quirion and Weiss 1983
		BAM-12 P /μ>κ>δ/[c]	Weber et al. 1983
		metorphamid /μ>κ>δ/[c]	Höllt 1986
	(D-Ala^2,D-Leu^5) enkephalin (DADL)		Chang et al. 1979
	(D-Thr^2,Leu^5,Thr^6) enkephalin (DTLET)		Zajac et al. 1983
	(D-Pen^2,D-Pen^5) enkephalin (DPDE)		Mosberg et al. 1983
δ		Leu^5-enkephalin	Lord et al. 1977
		Met^5-enkephalin	Rónai et al. 1977b
		Met^5-enkephalin-Arg^6, Phe^7	
		Met^5-enk-Arg^6, Gly^7,Leu^8	Wüster et al. 1981a
	Tifluadon		Römer et al. 1982
κ	U-50488H[d]		Piercey et al. 1982
		dynorphin A[c]	Wüster et al. 1981a
		dynorphin B[c]	Goldstein and James 1984
		α-neo-endorphin	
ε		β-endorphin[c]	Schulz et al. 1979

Table 2.2. cont.

	N-allylnormetazocine (SKF-10047)	Martin et al. 1976
σ		
	Phencyclidine (PCP)	Zukin and Zukin 1981

a) For the antagonists see Table 1
b) For recent reviews see Chang 1984, Höllt 1986, Goldstein 1987
c) For the structures see Chapter 5
d) U-50488H=trans-3,4-dichloro-N-methyl-(2-/1-pyrrolidine/-cyclohexyl) benzeneacetamide
e) The σ/PCP receptor is no longer considered as an opioid receptor; it is listed solely for historical reasons

2.6. SOME THEORETICAL AND METHODOLOGICAL ASPECTS OF RECEPTOR RESEARCH

The ideal experimental system for receptor research is one where both receptor occupation by the ligand and the first functional cellular response upon agonist binding to the receptor species can be determined. This ideal state is approached by the isolated and sealed vesicle prepared from homogenates of T o r p e d o electric organs (Neubig and Cohen 1980, Taylor and Sine 1982), where the measurements are made on the same receptor (nicotinic cholinergic), in which structural characterization is most complete, and the system can be studied in suspension which permits measurements of ion permeability and receptor occupation in a time frame approaching several milliseconds.

In general, from a methodological point of view, the ligand-receptor interaction can be monitored by classical pharmacological/biochemical means and also by ligand binding studies. The pharmacological methods have the advantage that although they characterize ligand affinities indirectly, they provide the opportunity of measuring tissue responses to agonists. The binding studies gather direct information on ligand affinities and have the unique opportunity of determining the concentration of receptors in a tissue sample. The comparison of results obtained in pharmacological and binding studies serves as a further source of information (Furchgott 1978).

2.6.1. THE VALUE OF RECEPTOR-RELATED PARAMETERS OBTAINED BY PHARMACOLOGICAL MEANS

Several theories were elaborated to relate biological responses to the interaction of ligands with the receptors (Paton 1961, Furchgott 1978, for review see Ariens et al. 1979).

To obtain conveniently primary data in the pharmacological experiments one should have selective receptor agonists and selective, competitive receptor antagonists (see Table 2.1, and 2.2). These criteria are seldom fully met, thus the interfering factors must be excluded by proper pharmacological manipulations (Furchgott 1978, Kenakin 1982a,b).

The proper choice of an experimental system is also of vital importance. Besides the drug-related factor, the experimental media should ensure that the measured effects be governed primarily by receptor-related parameters, and not be distorted by pharmacokinetic influences such as absorption, distribution, metabolism and excretion. For the kinetical treatment of primary data it is also important that the ligand-receptor interaction be studied at equilibrium. Although data obtained in properly conducted in vivo or in situ experiments are also of high value for kinetical analysis (Takemori et al. 1969, Szekely et al. 1978), the prerequisites are matched far better in simple isolated smooth muscle/nerve-smooth muscle preparations, cell cultures and homogenates or specially treated tissue samples prepared by biochemical techniques (Furchgott 1978, Taylor and Sine 1982).

The basic paradigm in pharmacological experiments is the construction of a dose-response curve or a series of dose-response curves with agonist(s) (Tallarida 1982). Typically, these curves are hyperbolas when plotted on linear scales; on a semilog scale the linearization of the steep portion can be achieved. The horizontal asymptote or upper limit, E_{max} , is a measure of efficacy, the power of the compound to produce the effect. Several calculated parameters such as efficacy (Stephenson 1956) intrinsic activity (Ariens 1954) and intrinsic efficacy (Furchgott 1972, 1978) have been introduced

to characterize more exactly this property of an agonist. The intrinsic efficacy (ε) as defined by Furchgott (1972, 1978) is a true indicator of receptor-dependent efficacy whereas the other two are strongly influenced by organ-related and not strictly receptor-related factors (Kenakin 1982a,b). The left to right position is an indicator of the compound's potency. It is convenient to express potency in terms of the concentration or dose that produces a half-maximal effect (EC_{50}). In quantal (all or none) dose-response relations the potency is characterized by the dose that produces a specific level of effect in 50% of the population. Some interpretations erroneously treat EC_{50} and the dissociation constant as interchangeable indicators: this is generally not valid. To determine the affinity of a "pure" agonist by pharmacological means requires an indirect approach such as the inactivation of a fraction of receptors by an irreversibly acting agent (Furchgott 1966, 1978, Furchgott and Bursztyn 1967, Tallarida 1982) or the use of functional antagonism (Broadley and Nicholson 1979). The affinities of antagonists, partial agonists and mixed agonists-antagonists to their respective receptors could be characterized more conveniently by pharmacological experiments using either the so-called Schild-plot (Arunlakshana and Schild 1959) or calculations based on the same principle (Gaddum et al. 1955, Kosterlitz and Watt 1968).

Using the pharmacological approach, the receptors are characterized first from the "agonist side". Historically, this was the case for the "muscarinic" and "nicotinic" acetylcholine receptors as well as for the α- and β-adrenoceptors (ref. in Ahlquist 1984). The peptide receptors are no exceptions in this respect; to give just two examples, such an initial strategy was used for opioid peptide (Lord et al. 1977, Wuster et al. 1979, 1981a,b) and neurokinin (for review see Dutta 1987) receptors.

2.6.2. RADIOLIGAND BINDING STUDIES

Over the past fifteen years sufficient expertise has been developed to reliably measure the binding of radioactively labeled probes to quantitatively small numbers of specific binding sites in tissue samples which, in most cases, represent pharmacologically/physiologically receptors of interest (Cuatrecasas and Hollenberg 1975, Dole et al. 1975, Snyder and Pert 1975, Hollenberg and Cuatrecasas 1979). There are several criteria to be met to designate these binding sites as pharmacologically/physiologically relevant receptors. The best probe is to correlate the binding and biological activities of closely related and also of structurally diverse ligand candidates of the receptor in question.

From a methodological point of view basically two kinds of binding studies can be performed. First the specific binding of a labeled ligand can be measured as a function of free labeled ligand concentration (the so-called "saturation" experiments). For the convenience of obtaining the parameters of binding, such as the equilibrium dissociation constant and the number of binding sites in the sample and also to characterize the binding process, several plotting procedures have been applied to the primary data (for review see Boynaems and Dumont 1975, Posner 1975, Henis and Levitzki 1976, De Lean and Rodbard 1979, Weiland and Molinoff 1981). For the simplest case of ligand-receptor interaction the plots are linear. The linearity facilitates the derivation of binding parameters on the one hand, and on the other the deviations from linearity (or changes in the slope) are indicators of more complex processes. Of these the most frequently used ones are the Scatchard (1949) and the Hill (1910) plots.

In the other type of experiments ("displacement" experiments) the competitive inhibition of the binding of a labeled ligand by unlabeled compounds can be used to indirectly characterize the interactions of the receptor with competing drugs (Weiland and Molinoff 1981). The selection of the proper concentration of the labeled ligand is crucial: the choice must accomodate the problem to be answered.

For the simplest case of competition, the equilibrium dissociation constant of the competing compound can be calculated by the Cheng-Prusoff (1973) equation. The applicability of the Cheng-Prusoff equation is restricted (Weiland and Molinoff 1981, Goldstein 1987). For the analysis of more complex cases of interaction Eadie-Hofstee plots (Eadie 1952, Hofstee 1952, for review see Weiland and Molinoff 1981) or a non-linear curve fitting resolution (De Lean et al. 1980, Minneman and Molinoff 1980) can be applied.

Receptor binding studies represent a powerful tool in characterizing receptors, receptor types and subtypes, especially when specific labeled ligands of high specific activity (Chang 1984) and competing ligands of high selectivity (Chang 1984, Goldstein 1987, see also Table 2.1 and 2.2) are at hand. The methodological pittfalls and other sources of interpretational error, however, are numerous in general and even more numerous in the case of peptide ligands/receptors (De Lean et al. 1979, Goldstein 1987). The incidence of nonspecific or quasi-sepcific interaction with biologically non-relevant targets is considerably higher when using peptide ligands; one should be aware also of the potential distorsion of data caused by degrading enzymes. Finally, the intra- and especially the intermolecular interactions within/between peptide ligands may present unexpected puzzles. Some of these interactions may be predicted, calculated and measured or modelled experimentally; these interactions, however, may take place in a hardly predictable manner in the environment presented by the vicinity of receptors.

2.6.3. CHEMICAL CHARACTERIZATION OF RECEPTORS

To determine the chemical characteristics of receptor molecules at least partial purification or, more desirably, solubilization of receptors must be achieved first (Laduron and Ilien 1982). To identify the isolated substance as the receptor, its biological (biochemical, pharmacological) relevance must be checked.

Cloning and sequencing of cDNAs encoding various receptor proteins revolutionized also the chemical characterization of receptors. Through the use of molecular cloning techniques, the complete amino acid sequences of various receptor proteins have recently become available. Besides the best-characterized one, the nicotinic acetylcholine receptor (Noda et al. 1982, for review see Luyten 1986), the primary structures of $GABA_A$, (Schofield et al. 1987) and a subunit of glycine receptor (Grenningloh et al. 1987), human LDL (Yamamoto et al. 1984), transferrin (McCleland et al. 1984), epidermal growth factor (Ullrich et al. 1984) and insulin (Ullrich et al. 1985) receptor, and also the substance K-(neurokin A)-type tachykinin/neurokinin receptor proteins (Masu et al. 1987) have been disclosed.

Of the neuropeptide receptors, the purification and partial chemical characterization of various opioid receptor types (Simonds et al. 1985, Cho et al. 1986, for review see Simon 1987), the substance P-(neurokin-1)-type tachykinin/neurokinin receptor (Dam et al. 1987) and the central and peripheral neurotensin receptors has been undertaken.

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CHAPTER 3

ENDOGENOUS PEPTIDES IN THE INTEGRATION OF ENDOCRINE FUNCTIONS, THE PEPTIDE HORMONES

B. VARGA and J. SZUCS

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3.1. PHYSIOLOGY OF HORMONAL PEPTIDES

According to the classic model the endocrine glands produce hormones and secrete them into the blood vessels in response to specific stimuli carried by the blood stream. Within this concept, the pituitary gland was considered the main and independent organizer of endocrine function.

In the past decades, endocrinology has gone through a fascinating development which has led to substantial changes in our previous concept of endocrinology. Current endocrinology is characterized by a tremendous increase in knowledge concerning the biochemical basis of endocrine functions or disfunctions, and the development of sensitive methods for the measurement of hormones. The field of endocrinology expanded and a new discipline, neuroendocrinology was developed. One of the basic concepts of neuroendocrinology is that the primary endocrine signal starts from the brain, passes through some transducers, and ends on the peripherial effector cells. It has become increasingly clear that hypothalamic neurons play a prominent role in organizing endocrine function by producing peptides with hormonal characteristics, and that the neuronal transmitter substances also have an important role in this process.

It has also become evident that endocrine functions are not restricted exclusively to the endocrine organs. According to the "classic" concept of endocrinology, the hormones originate from specific glandular tissues and exert their effects on

target organ cells. Recently, however, a number of extra glandular hormones as well as the effects produced by hormones on "non-target cells" have been observed (see e.g., Krieger 1985).

3.1.1. HYPOTHALAMIC-HYPOPHYSIOTROPIC PEPTIDE HORMONES

Hypothalamic peptide hormones regulate secretion of protein and peptide hormones from the pituitary gland. Because the hypothalamus is part of the brain region nearest to the pituitary gland, it was reasonable to suppose that the hypothalamus might contol pituitary functions (Harris 1955, Szentagothai et al. 1962). Hypothalamic regulation is based on a portal system of blood vessels between the median eminence and the pituitary. Hypothalamic neurons of different types release hormonal substances from their nerve endings into the primary capillary plexus, are subsequently transported by the portal circulation to the hormone-secreting cells of the pituitary gland where they stimulate or inhibit synthesis and/or release of anterior pituitary hormones. Neurosecretory granules located in the neurons of the median eminence may be related to the releasing hormones of the hypothalamus.

3.1.1.1. CORTICOTROPIN-RELEASING FACTOR (CRF) OR HORMONE (CRH): CORTICOLIBERIN

Direct evidence for the neurohormonal regulation of ACTH secretion was firstly presented by Saffran et al. (1955), who demonstrated the capacity of extract from both the posterior pituitary gland and the hypothalamus to stimulate ACTH release in vitro. Subsequently, a linear log-dose response was established for the release of ACTH into the medium under the influence of varying amounts of standardized hypothalamic extracts (Takebe et al. 1976). As vasopressin (and epinephrine) can also stimulate ACTH release, vasopressin was initially supposed to be the CRF. Finally ovine CRF was identified as a

41 amino acid peptide (Vale et al. 1981). Subsequently, the human CRH gene has been isolated and sequenced and a cDNA-derived sequence for human CRH was established (Fig.3.1., Shibahara et al. 1983). The human and rat sequences are identical. Mammalian CRH has homologies with two submammalian peptides, sauvagine and urotensin 2 (see Chapter 13), both having potent CRH activity.

```
                                   10                                  20
h: Ser-Glu-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-
o:  -  Gln  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
                                   30                                  40  41
h: Met-Ala-Arg-Ala-Glu-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Met-Glu-Ile-IleNH2
o:  -  Thr-Lys  -  Asp  -  -  -  -  -  -  -  -  -  -  -  -  -  -  Leu-Asp  -  AlaNH2
```

Fig. 3.1. cDNA-derived amino acid sequence of human (h), and chemically determined primary structure of ovine (o) corticotropin-releasing hormone

CRF containing samples can be injected into dexamethasone -chlorpromazine-pentobarbital-blocked rats, either intravenously or directly into the pituitary, and changes in the peripheral corticoid level can be measured. CRF can also be assayed in vitro using isolated anterior pituitary cells. Superfused anterior pituitary gland is also used as a test system. After CRF is added to an in vitro test system the released ACTH is determined (Jones and Hillhouse 1977). Recent availability of synthetic CRF allows to determine CRF by RIA (Bloom et al. 1982, Linton and Lowry 1982, Palkovits et al. 1983).

Acetylcholine added to isolated hypothalamus stimulates both the synthesis and the release of CRF (Kuhn 1974). 5-HT also increases the secretion of CRF (Buckingham and Hodges 1977, 1979) and this can be prevented by methylsergide, a specific 5-HT inhibitor. An inverse relationship has been established between plasma corticosterone concentrations and hypothalamic noradrenaline levels in the rat (Ganong 1977). Noradrenaline induces a reduction in CRF release in response to acetylcholine. Intraventricularly administrated GABA inhibits CRF activity, and this can be abolished by picrotoxin in rats (Makara and Stark 1974). In the hypothalamus, GABA is a

more potent inhibitory neurotransmitter than noradrenaline in vitro. The finding that response to both acetylcholine and 5-HT is inhibited by GABA indicates that GABA might act directly on CRF neurons. As the effect is abolished by picrotoxin, these neurons presumably contain specific GABA receptors (Jones et al. 1981). On the other hand, corticoids may influence GABA synthesis (Acs and Stark 1978). Melatonin has also been suggested as a neurotransmitter inhibiting CRF release (Jones et al.1981).

Adrenalectomy results in elevated blood levels of ACTH (Gemzell et al. 1981), an effect prevented by a physiological dose of corticosteroids (Dallman et al. 1972). The feedback inhibition of CRF secretion has been demonstrated by using hypothalamus or hypothalamic synaptosomes as in vitro test systems (Edwardson and Bennett 1974). Corticosteroids reduce CRF-induced release of ACTH from anterior pituitary cells (Sayers and Portanova 1974). It may be concluded that feedback inhibition is exerted at levels of both the hypothalamus and the anterior pituitary, reducing both CRF secretion and the pituitary response to CRF.

3.1.1.2. THYROTROPIN-RELEASING FACTOR (TRF) OR HORMONE (TRH): THYROLIBERIN

TSH release from the pituitary gland is regulated by the interaction of the hypothalamic TRH and the thyroid hormones. The former stimulates, whereas the latter counteract the release of TSH. The existence of TRH was first demonstrated by Schreiber et al. (1961) and Guillemin et al. (1962) by using purified hypothalamic TRH preparations. Both porcine and ovine TRHs were identified as a tripeptide: pGlu-His-ProNH_2 (Burgus et al. 1969, Schally et al. 1969, Bowers et al. 1970), and the structure confirmed by synthesis (Boler et al. 1969). The nucleotide sequence of a cDNA encoding the precursor to rat TRH and the predicted amino acid sequence of preproTRH has also been determined recently (Lechan et al. 1986, for review see Mandel and Goodman 1987). In mammals, plasma concentrations of

TSH was found to increase upon TRH administration, irrespective of the route of administration. In the anterior pituitary, TRH stimulates both synthesis and release of TSH. Thyroxine (T_4) and triodothyronin (T_3) block the stimulatory effect of TRH on TSH release. Since the latter phenomenon could be observed both in vivo and in vitro, thyroid hormones might exert their inhibitory actions at the pituitary level. Protein synthesis inhibitors do not prevent the TRH-induced TSH release, but they abolish the inhibitory effect of T_3 and T_4. Cold seems to be the physiological stimulus eliciting TRH release in hypophysectomized thyreoidectomized rats (Reichlin et al. 1978). The availability of synthetic TRH allowed the production of antibodies for RIA, although the rapid inactivation of TRH in the serum and its similarly rapid renal excretion presented some problems for TRH determination in the blood (Schally et al. 1979).

In the rat brain, the highest TRH concentration was found in the hypothalamus, but 70% of all TRH are localized extrahypothalamically. Relatively large amounts of TRH were also found in the septal and preoptic areas (Brownstein et al. 1974).

TRH also stimulates prolactin release from pituitary tumor cells in vitro (Tashijan et al. 1971), and increases serum prolactin levels in several species, including humans (Jacobs et al. 1971). In rats L-Dopa blocked prolactin release but TSH release remained unchanged in response to TRH (Chen and Meites 1975).

In clinical practice, TRH is used primarily for two purposes: (a) To test the pituitary reserve of TSH. Patients who do not respond to TRH by a sufficient release of TSH are suspected to suffer from pituitary deficiency. The lack of the response to TRH may support the diagnosis of pituitary tumor. (b) To test the principle that the major feedback of thyroid hormones is exerted rather at the pituitary than at the hypothalamic level. Excess of the thyroid hormones in hyperthyroidism pervents the TRH-induced release of TSH (Schally et al. 1979).

TRH also exerts extrapituitary effects in the brain. Preliminary observations suggest that TRH has both behavioral and

electrophysiologic effects which cannot be interpreted on the basis of its pituitary stimulating activity (Plotnikoff et al. 1974). TRH may have direct effects on the electrical activity of a single neuron, by depressing firing in the affected cell population (Dyer and Dyball 1974). Normal cellular firing is restored a few seconds after the termination of TRH application (Reichlin et al. 1978). This raises the question whether or not this peptide functions as a neurotransmitter. Final proof for this hypothesis requires the demonstration that TRH is present in, and is released from, the nerve terminals in the brain regions where it has been localized.

3.1.1.3. GROWTH HORMONE RELEASE-INHIBITING HORMONE (GH-RIH): SOMATOTROPIN RELEASE-INHIBITING FACTOR (SRIF), SOMATOSTATIN

In studies concerned with the effect of the hypothalamus on pituitary GH secretion, both stimulatory and inhibitory activities could be separated from hypothalamic extracts using isolated pituitary gland as test systems. The inhibitory activity was found to be localized to the posterior median eminence and the preoptic region (Krulich et al. 1969). In the early seventies, Brazeau et al. (1973) succeeded in isolating a peptide inhibitor of GH release from ovine hypothalamus. The inhibitor called somatostatin was identified as a cyclic tetradecapeptide with a 14-membered ring structure and with a disulfide bridge between residues at positions 3 and 14:
Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys. The structure was confirmed by synthesis. (Rivier et al. 1973, see also Chapter 6).

Other molecules with somatostatin activity, but composed of 22 and 28 amino acids, were also reported. Structure-activity studies with synthetic analogues demonstrate that neither the disulfide bridge nor the N-terminal dipeptide are necessary for biological activity (Sandow and Konig 1978). Somatostatin was identified in discrete brain areas by immunohistochemistry (Brownstein et al. 1976). Immunoreactive somatostatin of dif-

ferent molecular sizes was also found in the pancreas, stomach and gut (Arimura at el. 1975, see also Chapter 6).

Somatostatin added to isolated rat anterior pituitary cells inhibits cAMP accumulation during the first minutes of incubation, and prevents the stimulatory effect of theophylline and dibutyryl-cAMP on GH synthesis (Sandow and Konig 1978). The inhibitory action of somatostatin can be prevented by pentobarbital, morphine and PGE_2. Depending on the dose, somatostatin inhibits the release most of the peptide hormones from the anterior pituitary gland (McCann 1982).

The effects of somatostatin are short-lived. When infusion is stopped, a rebound in GH, insulin and gastrin secretion can be observed. When administered in high doses, side effects have been noted. In rats e.g., sedative and pronounced vasodepressor effects and somnolence were observed. In baboons, prolonged treatment with somatostatin proved to be toxic due to thrombocytopenia and reduced platelet aggregation (Koerker et al. 1975).

3.1.1.4. GROWTH HORMONE-RELEASING HORMONE/FACTOR (GRH/GRF): SOMATOLIBERIN, SOMATOCRININ

The existence of a GRH has long been suggested by physiological evidences, such as demonstrating GH-releasing activity in hypothalamic extracts (Deuben and Meites 1964). Although several agents with GH-releasing activity were detected in the past (Schally et al. 1979, Muller 1979), none of them was potent enough to regard them as a physiological GRH.

GH-releasing activity observed in the extracts of carcinoid and pancreatic islet tumors removed from patients with GH hypersecretion, acromegaly, and pituitary adenoma or hyperplasia, has been partially characterized. A substance with pronounced GRH activity was isolated from human pancreatic tumor and identified as a 44 amino acid peptide (Guillemin et al. 1982) which is identical with the GRH isolated from human hypothalamus (Ling et al. 1984):

Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-LeuNH_2. Others have reported 40 and 37 amino acid forms of the same sequence from a human pancreatic tumor with similar activity and demonstrated that the 1-29 residue already had full activity and potency in vitro (Rivier et al. 1982), but the 1-27 fragment had no effect.

3.1.1.5. MSH RELEASE-INHIBITING FACTOR (MIF) AND MHS-RELEASING FACTOR (MRF)

Fractions separated from hypothalamic extracts were found either to promote or to block MSH release from the pars intermedia of the pituitary gland in lower vertebrates and mammals. The inhibitory effect was more pronounced. MIF was identified as a tripeptide: Pro-Leu-GlyNH_2, that was found to be active both in vivo and in vitro in inhibiting MSH secretion from pituitary gland (Celis et al. 1971a, Nair et al. 1971).

On the other hand, MRF proved to be a pentapeptide: Cys-Tyr-Ile-Gln-Asn, corresponding to a partial sequence of the oxytocin molecule. The peptide induces MSH release with a concomitant rise in serum MSH levels and a depletion of the pituitary MSH content (Celis et al. 1971b).

Enzymes present in the hypothalamus are involved in the formation of both RMF and MIF, and oxytocin and vasopressin may equally serve as substrates.

A dual control mechanism for MSH secretion has been supposed. Two populations of neurons have been described in the pars intermedia: a light sensitive and a light insensitive population. It is believed that they act oppositely on MSH secretion. The facilitatory mechanism appears to be mediated by catecholamines through β-adrenergic receptors, whilst the inhibitory mechanism apparently depends on α-adrenergic receptor stimulation. Catecholaminerg neurons may affect MSH secretion by acting directly on the cells of pars intermedia as well as on the MIF-secreting hypothalamic neurons. Local application

of noradrenaline or dopamine inhibits MSH secretion in the isolated pituitary gland. On the other hand, reserpine treatment increases MIF content of the hypothalamus. The observed depletion of MIF by noradrenaline injection into the third ventricle substantiates a hypothalamic site of action (Taleisnik 1978).

MIF or MRF receptors have not been identified in the pituitary. Circulating levels of MIF and MRF remain undetermined for the time present. Plasma elimination of these peptides is rapid with a half-life of 3-13 minutes. Little is known of the mechanism of their action. Involvement of the adenylate cyclase system in the effect of MIF is unlikely.

Very high doses of MIF, administered alone or in combination with L-Dopa, seem to have a beneficial effect on tremor and rigidity in Parkinson's disease. The neuropharmacological effect of MIF and its analogues are probably unrelated to its physiological effects, as is indicated by the extremely high doses required (Sandow and Konig 1978).

3.1.1.6. PROLACTIN RELEASE-INHIBITING FACTOR (PIF) OR PROLACTOSTATIN AND PROLACTIN-RELEASING FACTOR (PRF): PROLACTOLIBERIN

Prolactin secretion is predominantly under inhibitory control by the hypothalamus in mammals, and under stimulatory control in birds.

In hypothalamic extracts from different species, a PRF activity was also demonstrated (Meites and Nicoll 1966). Beside releasing TSH, TRH is also able to release prolactin, though TRH is not identical with PRF: the nocturnal TSH peaks and prolactin secretion are dissociated in man (Vanhaelst et al. 1973). PRF activity isolated from the hypothalamus-posterior pituitary complex was found to be distinct from TSH (Dular et al. 1974).

The functions of PIF and PRF in various animals may be different. In the rat, ether stress causes a rapid but relatively small release of prolactin, while the suckling stimulus

results in a slightly delayed but much larger release. It was supposed that two separate mechanisms exist for prolactin release. A specific PRF may be involved in the immediate though relatively minor prolactin release induced by stress, while the delayed but more marked suckling-triggered release may be due to a decreased PIF activity (Tindal 1978). Serotoninergic mechanisms may also have a role in the release of prolactin. 5-HT or melatonin injected into the third ventricle causes prolactin release in rats.

PIF activity was purified from crude hypothalamic extracts and its activity demonstrated in vivo (Meites and Nicoll 1966). Noradrenaline and dopamine were found to have PIF activity in vitro and in vivo, indicating that chatecholamines have direct effects on prolactin release from the pituitary gland. The peptide nature of PIF has been demonstrated by Greibrokk et al. (1974).

It has long been known that cathecholamines play a role in the control of prolactin release. In the rat intraventricular injection of dopamine results in an increased PIF activity in pituitary stalk plasma, and reduced prolactin levels in peripheral plasma. Injection of dopamine directly into the portal vessels has no effect (Kamberi et al. 1971). L-Dopa, the precursor of dopamine which can pass the blood-brain barrier, induces an increased hypothalamic PIF content and causes a remission of galactorrhoea in humans (Turkington 1972a,b). Other studies indicate that a cholinergic mechanism might be involved in the inhibition of prolactin release, which appears to precede the dopaminergic link (Libertun and McCann 1974). GABA inhibits the release of prolactin from anterior pituitary in vitro. GABA suppresses prolactin release in monolayer cultures of anterior pituitary cells and inhibits TRH-stimulated prolactin secretion. Although catecholamines or GABA inhibit the release of prolactin by acting directly on the pituitary gland it remained to be determined for long whether catecholamines or/and GABA represent a physiological PIF (Schally et al. 1979).

Under such conditions it was an important step forward when Nikolics and associates (1985) and Adelman and coworkers

(1986) have isolated a gene and a cloned cDNA from a library derived from human and rat hypothalamic mRNAs that encoded a precursor protein common both to Gn-RH and PIF, also termed Gn-RH associated peptide (GAP). The 92 amino acid precursor having a 23 amino acid signal peptide is processed to yield the Gn-RH decapeptide and the 56 amino acid GAP, while a tripeptide (Gly-Lys-Arg) flanking the Gn-RH sequence serves as a site for enzymatic cleavage and C-terminal amidation of Gn-RH. The primary structure of GAP/PIF is shown in Fig.3.2.

```
                                  10                                  20
h: Asp-Ala-Glu-Asn-Leu-Ile-Asp-Ser-Phe-Gln-Glu-Ile-Val-Lys-Glu-Val-Gly-Gln-Leu-Ala
r: Asn-Thr - His - Val - - - - - - Met-Gly - - Glu-Asp - Met -
                                  30                                  40
h: Glu-Thr-Gln-Arg-Phe-Glu-Cys-Thr-Thr-His-Gln-Pro-Arg-Ser-Pro-Leu-Arg-Asp-Leu-Lys
r: - Pro - Asn - - - - Val - Trp - - - - - - - - Arg
                                  50                            56
h: Gly-Ala-Leu-Glu-Ser-Leu-Ile-Glu-Glu-Glu-Thr-Gly-Gln-Lys-Lys-Ile
r: - - - - Arg - - - - - - Ala - - - - Met
```

Fig. 3.2. cDNA-derived sequences of human (h) and rat (r) Gn-RH-associated peptide/prolactin release-inhibiting factor

While Gn-RH portion of the human and rat precursors is fully conserved, the GAP/PIF portion displays an about 70% homology. GAP/PIF produced by recombinant DNA technology in E. c o l i proved to be an energetic inhibitor of prolactin release and a moderate stimulator of LH and FSH release. The occurrence of Gn-RH and PIF within a single precursor suggests that regulation of the two peptides is coupled. On the other hand, the established identity of the hypothalamic and placental Gn-RH/PIF precursors indicates that this particular protein is orchestrating both cental and peripheral reproductive functions (for further details and references see Nikolics et al. 1985, Adelman et al. 1986).

3.1.1.7. LUTEINIZING HORMONE-RELEASING HORMONE (LH-RH)

is discussed in Chapter 4.

3.1.2. PITUITARY PEPTIDE HORMONES

The pituitary gland is divided into three parts: (1) the adenohypophysis (anterior lobe, pars anterior), (2) pars intermedia (in human poorly developed), and (3) the neurohypophysis (posterior lobe, pars posterior).

3.1.2.1. PEPTIDE HORMONES FROM THE ADENOHYPOPHYSIS

3.1.2.1.1. ADRENOCORTICOTROPIC HORMONE (ACTH): CORTICOTROPIN

Smith (1930) first demonstrated a pituitary factor affecting the adrenal gland. ACTH was first isolated and its protein nature determined by Li et al. (1943) and Sayers et al. (1943). Subsequently this factor was identified as a low molecular weight peptide comprising 39 amino acids (Bell 1954). The revised structures of bovine and human ACTH were later reported by Graf et al. (1971) and Riniker et al. (1972), respectively. The human peptide has the following sequence:

Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe. Amino acid composition and sequence of ACTH display slight species differences. The porcine molecule differs in Leu(31) whereas the identical ovine and bovine ACTH in Gln(33) from their human counterpart. ACTH is synthesized by the corticotroph cells in the adenohypophysis, or by the melanotroph cells in the pars intermedia of the pituitary gland as a part of a precursor molecule (proopiocortin), which also contains the sequence of β-lipotropin (β-LPH) (Graf et al. 1977). For a more detailed discussion of the peptides originating from proopiocortin, and their relation to each other as well as to ACTH see Chapter 5.

In the adult human pituitary, ACTH and β-LPH are located within the same cells and granules, and are present in equimolar concentration. The steroidogenic N-terminal region of ACTH is identical in all mammals, and the residues primarily responsible for the species differences are those at position 25-39 (Hope and Lowry 1981).

Bioassay: ACTH decreases ascorbic acid content of adrenocortical slices, a phenomenon on which the earliest bioassay was based (Sayers et al. 1948). The steroidogenic response can also be used as a bioassy; in hypophysectomised rats, the adrenal vein is cannulated and corticosterone mesaured in the effluent blood. Corticosterone production can also be assayed using isolated adrenocortical cells.

RIA: various types of anti-ACTH antisera have been produced for measuring ACTH by RIA which recognize the N-terminal, mid-portion or the C-terminal regions of the molecule. The antibodies used most frequently are raised against synthetic ACTH (1-24) or porcine ACTH.

Radioreceptor assay: Receptors prepared from the adrenal gland, and ^{125}I-ACTH can be used in competitive binding tests (Aronin and Krieger 1981).

ACTH synthesis and release are under hypothalamic regulations. Following a CRF stimulus, ACTH is rapidly released into the circulation. ACTH plasma levels undergo a circadian variation, with highest levels in the early morning. The episodic secretion is superimposed upon the circadian patterns. The half-life of the different ACTH peptides in the blood of different mammals is within a range of 5-20 minutes.

The major functions of ACTH are the acute stimulation of steroidogenesis in the adrenal gland (Koritz et al. 1977), and long-term maintenance of structural integrity of the adrenal cortex (Ramachandran et al. 1977). The blood circulation of the adrenal gland is also regulated by ACTH (Stark et al. 1965).

The cell membrane plays a prominent role in the function of adrenocortical cells. Its complex lipid and protein structure receives and recognizes hormonal (ACTH) signals and transfers them via adenylate cyclase and cyclic AMP into the cell inte-

rior. One of the important results of the intracellular message is an increased availability of the cholesterol substrate for pregnenalone formation in the mitochondria, which is the first step in steroidogenesis (Ontjes et al. 1977). ACTH-induced steroidogenesis may require an increased protein synthesis. This is supported by the observation that protein synthesis inhibitors, e.g., puromycin or cycloheximide, abolish the sterodiogenetic effect of ACTH (Ferguson 1963, Stark and Varga 1968). However, no increase in protein synthesis could be detected when steroidogenesis had already been stimulated by ACTH. Available data indicate that a "rapid turnover protein" might be required for the acute steroidogenetic effect of ACTH. This protein may be necessary for transforming cholesterol into pregnenalone.

ACTH and its fragments, ACTH(1-10) and ACTH(4-10), but not ACTH(11-24), appear to have an effect on avoidance acquisition in hypophysectomized rats. The behaviorally active site is located in the N-terminal region of the molecule, presumably in the 4-10 sequence of ACTH, which is also present in α-MSH, β-MSH and β-LPH (DeWied 1977, DeWied and Jolles 1982).

ACTH has a lipolytic activity in adipose tissue cells and stimulates adenylate cyclase in fat cell membranes through specific receptors. This effect may be mediated by the interaction of ACTH and other metabolically active hormones (insulin, glucagon), or by a direct action on the cellular metabolism (Schwandt 1981).

ACTH induces hyperemia, not only in the adrenal gland but also in the ovary of both intact and adrenalectomized dogs (Stark et al. 1967). The ACTH-induced hyperemia in the ovary (Stark and Varga 1975) and the adrenal gland (Varga et al. 1979) can be abolished by indomethacin. Exogenous ACTH increases oestrogen secretion in HCG-primed dogs (Varga et al. 1973), and ovarian oestradiol and progesterone secretion in the proestrous hamster indicating that ACTH may potentiate the effects of gonadotropic hormones (Varga and Greenwald 1979).

3.1.2.1.2. GROWTH HORMONE (GH): SOMATOTROP HORMONE (STH), SOMATOTROPIN

GH is a polypeptide synthesized by specific acidophil cells in the anterior pituitary. Typical somatotroph cells contain numerous large secretory granules of 300-400, µm diameter. The primary structure of GH displays species differences. Human GH contains 191 amino acids with two intramolecular disulfide bonds between residues 53-165 and 182-189, respectively and has a primary structure as follows:
Phe-Pro-Thr-Ile-Pro-Leu-Ser-Arg-Leu-Phe-Asp-Asp-Ala-Met-Leu-Arg-Ala-His-Arg-Leu-His-Gln-Leu-Ala-Phe-Arg-Thr-Tyr-Gln-Glu-Phe-Glu-Glu-Ala-Tyr-Ile-Pro-Lys-Glu-Gln-Lys-Tyr-Ser-Phe-Leu-Gln-Asp-Pro-Gln-Thr-Ser-Leu-Cys-Phe-Ser-Glu-Ser-Ile-Pro-Thr-Pro-Ser-Asp-Arg-Glu-Glu-Thr-Gln-Gln-Lys-Ser-Asp-Leu-Gln-Leu-Leu-Arg-Ile-Ser-Leu-Leu-Leu-Ile-Gln-Ser-Trp-Leu-Glu-Pro-Val-Gln-Phe-Leu-Arg-Ser-Val-Phe-Ala-Asp-Ser-Leu-Val-Tyr-Gly-Ala-Ser-Asp-Ser-Asp-Val-Tyr-Asp-Leu-Leu-Lys-Asp-Leu-Glu-Glu-Gly-Ile-Gln-Thr-Leu-Met-Gly-Arg-Leu-Glu-Asp-Gly-Ser-Pro-Arg-Thr-Gly-Gln-Ile-Phe-Lys-Gln-Thr-Tyr-Ser-Lys-Phe-Asp-Thr-Asp-Ser-His-Asp-Asp-Asp-Ala-Leu-Leu-Lys-Asp-Tyr-Gly-Leu-Leu-Tyr-Cys-Phe-Arg-Lys-Asp-Met-Asp-Lys-Val-Glu-Thr-Phe-Leu-Arg-Ile-Val-Gln-Cys-Arg-Ser-Val-Glu-Gly-Ser-Cys-Gly-Phe (Li and Dixon 1971). The hormone is structurally similar to both prolactin and placental chorionic somatomammotropin (HCS, HPL). Attempts to delineate an active core in the GH molecule have essentially failed so far. It seems very likely that biological activity is bound to the 134 amino terminal residues (Merimee 1979). For further information and references on the chemistry, gene and gene-structure of GH see Underwood and Van Wyk (1985).

GH secretion is regulated by somatostatin and somatoliberin, the hypothalamic hormones which, on the other hand, are under the control of different brain structures and neurotransmitters. Stress might provoke GH release, but "spontaneous" episodic increase in GH secretion may also occur apparently without any recognized exogenous stimulus. Basal GH plasma levels are higher in females than in males. A decrease in plasma glucose concentration induces an increase in GH levels (Pecile and Olgiati 1978).

GH is necessary for longitudinal growth of the skeleton. Growth of the mammalian bones takes place at the epiphyseal growth plate, where condrocytes proliferate and synthesize a matrix composed of collagen and sulphated polysaccharides. Ellis et al. (1953) reported that sulphate incorporation in vivo into chondroitin sulphate of growing cartilage was reduced by hypophysectomy, and restored by GH administration. Salmon and Daughaday (1957) showed that sulfate incorporation into the organic matrix of the cartilage was stimulated by a normal serum component. The latter disappears from the serum of hypophysectomized animals, and reappears following GH administration. This component, initially called sulfation factor, and now termed somatomedin/insulin-like growth factor exerts a wide-scale influence on cartilage metabolism. Somatomedin is not GH, or its altered form, and is not a GH degradation product either. It is a second hormone released after GH stimulation. Somatomedin production was observed both in liver and skeletal muscle. Some other substances, e.g., amino acids and insulin also have somatomedin-like activities. Bioassays, RIA and receptor assay have equally been developed for assessing various somatomedins (Merimee 1979; for more details see Chapter 8).

GH has both insulin-like and anti-insulin effects. When injected intravenously, blood glucose levels decreases whithin 20 minutes, but these early insulin-like effects are not associated with changes in plasma levels of immunoreactive insulin. GH also has an "insulinotrop" effect. Exogenous GH administered to normal subjects stimulates insulin release. In vitro data support a direct effect of GH on the pancreatic β-cells.

GH has a well-pronounced anabolic effect. It increases the incorporation of amino acids into proteins probably indirectly, by somatomedin liberation. Lipid metabolism is also affected by GH. Chronic administration of GH to experimental animals leads to depletion of adipose stores, elevation in plasma-free fatty acid levels, and to an increased transfer of lipids to the liver. The respiratory quotient decreases simul-

taneously, indicating enhanced lipid oxidation. Ketosis may also occur (Merimee 1979).

The most commonly used bioassay is based on the observation that the width of the tibial epiphyseal growth plate increases when GH is injected into young hypophysectomized rats. In experimental and clinical practice, GH is measured by RIA. Prolactin does not appear to cross-react with GH antibodies, whereas placental GH may interact in late pregnancy. Normal basal levels in serum range between 1 and 5 ng/ml. This value is higher in females, probably due to the stimulatory effect of oestrogens. GH may also be measured by radioreceptor assay (Retetoff et al. 1979).

Insulin hypoglycemia induces about a 100% elevation in plasma GH levels. Arginine, but also other amino acids, as well as dopaminergic stimulation, glucagon, glucose, sleep and exercise also increase plasma levels of GH. Hyporesponsiveness is characteristically seen when GH secretion is impaired due to direct physical damage of the pituitary, hypothalamus, or some higher CNS structures, or to idiopathic hypopituitarism. Blunted or depressed responses, and occasionally increased GH secretion, may be seen in several nonpituitary diseases.

3.1.2.1.3. PROLACTIN (PRL): LACTOTROP HORMONE

Prolactin was first observed in 1928 as a component of pituitary extracts capable of inducing lactation in pseudopregnant rabbits (Stricker and Grueter 1928).

Riddle et al. (1933) used pigeons' crop sacs to characterize this substance and named it "prolactin". Prolactin has 198 amino acid residues in most species. It is a distinct molecule from both GH and placental lactogen in all species. Human prolactin is more similar to prolactins in other species than to human GH or placental lactogen. Its primary structure is the following:

Leu-Pro-Ile-Cys-Pro-Gly-Gly-Ala-Ala-Arg-Cys-Gln-Val-Thr-Leu-Arg-Asp-Leu-Phe-Asp-Arg-Ala-Val-Val-Leu-Ser-His-Tyr-Ile-His-Asn-Leu-Ser-Ser-Glu-Met-Phe-Ser-Glu-Phe-Asp-Lys-Arg-Tyr-Thr-His-Gly-Arg-Gly-Phe-Ile-Thr-Lys-Ala-Ile-Asn-Ser-Cys-His-Thr-Ser-Ser-Leu-Ala-Thr-Pro-Glu-Asp-Lys-Glu-Gln-Ala-Gln-Gln-Met-Asn-Gln-Lys-Asp-Phe-Leu-Val-Ser-Ile-Leu-Ile-Leu-Arg-Ser-Trp-Asn-Glu-Pro-Leu-Tyr-His-Leu-Val-Thr-Glu-Val-Arg-Gly-Asx-Gln-Glu-Ala-Pro-Glu-Ala-Ile-Leu-Ser-Lys-Ala-Val-Glu-Ile-Glu-Glu-Gln-Thr-Lys-Arg-Leu-Leu-Glu-Gly-Met-Glu-Leu-Ile-Val-Ser-Gln-Val-His-Pro-Glu-Thr-Lys-Glu-Asp-Glu-Ile-Tyr-Pro-Val-Trp-Ser-Gly-Leu-Pro-Ser-Leu-Gln-Met-Ala-Asp-Glu-Ser-Glu-Arg-Leu-Ser-Ala-Tyr-Tyr-Asn-Leu-Leu-Lys-Cys-Leu-Arg-Arg-Asp-Ser-His-Lys-Ile-Asp-Asn-Tyr-Leu-Lys-Leu-Leu-Lys-Cys-Arg-Ile-Ile-His-Asn-Asn-Asn-Cys (Shome and Parlow 1977).

Prolactin is produced by the eosinophyl cells in the anterior pituitary whose distinction from GH-containing cells proved to be rather difficult and required the use of sensitive immunological methods (Pasteels et al. 1972). Prolactin release is regulated by PIF and/or PRH, but it is influenced by catecholamines and other neurotransmitters as well (Tindal 1978).

In human plasma, prolactin is present in several molecular sizes. Using chromatography, at least two distinct immunoreactive forms could be detected: the one present in the major peak was termed "little" prolactin (M_r 23,000), and another in a minor peak was called "big" prolactin (M_r 56,000). Following prolonged storage or repeated freezing, "big" prolactin may be converted into the "little" one, but no conversion of the little to big has been observed. This suggests that the "big" is not formed by binding the "little" to a carrier protein. This is in agreement with the finding that prolactin from the pituitary has an immunoreactivity pattern similar to that found in the plasma (Suh and Frantz 1974). Half-life of prolactin is about 20 minutes in human plasma. Prolactin levels in human amniotic fluid were found to be 2-200 times higher than in maternal serum (Fang and Kim 1975). Prolactin was detected in the cerebrospinal fluid, milk and also in the semen. Relatively high prolactin binding was found in the rat adrenal gland,

breast, ovary, kidney, and prostate. Other species have different relative amounts of prolactin bound in these tissues (Posner et al. 1974).

In concert with oestrogens and adrenal steroids, prolactin has a prominent role in pre- and postpubertal development of the ducts of the mammary gland. During pregnancy, prolactin is essential for lactogenesis. The action of prolactin on the breast does not appear to require activation of the adenylate cyclase in the cell membrane, and cAMP does not mimic the effect of prolactin.

In the rat ovary, prolactin was shown to have a luteotropic effect, and together with LH, prolactin maintains progesterone secretion in the corpora lutea. Its luteolytic or antigonadal effect is dependent on the timing of administration and on the dose. In human, excessively high prolactin levels may be associated with a reduced testicular androgen response to exogenous gonadotrophins: reduction of prolactin levels by ergot derivates may normalize the response, and restore both fertility and libido.

Metabolic effects of large doses of prolactin resemble those of GH (i.e., nitrogen retention, insulin antagonization, lipid mobilization) in a number of species.

Mean prolactin serum concentrations range between 5-10 ng/ml, both in girls and boys. In puberty, there is a slight increase in girls, whilst the levels remain unchanged in the boys (Aubert et al. 1974). In a 24-hour period, there is a sleep-associated night-time rise in prolactin levels. All kinds of stress release prolactin. There is a steady rise in maternal prolactin serum levels during gestation, reaching a peak value (200 ng/ml) at term, which may be due to the elevated serum oestrogen levels. After the start of suckling, maternal prolactin serum levels tend to rise within a few minutes, and reach 6-10-fold increase by the end of the nursing episode. In the midcycle, or in proestrous animals, prolactin levels are elevated (Frantz 1979).

3.1.2.1.4. THYROID-STIMULATING HORMONE (TSH): THYROTROPIN

TSH is the principal hormone which maintains the structure of the thyroid gland, as well as biosynthesis and secretion of the thyroid hormones, tyroxine (T_4) and triidothyronin (T_3).

TSH is a glycoprotein containing 15% carbohydrate. Its estimated molecular weight is 28,300 daltons. It is composed of two peptide chains designated as α and β subunits both having multiple disulfide bonds (Fig.3.3; Liao and Pierce 1971).

a.

10 20
Phe-Pro-Asp-Gly-Glu-Phe-Thr-Met-Glx-Gly-Cys-Pro-Glx-Cys-Lys-Leu-Lys-Glu-Asn-Lys-
30 40
Tyr-Phe-Ser-Lys-Pro-Asx-Ala-Pro-Ile-Tyr-Gln-Cys-Met-Gly-Cys-Cys-Phe-Ser-Arg-Ala-
50 60
Tyr-Pro-Thr-Pro-Ala-Arg-Ser-Lys-Lys-Thr-Met-Leu-Val-Pro-Lys-Asn-Ile-Thr-Ser-Glx-
70 80
Ala-Thr-Cys-Cys-Val-Ala-Lys-Ala-Phe-Thr-Lys-Ala-Thr-Val-Met-Gly-Asn-Val-Arg-Val-
90 96
Glx-Asn-His-Thr-Glu-Cys-His-Cys-Ser-Thr-Cys-Tyr-Tyr-His-Lys-Ser

b.

10 20
Phe-Cys-Ile-Pro-Thr-Glu-Tyr-Met-Met-His-Val-Glu-Arg-Lys-Glu-Cys-Ala-Tyr-Cys-Leu-
30 40
Thr-Ile-Asn-Thr-Thr-Val-Cys-Ala-Gly-Tyr-Cys-Met-Thr-Arg-Asx-Val-Asx-Gly-Lys-Leu-
50 60
Phe-Leu-Pro-Lys-Tyr-Ala-Leu-Ser-Gln-Asp-Val-Cys-Thr-Tyr-Arg-Asp-Phe-Met-Tyr-Lys-
70 80
Thr-Ala-Glu-Ile-Pro-Gly-Cys-Pro-Arg-His-Val-Thr-Pro-Tyr-Phe-Ser-Tyr-Pro-Val-Ala-
90 100
Ile-Ser-Cys-Lys-Cys-Gly-Lys-Cys-Asx-Thr-Asx-Tyr-Ser-Asx-Cys-Ile-His-Glu-Ala-Ile-
110 113
Lys-Thr-Asn-Tyr-Cys-Thr-Lys-Pro-Gln-Lys-Ser-Tyr-Met

Fig. 3.3. Amino acid sequences of α(a)-and β(b)-subunits of bovine thyroid-stimulating hormone. Underlined residues indicate possible glycosylation sites

The α-subunit is a 96 amino acid polypeptide (M_r 14,700), and is virtually identical with the α-subunits of LH and FSH in amino acid composition and immunological properties, however, the polysaccharide side chains of these subunits are quite different. The β-subunit is somewhat larger, containing 113 amino acid residues (M_r 15,600). The β-subunit of human TSH is structurally distinct from those of human LH, FSH and HCG. Antisera were raised against both the intact TSH and the subunits. Most of the immunoterminant sites reside in the hormone-specific β-chain (Pierce 1974).

TSH-secreting cells represent about 3-5% of the pituitary cell population in euthyroid subjects. In the rat, experimental hypothyroidism caused a three-fold incerease in thymidine uptake, and a six-fold increase in the mitotic index of TSH secreting cells.

Secretion of TSH is initiated by TRH-induced activation of the adenylate cyclase system in the plasma membrane of the thyrotroph cells. TSH secretion can be blocked by thyroid hormones in vitro, or by calcium deprivation, without completely abolishing the TSH synthesis-enhancing effect of TRH. TRH may stimulate TSH synthesis independently of TSH secretion. TSH production has been estimated to be roughly equivalent to one pituitary TSH pool per day. The site of TSH degradation in humans is unknown at present. Experiments in dogs suggested the kindneys as the primary site of TSH elimination (Ridgeway et al. 1974). TSH in the peripheral plasma represents a heterogenous population of TSH molecules. In blood plasma of euthyroid individuals, at least three forms could be identified: two forms represent intact TSH molecules, whilst the third one represents the α-subunit of TSH. The role of the subunit in the plasma is unclear at present. In euthyroid subjects the relative amount of the α-subunit in plasma is similar to that found in the pituitary gland.

Concerted efforts to improve existing assays for TSH resulted in the elaboration of numerous bioassays, but unfortunately none of them proved to be sensitive enough to allow routine determinations of TSH in the serum. Currently serum TSH is measured by RIA. Antibodies raised againts intact TSH usually react with the α-subunit common to TSH, LH, FSH or HCG. However, immune sera reacting with the uncommon β-subunit are also available for RIAs.

Since circulating TSH levels are regulated by the plasma concentration of free T_4 and T_3, an elevated peripheral TSH level is a sensitive marker of primary hypothyroidism. In myxedema, the high serum TSH levels are often associated with low T_4 and normal T_3 levels, the peripheral level of T_4 is thought to contribute to the pituitary suppression. On the other hand, TSH blood levels were found to be low, or even undetectable in

all forms of hyperthyroidism, except the rare secondary and tertiary forms (Retetoff et al. 1979).

3.1.2.1.5. LUTEINIZING HORMONE (LH) AND FOLLICLESTIMULATING HORMONE (FSH)

are separately discussed in Chapter 4.

3.1.2.2. PEPTIDE HORMONES FROM THE PARS INTERMEDIA OF THE PITUITARY GLAND

3.1.2.2.1. MELANOCYTE-STIMULATING HORMONE (MSH)

α-MSH is a 13 amino acid peptide, whilst β-MSH has 18 amino acid residues in most mammals but 22 in humans. Both types of MSH originate from a common precursor molecule, named proopiocortin (Krieger et al. 1980, for more details on the chemistry of MSHs see Chapter 5). Both peptides produce a dispersal of melanin pigment granules in the melanophores of amphibians, replites and some invertebrates (Dahlberg 1961).

The role of MSH in human physiology is unclear at present (Thody 1980). β-MSH and ACTH levels increase parallel in man receiving insulin or methyrapone (Hirata et al. 1975). Diseases characterized by an increased skin pigmentation are associated with hypersecretion of ACTH (Addison's and Cushing's diseases), and increased β-MSH blood levels were also reported in such diseases (Abe et al. 1969), while others pointed out that serum components corresponding in molecular size to β-MSH could not be detected in the blood of patients with Addison's and Cushing's diseases (Bachelot et al. 1977).

3.1.2.3. PEPTIDE HORMONES FROM THE NEUROHYPOPHYSIS

3.1.2.3.1. ANTIDIURETIC HORMONE (ADH): VASOPRESSIN

Histochemical studies have provided evidence that cells in the supraoptic and paraventricular nuclei are involved in the synthesis of vasopressin in the hypothalamus (Sachs et al. 1969). Vasopressin-synthesizing neurons are different from the

oxytocin-synthesizing ones. Vasopressin is synthesized initially as a part of a larger molecule in the neurosecretory granules (Swaab and Pool 1975). The rate of vasopressin biosynthesis in vivo is estimated to be 10^{-4}-10^{-6} µ mol/hour. Formation of the final form of vasopressin from the precursor molecule whithin the neurosecretory granules is accomplished during axonal transport to the neurohypophysis, the site of storage. The speed of axonal transport may be several millimeters per hour.

It was supposed that vasopressin and its associated "carrier protein", neurophysin, are synthesized together as part of a common precursor protein (Sachs et al. 1969). Oxytocin and its neurophysin are also co-manufactured (Pickering 1978). These peptides were described by vanDike et al. (1942) without recognizing their relationship to the posterior pituitary hormones. The neurophysins stored in neurosecretory cells are believed to be the carrier proteins for the intraneuronal transport of oxytocin and vasopressin from the site of synthesis to the posterior lobe. Two types of neurophysins have been found in each mammalian species which seem to correspond to oxytocin and vasopressin. The two neurophysins are single-chained polypeptides of 93-95 residues and have 7 disulfide bridges. The two types have also been recognized in man, horse, dog and rat, so that duality seems general in mammals. Neurophysins bind all the neurohypophyseal hormones, and both types bind oxytocin and vasopressin equally well, suggesting a lack of binding specificity. There are a number of arguments suggesting that each neurophysin shares a common precursor with a neurohypophyseal hormone. Pre-proneurophysin with a molecular weight of 23,000-25,000 and proneurophysin with a molecular weight of 17,000 were also identified (Acher 1981).

The structure of arginine vasopressin was elucidated by Du Vigneaud et al. (1953a) and subsequently synthesized (Du Vigneaud 1956). It is a 9 amino acid peptide with multiple molecular forms in various species. (Fig.3.4). Arginine vasopressin (AVP) is present in most mammals whilst lysine vasopressin (LVP) was found in few mammalian species only. On the other

hand, arginine vasotocin fullfils the role of vasopressin in all nonmammalian vertebrates (Fig.3.4).

Common structure with variable amino acids in positions 3,4 and 8 denoted by X: Cys-Tyr-(X)-(X)-Asn-Cys-Pro-(X)-GlyNH_2 (9)

	Amino acids in positions 3	4	8	Probable phyletic distribution
Basic principles:				
Arginine vasopressin	Phe	Gln	Arg	Most mammals
Lysine vasopressin	Phe	Gln	Lys	Some mammals (pigs, etc.)
Arginine vasotocin	Ile	Gln	Arg	All nonmammalian vertebrates
Oxytocin-like ("neutral") principles:				
Oxytocin	Ile	Gln	Leu	Mammals, birds (reptiles?) amphybians lungfishes, holocephalians
Mesotocin	Ile	Gln	Ile	Reptiles, amphybians,lungfishes
Isotocin	Ile	Ser	Ile	Actinopterygian fishes
Glumitocin	Ile	Ser	Gln	Some elasmobranchs

Fig. 3.4. Amino acid sequences of the most important natural neurohypophyseal hormones

All these peptides produce vascular constriction as well as antidiuresis, the main effects of vasopressin. It has repeatedly been shown that vasopressin-releasing stimuli modify the firing patterns of supraoptic neurons, but a causal releationship between recorded electrical activity and hormone release has not been established. Increased osmotic pressure in the cerebral circulation decreases renal water excretion, and injection of a hypotonic solution into the carotid artery causes a promt diuresis, suggesting the exsistence of very sensitive central osmotic receptors. Vascular functions may significantly affect urine flow, and the role of blood volume in the regulation of vasopressin secretion has been widely recognized. The chest and the left atria were shown to be the sites of the volume receptors, and parasympathetic fibers are the most likely pathways carrying the stimulus induced by volume changes to the hypothalamus. Under physiological conditions, volume and osmotic stimuli act synergically, but sensitive RIA measurements (Dunn et al. 1973) suggested that osmoreceptors were more sensitive than the volume receptors. Baroreceptor signals from the carotid sinus via the glossopharyngeal nerve, and from the aortic arch via the vagus nerve, influence va-

sopressin release. Adrenergic mechanisms, prolactin, adrenocortical hormones, thyroxin, temperature, physical and emotional stress, and numerous pharmacological agents are also able to affect vasopressin release.

Most of the evidence suggests that vasopressin circulates in a free form. Half-life of the circulating vasopressin was found to be 10-20 minutes in man. In rats, mainly the kidneys (43%) and the liver (50%) contribute to the removal of both vasopressin and oxytocin from the circulation. About 6-30% of endogenous vasopressin is excreted in the urine in a biologically active form. In pregnancy the enzyme systems (oxitocinase) involved in the inactivation of both vasopressin and oxytocin are identical (Sandow and Konig 1978).

Failure of the hypothalamo-neurohypophyseal system to secrete vasopressin in response to appropriate stimuli leads to a disturbed water metabolism. This is characterized by excretion of a large volume of hypotonic urine, which obligates the intake of an identical amount of fluid to prevent hyperosmolarity of body fluids, as well as dehydration (diabetes insipidus). About 50% of the patients with diabetes insipidus belong to the idiopathic group, and the other 50% to the secondary types associated with tumor, the posthypophysectomy state, vascular lesions, inflammation, trauma, etc. In clinical practice various vasopressin preparations are available for the therapy of diabetes insipidus (Kleeman and Berl 1979).

3.1.2.3.2. OXYTOCIN

Oxytocin is produced in the hypothalamus by the neurons in the paraventricular and supraoptic nuclei. Its structure was elucidated by du Vigneaud et al. (1953b) and Tuppy (1953). Like vasopressin oxytocin is a nonapeptide with a structure shown in Fig.3.4. This form of oxytocin is found both in mammals and birds. Biosynthetic mechanisms of oxytocin are similar to those of vasopressin. In addition to producing neurosecretory materials, and transporting them along the axons (speed of the axonal flow is approximatelly 2-3 mm/day) into the posterior pituitary for storage, these neurons also

transmit neural inpulses to the posterior lobe to induce immediate hormonal release. Following a secretory stimulus, hypothalamic synthesis of oxytocin is initiated after a lag of an hour or more. Calcium is essential for oxytocin release from the neurophysin-oxitocin complex. In humans, pronounced oxytocin release is seen during suckling (Vorherr 1979). Since vasopressin and oxytocin may be released independently, some of the nerve terminals must contain neurovesicles loaded exclusively with oxytocin or vasopressin.

Oxytocin released upon a suckling stimulus causes contraction of myoepithelial cells surrounding mammary alveoli, and resultant milk ejection. In the absence of oxytocin (e.g., after hypophysectomy) women fail to lactate. Sensitivity of the myometrium to exogenous oxytocin increases as pregnancy advances. However, the observed oxytocin-independent, intrinsic myometral activity, and the normal labor of hypophysectomized women (no postpartum lactation!) fail to support the assumption which regards oxytocin as the main factor involved in the initiation of labor. However, the observation that contractility of the postpartal uterus increases upon suckling indicates a close correlation between suckling-induced oxytocin release, myometrial activity and postpartum uterine involution (Vorherr 1979).

A small amount of oxytocin injected into the mammary artery elicits milk ejection, an effect which is utilized in oxytocin bioassay. Currently, however, sensitive and specific RIA methods are also available for measuring oxytocin in the plasma.

In the human (Wathes et al. 1982) and pig ovary (Pitzel et al. 1984) more oxytocin and vasopressin has been determined than was expected on the basis of peripheral blood levels. Oxytocin is probably produced by corpus luteum (Khan-Dawood et al. 1984), however, its role in the function of the ovary is not yet known (Varga et al. 1985).

Plasma half-life of oxytocin is short; 50% of injected oxytocin disappears from the circulation within 5 minutes. The kidneys and the liver seem to be the main organs responsible for oxitocin removal from the circulation. However, the uterus

and the mammary gland, the target organs of oxytocin, may also trap and remove a considerable amount of oxytocin from the circulation. Unlike normal plasma, plasma from pregnant women is able to inactivate oxytocin and vasopressin in vivo, due to the presence of the oxytocinase enzyme in syncytiotrophoblasts of the placenta.

In clinical practice oxytocin is used to induce, or to augment myometrial concentration during labor, or to treat postpartum hemorrhage. Decreased secretion of oxytocin during the lactation period, which might be due to cerebral trauma or tumor, or to a decreased responsiveness of mammary myoepythelia to oxytocin, may lead to nursing difficulties. In such cases and if the process of milk synthesis is unimpaired, administration of oxytocin promptly elicits milk ejection.

3.1.3. PEPTIDE HORMONES IN THE REGULATION OF CALCIUM METABOLISM

All vertebrate species are able to regulate calcium levels in their body fluids with remarkable precision. In mammals, calcium reservoires of the bone, absorption of calcium from the gut, and excretion of calcium via the kidneys are the main factors and mechanisms involved in the regulation. These factors and mechanisms are modulated by three hormones, two of which, the parathyroid hormone and thyrocalcitonin are peptides, whilst the third is 1,25-dihydroxycholecalciferol derived from vitamin D_3. Therefore, the latter will not be discussed here.

3.1.3.1. PARATHYROID HORMONE (PTH): PARATHORMONE

PTH is generally a 84 amino acid polypeptide. In man PTH was also synthesized and characterized as a peptide of 84 amino acids that has the following primary structure:

Ser-Val-Ser-Glu-Ilu-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn-Phe-Val-Ala-Leu-Gly-Ala-Pro-Leu-Ala-Pro-Arg-Asp-Ala-Gly-Ser-Gln-Arg-Pro-Arg-Lys-Lys-Glu-Asp-Asn-Val-Leu-Val-Glu-Ser-His-Glu-Lys-Ser-Leu-Gly-Glu-Ala-Asp-Lys-Ala-Asp-Val-Asp-Val-Leu-Thr-Lys-Ala-Lys-Ser-Gln (Fairwell et al. 1983). It is produced by two successive cleavages of N-terminal sequences of a larger precursor molecule, the 115 amino acid pre-proparathyroid hormone (pre-proPTH) in the parathyroid gland. The earliest cleavage occurs in the rough endoplasmic reticulum, and results in the removal of 25 N-terminal amino acids, forming an intermediate precursor, the proparathyroid hormone (proPTH). The second cleavage occurs in the Golgi complex and removes additional 6 amino acid residues from the N terminus (Habener 1981). After the hormone is released from the gland and enters the circulation, a third highly specific cleavage occurs in the liver (D'Amour et al. 1979) and the kidneys (Martin et al. 1979). This is a proteolytic cleavage between residues 33-34, but also at several other sites in the middle region of the molecule. The peripheral cleavage might represent an activation step prior to action of the secreted peptide on the target cells, since it yields a minimum amino acid sequence required for a proper biological activity. For further information on the chemistry and biosynthesis of PTH see Aurbach et al. (1985).

Intracellular levels of cAMP change parallel with PTH secretion, when secretion is stimulated by secretagogues, such as epinephrine, isoproterenol, dopamine, secretin, prostaglandin E_2 and hypocalcemia. Whether or not the hydroxylated metabolites of vitamin D actually exert any regulatory effects on the parathyroid gland remains undetermined. Cortisol may also influence PTH secretion by a mechanism independent of changes in blood calcium levels. Growth hormone should also be considered as a potential regulator of parathyroid gland activity (Habener 1981; for a human parathyroid hormone-like peptide of tumor origin see Mangin et al. 1988).

3.1.3.2. CALCITONIN (CT): THYROCALCITONIN

Copp et al. (1962) observed first that perfusion of the thyroid-parathyroid complex with hypercalcemic plasma resulted in a significant fall in peripheral calcium levels, and the fall was larger than expected on the basis of suppression of parathyroid hormone. This suggested a hypocalcemic substance liberated in response to hypercalcemia. Hirsh et al. (1966) demonstrated the thyroid origin of this substance, called calcitonin. The amino acid sequence of calcitonin has been determined in about ten different species. Calcitonins from different species proved to have similar structure with only minor differences in the sequence of the straigh-chain, 32 amino acid peptide:
Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-Gly-Thr-Tyr-Thr-Glu-Asp-Phe-Asn-Lys-Phe-His-Thr-Phe-Pro-Glu-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro. Nonmammalian calcitonins are more stable and more active than are mammalian ones (Copp 1979).

Peripheral calcitonin levels are elevated in a variety of diseases including medullary carcinoma of the thyroid gland, islet cell tumors, malignant tumors, renal failure, etc. In these conditions, the immunologically detectable calcitonin proved to be heterogenous by chromatographic criteria. The heterogeneity has a significance in calcitonin measurements, and also in the understanding of secretory events. The fact that secreted calcitonin is more heterogenous than that in the glandular tissue suggests that some of the heterogeneities result from postsecretory events (Pont 1979; for further information on, and recent advances in, the chemistry of calcitonin see Aurbach et al. 1985).

Calcitonin secretion is regulated by plasma calcium levels. It is now believed that the principal role of calcitonin is to prevent hypercalcemia. However, thyroidectomy does not elicit hypercalcemia. Gastrointestinal hormones, like gastrin, pancreozymin (cerulein) and glucagon increase calcitonin blood levels. Calcitonin may also contribute to the regulation and faciliation of calcium absorption and distribution (Talmage and Cooper 1979).

3.1.3.3. CALCITONIN GENE-RELATED PEPTIDES (CGRP)

α-CGRP, a 37 amino acid peptide, is the result of alternative processing of RNA transcribed from the calcitonin gene Its primary structure was established to be:
Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-Hys-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Glu-Ala-PheNH_2 (Amara et al. 1982).

α-CGRP occurs abundantly in neural elements and is likely to have a neuropeptide role (Mason et al. 1984, see also Chapter 5.). An mRNA product of a gene related to the one encoding calcitonin/α-CGRP has been identified in rat brain and thyroid gland (Amara et al. 1985). The peptide, designated as β-calcitonin gene-related peptide differs from α-CGRP only in one amino acid: it contains Lys instead of Glu in position 35.

3.1.4. PEPTIDE HORMONES IN THE REGULATION OF CARBOHYDRATE METABOLISM

3.1.4.1. INSULIN

Mering and Minkowsky (1890) demonstrated that the pancreas plays an important role in the prevention of diabetes. Banting and Best (1922) produced the first efficient insulin preparation. Preparative methods based on Banting and Best's procedure developed rapidly and yielded commercially utilizable insulin preparations. As a result, within a one year period following its discovery, insulin was applied in clinical practice. However, the elucidation of the structure of insulin proved to be a more difficult problem. Although the early observation that insulin was destroyed by proteolytic enzymes suggested the protein character of the active agent, it was initially not appreciated that proteins might function as hormones. Insulin may be found in the whole vertebrate kingdom, but immunologic and biologic evidence also indicates occurrence in the digestive system of some invertebrate species.

The primary structure of bovine insulin was determined first (Ryle et al. 1955). Since then, the structure of insulin has been identified in about 30 species. The insulin molecule consists of an "A" chain (21 amino acids) and a "B" chain (30 amino acids). The two chains are linked together with two disulfide bonds, and a third intrachain disulfide bridge is found inside the "A" chain. (Fig.3.5). Amino acid substitutions may be accomplished at many positions without a significant change in biological activity of the molecule.

```
                                  10                                  20
Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-
                                  30                                  40
Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-Arg-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Val-Gly-
                                  50                                  60
Gln-Val-Glu-Leu-Gly-Gly-Gly-Pro-Gly-Ala-Gly-Ser-Leu-Gln-Pro-Leu-Ala-Leu-Glu-Gly-
                                  70                                  80
Ser-Leu-Gln-Lys-Arg-Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-Gln-
                              86
Leu-Glu-Asn-Tyr-Cys-Asn
```

Fig. 3.5. Amino acid sequence of human proinsulin. The N-terminal 30 residues and the C-terminal 21 residues of proinsulin constitute the B- and A-chain of mature insulin, respectively. The two chains are linked with a 35 amino acid connecting peptide (C-peptide) within the proinsulin molecule. Cysteine residues at positions 7 (B-chain) and 72 (A-chain) and those at positions 19 (B-chain) and 85 (A-chain) are disulfide-bonded, respectively, while an additional (intramolecular) disulfide bridge is present between cysteine residues at positions 71 and 76 within the A-chain

On the other hand, certain structural features are conserved throughout the vertebrate evolution. Insulin is formed as a part of proinsulin, containing the "A" and "B" chains in a single 9000 dalton peptide (Steiner et al. 1969). Proinsulin is synthesized in the rough endoplasmic reticulum of pancreatic β-cells and is subsequently cleaved during its intracellular transportation yielding an intact insulin molecule and a peptide fragment, known as the connecting or C-peptide (Fig.3.5). Both insulin and the C-peptide are then stored in the secretory granules along with small amounts of residual proinsulin. Length of the proinsulin molecules in various mammals ranges from 78 (dog) to 86 (human, horse, rat) amino acid residues. Proinsulin is similar to insulin in many properties, including solubility, isoelectric point and reactivity with

insulin antisera. This suggests that conformation of the insulin moiety within the proinsulin might be identical to that of the authentic insulin. The intracellular conversion of proinsulin to insulin also continues in the presence of protein synthesis inhibitors, indicating that continuous protein synthesis is not a prerequisite of the transformation process. The conversion of proinsulin to insulin in intact rat islet cells takes place in about one and a half hours, and is probably initiated in the Golgi apparatus.

Translation of islet nucleic acids in a wheat germ ribosomal system gives rise to preproinsulin, a major immunoreactive peptide with a molecular weight of 11,500, which consists of proinsulin and a 23 amino acid long N-terminal extension (Steiner and Tager 1979).

Morphological studies on newly formed secretory granules in a variety of cells suggest that they undergo biochemical maturation after their formation in the Golgi apparatus. In the β-cells, the "progranules" are less dense than the mature granule inclusions. A variety of biochemical changes may occur in these granules, including the proteolytic conversion of proinsulin to insulin. The dense central inclusions in the mature insulin-secreting granules are crystallized, and these crystals are closely similar to those observed in the ordinary zinc-insulin preparations (Lange et al. 1972). It appears that insulin tends to crystallize with zinc when the insulin is liberated from proinsulin. The role of zinc in secretion granule formation is not fully understood. It has been suggested that the microtubular-microfilamentous system of the β-cells is involved in the intracellular transport of granules (Lacy and Malaisse 1973).

During the process of proinsulin conversion within the secretion granules, the C-peptide and insulin accumulate in equimolar quantity and are cosecreted by exocytosis. Proinsulin comprises 2-9% of the immunoreactive insulin-like materials of the pancreas. Similar values are found in the portal vein in man. This value is, however, lower than that found in the peripheral serum, because in the periphery metabolism of proinsulin is very slow.

Glucose is the most important insulin release-stimulating physiologic substance. Amino acids have also been shown to stimulate insulin release in the absence of glucose. Epinephrine inhibits insulin release triggered by a variety of agents, an effect prevented by α-adrenenrgic blockers (Porte 1967). Conversely, cholinergic agents stimulate insulin release, and this effect can be blocked by atropine. Growth hormone, glucocorticoids, placental lactogen and sex hormones also affect insulin release. High levels of growth hormone or corticoids, present in acromegaly or in Cushing's syndrome, markedly enhance insulin secretion. Placental lactogen, progesterone and estrogens seem to participate in the enhanced insulin output in the second half of pregnancy (Rubenstein 1979).

Insulin is distributed in a fluid space larger than the plasma volume and has a short (3-5 minutes) half-life. Liver and kidneys appear to be the major sites of both uptake and degradation of insulin (Kaden et al. 1973, Rubenstein et al. 1975). Two enzyme systems were implicated in the degradation of insulin. One is the gluthation-insulin transhydrogenase (Varandani 1972) which catalyses the cleavage of interchain disulphide bonds by gluthation, with a consecutive liberation of intact "A" and "B" chains. The other is an insulin-specific protease with highest activity in liver, pancreas and the kidney, which accomplishes a rapid degradation of both chains into small molecular weight peptide fragments.

Primary or idiopathic diabetes mellitus is a genetically determined metabolic disorder associated with insulin insufficiency. Clinically it had two major types; the "juvenile-onset" type is characterized by a complete insulin insufficiency. Patients suffering from this type of diabetes are dependent on exogenous insulin for survival. On the other hand, the "maturity-onset" type is "insulin independent", where ketosis does not develop without insulin therapy. Therefore it is also designated as "ketosis-resistant" diabetes. In the latter form, the therapeutic goals can be achieved with diet, or diet plus oral drugs, but exogenous insulin may be required for the correction of fasting hyperglycemia. Initial-

ly it was thought that these two types of diabetes differ from each other only in the "quantity" of the defect ensuing from the secretion and action of insulin. However, more recent evidence suggests the heterogeneity of "idiopathic diabetes mellitus" in terms of inheritance, enviromental factors, insulin response to glucose and prevalence of vascular disease. It appears that "idiopathic diabetes mellitus" is not a specific disease entity, but a syndrome associated with a number of diseases.

3.1.4.2. GLUCAGON

Kimball and Murlin (1923) first suggested the existence of a pancreatic hormone exerting an effect opposite to that of insulin. The assumed active agent was referred to as hyperglycemic, or glycogenolytic factor, later called glucagon. Glucagon was isolated and crystallized (Staub et al. 1955), and subsequently its amino acid sequence was also reported (Bromer et al. 1957). Accordingly, glucagon is a 29 amino acid peptide which exhibits minor species variations. The primary structure of the human peptide is the following:
His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr (Steiner and Tager 1979).

Glucagon is secreted by the pancreatic α-cells into the intercellular space. This peptide is known to increase glucose production through stimulating both glycogenolysis and gluconeogenesis. The main function of the α-cells, and thus glucagon, is to prevent hypoglycemia. Hypoglycemia virtually never occurs in normal individuals, not even after a prolonged starvation or hard exercise. Insulin-induced hypoglycemia is accompanied by a rapid rise in plasma glucagon levels. In hypoglycemia accompanied by a low insulin level, α-cell secretion rises dramatically. After a carbohydrate meal, or after glucose administration, plasma insulin levels increase and glucagon levels decrease, and liver is converted from a glucose-producing organ to a glucose-storing one. Ingestion of a

pure protein meal elicits a prompt and significant increase in plasma glucagon levels. Upon exercise, normal islet cells respond with an increased glucagon and decreased insulin secretion. This bihormonal system increases hepatic glucose production and allows an increased glucose utilization in skeletal muscles without reducing arterial glucose level (Unger and Orci 1979). Severe stress conditions are accompanied by absolute hyperglucagonemaia, and by absolute or relative hypoinsulinemia. Under such conditions, this hormonal setting increases glucose production and minimizes glucose utilization in muscle and fat cells, thereby conserving endogenously produced glucose for the brain (Lindsay et al. 1975).

Available evidence suggests that pancreatic α-cells function abnormally in all forms of diabetes mellitus. It has been proposed that the consequences of insulin deficiency on the underutilization and overproduction of glucose by the liver largely depend on the absolute or relative excess of glucagon (Unger and Orci 1979). Glucagon rises promptly following insulin withdrawal, and this is associated with a rise in the blood ketone levels. Ketosis can be prevented by suppressing glucagon levels with somatostatin.

Disappearance of glucagon from the circulation is rapid; its half-life is estimated to be about 6 minutes. Glucagon levels in the portal blood was estimated to range between 300-5,000 pg/ml, which is compatible with its binding to hepatic receptors. Glucagon is mainly degraded in the liver and kidneys. Kidneys are the major posthepatic site of glucagon clearence. It has been suggested that glucagon is filtered through the glomerulus and reabsorbed in the proximal tubules, where it undergoes intrarenal degradation.

As a gross disorder of glucagon secretion, a separate glucagon producing pancreatic tumor, "glucagonoma" has been reported (Mallinson et al. 1974).

3.1.5. THE AMINE PRECURSOR UPTAKE AND DECARBOXYLATION (APUD) SYSTEM

Isolated endocrine cells dispersed within the body have been suggested to represent a separate system, called the diffuse endocrine system (Feyrter 1969). The essential features of these cells are their capability to take up amine precursors (like dopa or 5-HT), to decarboxylate them and to produce biogenic amines, which may be utilized as precursors for the production of more complex peptides (Pearse 1969). The APUD system includes all peptide-producing cells dispersed in the stomach, duodenum, intestine, pancreatic islets, adrenal medulla, extradermal paraganglia cells, adenohypophysis, parafollicular thyroid cells and melanoblasts. Cells with APUD characteristics were also found in the respiratory system, gastrointestinal and urogenital tracts, but some of their secretory products are unidentified even today (Pearse 1969 1980).

The APUD system may have originated from primitive neural transmitter cells, which migrated from their original location to populate other organs and tissues as the environmental requirements changed. As their capability to synthesize biogenic amines remained unchanged, some components of the system acquired the capacibility of producing more complex peptides (Pearse 1980).

A number of hormone-producing tumors are derived from APUD cells (i.e., ACTH-producing oat cell carcinoma of the lung). The APUD concept may be useful for explaining the pathogenesis of the multiple endocrine neoplasia syndromes. The neoplastic transformation of these cells may result in severe structural and functional changes in the secretory apparatus (Sherwood and Gould 1979). The name apudoma was introduced by Szijj et al. (1969), and is used to designate a variety of endocrine tumors originating from APUD cells.

The APUD concept is supported by the fact that gastrointestinal peptides were found in different regions of the brain, and vice versa, some hypothalamic/brain peptides were observed in some cells of the gastrointestinal tract. The concept

offers a rational, but at present, largely speculative explanation for many obscure features of tumor-associated hormone production. However, the APUD concept will apparently not resolve all the problems connected with the abnormal activity of related tumors. Many neoplasms associated with abnormal peptide production cannot be included in this system. It appears likely that APUD characteristics may not be the exclusive domain of neuroectodermal cells, but can also be found in endodermal cells. One should keep in mind, however, that even unicellular organisms are capable of producing messenger substances such as transmitters, modulators and hormones, including substances of sophisticated structure like the peptide messengers (LeRoith and Roth 1984, Krieger 1985). Each cell in a higher organism has the genetic information and thus capable of evolving the underlying biochemical machinery to produce the same messenger substances as do the cells specialized for intercellular communication. The property which is unique to the cells specialized for organized communication is to produce and store the messenger substances in proper intracellular compartment in proper quantities which enables the cell to liberate the messenger substance(s) in a patterned fashion upon appropriate stimuli.

3.2. SELECTED TOPICS ON THE PATHOLOGY OF SOME PEPTIDE HORMONES

In dealing with the clinical-endocrinological aspects of the endopeptides it must be emphasized that the theme is so widespread that only selected topics can be discussed in some detail.

The disturbances of carbohydrate metabolism (diabetes mellitus syndrome, the hypoglycemia syndrome) are reviewed in monographs (Magyar and Tamas, eds. 1979, Ellenberg and Rifkin 1983) and recent reviews (Unger and Orci 1977, Salans 1982).

The endocrinology of the placenta (Everett and MacDonald 1979), the role of gastrointestinal hormones in pathological processes (Guigan 1978), or some humoral syndromes associated with cancer (Odell and Wolfsen 1978) are beyond the scope of

this review. Therefore, this chapter is limited to the discussion of diseases in connection with the hormones of the adenohypophysis (except TSH, because of its limited role in human pathophysiology, Mihalovic et al. 1980), and LH-FSH, which are discussed in Chapter 4, and to diseases related to the calcium-regulating hormones.

3.2.1. GROWTH HORMONE (GH)

In 1886 Pierre Marie reported 2 patients with "acromegaly". Minkowsky observed the frequent association of acromegaly and pituitary enlargement in 1887, and shortly thereafter Benda (1900) described the acidophil adenoma. The first successful hypophysectomy was performed by Schloffer in 1907 and Li and coworkers confirmed increased growth hormone (GH) secretion in acromegalic patients in 1948.

3.2.1.1. EXCESS SECRETION: ACROMEGALY, GIGANTISM

GH, an important peptide-hormone of the adenohypophysis, is essential in facilitating normal growth. Its secretion is under the stimulating and inhibiting control of the hypothalamus. Before puberty GH overproduction causes gigantism, while after puberty the excess secretion of GH results in acromegaly, i.e., gross disfigurement of the face, hands, feet and other parts of the body, crippling arthritis, heart disease, hypertension, lung disease, a number of dermal changes and abnormalities in carbohydrate, lipid and mineral metabolism. On the skeleton, excess GH produces an increase in linear growth before the epiphyses are closed and an increase in linear bone formation after closure (Raisz and Kream 1981). Formerly, the disease was considered relatively uncommon; in Zurich 45 cases were found among 36,000 patients, almost equally in both sexes. A survey in the Newcastle region found a prevalence of 40 cases per million population (Thomas 1983). Generally in acromegaly or in gigantism GH overproduction is

due to an acidophil adenoma of the pituitary. Recently the improved methods (RIA, sellar tomography, etc.) disclose far more patients.

Nowadays it is generally accepted that in acromegaly the cause of GH overproduction is, in contrast to other hypersecretory pituitary tumors, an adverse reactivity of the somatotrophs (Werner 1978). The adverse reactivity may present an abnormal response mechanism in the hypothalamus, but intrinsic anomalies of the tumor cells are suggested as well. It was postulated (Goldfine 1978) that in certain cases of acromegaly the GH secretion by the pituitary is not autonomous, but to some extent can be regulated by the hypothalamus. Even an overstimulation by GH-releasing factor(s) can occur. In active acromegaly GH hypersecretion has variable diurnal fluctuations but the nocturnal rise is characteristically absent (Jacquet et al. 1980). Serotonin antagonists inhibit both normal and pathological GH secretion in certain cases (Delitala et al. 1976).

On the other hand, numerous studies suggest qualitative differences. In acromegaly certain stimuli provoke paradoxical responses. Sometimes induced hypoglycemia produces a fall, hyperglycemia produces a rise in plasma GH. Response to arginine, or exercise might be normal, absent, or paradoxical. TRH and GnRH have no effect on GH in healthy subjects, but in acromegaly they stimulate its release (Goldfine 1978), acting as an adenylate cyclase activator (Irie and Tsushima 1972, Matsukura et al. 1977). Under normal conditions the dopamine agonist L-Dopa, ergot alkaloids and apomorphine increase GH release, but become depressors in acromegaly (Liuzzi et al. 1974a,b, Thorner et al. 1975, Camanni et al. 1977). In vitro studies demonstrated that the abnormal response to glucose, TRH and dopaminergic drugs are related to intrinsic anomalies of the tumor cells (Mashiter et al. 1977, Ishibashi and Yamaj 1978). It is concluded that in acromegaly the somatotrophs become fundamentally similar to lactotrophs. All or some GH-producing cells become less differentiated, with multiple hormone receptors on cell surface. Therefore, acromegaly is considered to be a receptor recognition disease of all GH pro-

ducing cells or sometimes of a clone of cells, forming pituitary adenomas (Werner 1978). Matsukura et al. (1977) and Camanni et al. (1977) strenghtened the hypothesis that GH-releasing dopaminergic agents act normally via central neurons, while in acromegaly the dopamine receptors develop within the tumor cells themselves. Plasma GH seems to be heterogenous in healthy subjects. In acromegaly "large GH" without remarkable biological activity is found in smaller percentage of the total (Van den Brande 1979).

Patients with acromegaly have reduced life expectancy: 50% die before the age of 50 and 89% before 60 years, which is almost twice the expected. The deaths are mainly due to cardiovascular and respiratory diseases in man and cerebrovascular and respiratory diseases in women (Wright et al. 1970). Patients with gigantism seldom reach age 30 without treatment.

Enlargement of the hands and feet, disfigurement of the face, visceromegalias are well-known consequences. The most serious complaints are perhaps those connected with joint abnormalities. Destruction of the hip sometimes urges replacement therapy with prostheses. Profuse sweating is almost always present, and impotence or amenorrhoea are common and not only in cases with coexistent hyperprolactinemia.

Plasma prolactin (PRL) levels are often elevated, sometimes galactorrhoea develops. After sulpiride or TRH loading, blunted PRL response was observed, irrespective of basal levels (Zseli et al. 1980a). Urinary free cortisol output often increases (Lindholm et al. 1980). Coexistent autonomous thyroid adenoma with hyperthyroidism seems to be a common finding ("Troel-Junet syndrome", Horvath et al. 1981).

GH excess is associated with a spectrum of carbohydrate abnormalities: impaired insulin release, chronic stimulation of insulin production, decreased peripheral insulin sensitivity are suggested (Lippe et al. 1981) resulting in a tendency toward or in actual diabetes mellitus in acromegalics. Diabetes mellitus is more frequent and severe in patients with higher GH values (Jadresic et al. 1982).

Serum phosphorus is generally elevated due to metabolic processes and increased renal tubular reabsorption. Hypercal-

ciuria and elevated serum calcium are found in 10% (West 1981). GH-stimulated intestinal calcium hyperabsorption or increased parathormone (PTH) action can be involved. Recently elevated serum levels of both 1,25- and 24,25-dihydroxy-cholecalciferol were found (Lund et al. 1981). In the kidney glomerular filtration rate and creatinine clearance also increase (Eskildsen et al. 1979).

Conventional irradiation therapy or megavoltage therapy is rarely followed by total cure, even ten years afterwards (Thomas 1983, Wass and Besser 1983). External irradiation with heavy particles with or without the utilization of Bragg peak effect is available only in certain centers. At present radioactive yttrium or gold implantation seems to have minor significance (Quabbe 1982, Thomas 1983).

Bromocriptine treatment seems to be a reliable method, although in much higher doses than recommended for prolactinomas. In Besser's series (1978) it was clinically effective in 71 out of 73 patients, but GH levels were normalized in only 15 of them. The formerly elevated PRL levels became undetectable, glucose tolerance improved. If initially higher, somatomedin A levels were normalized, even in one case where GH failed to decrease (Werner et al. 1978).

At present transsphenoidal microsurgery seems to be the optimal form of therapy (Hardy et al. 1978, Pasztor et al. 1981, Quabbe 1982), but the results are not as good as with other pituitary tumors. When extrasellar extension exists, cure cannot be achieved (Arafah et al. 1980). Adjuvant bromcriptine therapy is suggested and sometimes even tumor-size reduction is observed (Spark et al. 1982). After successful microsurgery, physiological regulation baseline and dynamic GH secretion can be achieved, but bromocriptine therapy does not induce the resumption of physiological GH secretion (Jacquet et al. 1980).

Bromocriptine mainly inhibits GH release and not its synthesis (Sachdev et al. 1981) perhaps without or only with a slight effect on tumor growth.

Many effects cannot be attributed to GH itself but to one or several factors that are formed or activated under the in-

fluence of GH: the somatomedins (Megyesi 1978, Nevo and Laron 1979, Philips and Vassilopoulou-Sellin 1980). The Daughaday group (Salmon and Daughaday 1957, Daughaday 1984) disclosed that GH does not mediate directly the sulfation of hypophysectomized rat cartilage, but via anabolic, insulin-like peptides. Disease states connected with excess or deficient GH have been associated with increased or decreased somatomedin levels (Van Wyk and Underwood 1975). Generally good correlation exists between somatomedin concentrations, GH concentrations and growth rate, but exceptions definitely exist, mainly in different types of dwarfisms. In bromocriptine-treated acromegalics, however, discordance was observed in disease activity, GH and somatomedins (Werner et al. 1978). In Stonesifer's series (1981) the changes in plasma GH or somatomedins correlated poorly with clinical response. However, somatomedin-C determination seems to be very useful in the diagnosis and evaluation of activity in (untreated) acromegaly (Van Wyk and Underwood 1975).

3.2.1.2. GROWTH HORMONE DEFICIENCY

Human growth hormone deficiency is characterized by inhibition of growth, retarded bone development, discrepancy in "age and bone age", anomalies in carbohydrate metabolism ranging from hypoglycemia to carbohydrate intolerance (Lippe et al. 1981). Both increased insulin sensitivity and relative endogenous insulin deficiency may be present. Erythrocyte and monocyte receptor binding seems to be normal.

When dealing with the effects of endopeptides, GH deficiency should not be discussed in detail. However, certain aspects have to be mentioned. The causes leading to GH deficiency are the following: (1) organic changes in the hypothalamus or pituitary (craniopharingeomas or other rare tumors and infections); (2) Idiopathic forms with male predominance (in certain cases hereditary factors or obstetric traumas are involved). Two genes, involved in the biosynthesis of human GH are located on chromosome 17. The alterations of these genes

may cause GH deficiency and growth failure (Chawla et al. 1983); (3) Malnutrition and emotional depression (Van den Brande 1979); (4) Special forms of reduced efficiency; (a) The so-called Laron's dwarfism (Laron et al. 1971) is characterized by a very low somatomedin activity despite elevated serum GH, implying a hereditary defect in the GH receptor. GH therapy is ineffective; (b) In the cases of Kowarsky et al. (1978) GH secretion was normal, but the levels of plasma somatomedins were low. GH administration restored somatomedin levels and induced growth. In this disease the defect may be due to the secretion of a biologically ineffective GH; (c) In the case of Lanes et al. (1980) GH was normal, but somatomedin was elevated: a previously unrecognized defect in somatomedin responsiveness was suspected; (d) In pigmies, insulin-like growth factor I deficiency was obsreved, while GH was normal (Merimee et al. 1981).

Nowadays we are able to treat patients with GH deficient hypopituitary with human GH. Neither baseline, nor human GH-stimulated somatomedin-C levels correlated with growth response (Rosenfeld et al. 1981). Somatomedin-C response to treatment remained constant, while growth rate declined in the same patient. In the series of Kemp et al. (1981) somatomedin levels remained low despite adequate growth. Recently GH treatment again suggested for short stature in children with normal GH levels (Van Vliet et al. 1983).

3.2.2. PROLACTIN

3.2.2.1. PROLACTIN EXCESS: HYPERPROLACTINEMIA

A special form of hypothalamic dysfunction, leading to the syndrome of postpartum amenorrhea, persistent galactorrhea, reduced gonadotropin and estrogen levels often with moderate obesity was described by Chiari in 1852, and Frommel in 1882 (Chiari-Frommel syndrome). In 1953 Argonz and del Castillo characterized a syndrome now bearing their name as a hypothalamopathy leading to amenorrhea and galactorrhea with-

out (remarkable) organic changes in the hypothalamus or pituitary. Forbes and coworkers described the so-called amenorrhea-galactorrhea with a pituitary tumor (Forbes-Albright syndrome) but without pregnancy in 1954. In 1965 Canfield and Bates presented bioassay evidence of excessive lactogenic activity in the sera of patients with pituitary tumors and galactorrhea. Fluckiger and Wagner in 1968 disclosed that the ergot alkaloide 2-bromo-α-ergocryptine (bromocriptine) is a potent inhibitor of prolactin (PRL) secretion. The radioimmunoassay for human PRL (Hwang et al. 1971, Turkington 1972) has markedly expanded our knowledge of the most common human pituitary tumor, the prolactinoma and of other disorders in connection with the impaired secretion of PRL.

From a practical point of view two main types of hyperprolactinemic states can be distinguished: primary hyperprolactinemia mainly due to an autonomous (?) pituitary micro- or macroadenoma, and secondary hyperprolactinemia observed in certain metabolic and endocrine disorders (chronic renal failure, hypothyroidism, etc.) or as a result of taking certain drugs or consuming alcohol.

The most important and most frequent hyperprolactinemia is the one caused by a pituitary micro- or macroadenoma, the prolactinoma. Currently, prolactinomas are considered to be the most frequent pituitary tumors with a female predominance: from more than 400 surgically treated pituitary adenomas 180 cases (160 females, 20 males) were found (Hardy et al. 1978). Pasztor et al. (1981) found it in 59% of 117 patients. Generally the adenomas are small, so they can be detected only by the polytomography of the sella turcica (Vezina 1978, Kollin 1982) or by computed tomography (McGregor and Ginsberg 1981, McGregor et al. 1978, Chiodini et al. 1981), but macroadenomas, mainly in men (Prescott et al. 1982) occur as well. The main cell type, the chromophobe, was considered to be without endocrine function before hormone determinations or prior the use of special staining or electron microscopy.

PRL is the only hormone of the adenohypophysis which is mainly under the inhibitory control of the hypothalamus. The

most important if not the only PRL inhibitory factor (PIF) is dopamine. After pituitary stalk damage PRL secretion as well as plasma PRL levels increase. A nonfunctioning pituitary tumor can obstruct the transport of PIF, but, considering the weak correlation between the size and place of the tumor and the observed hyperprolactinemia, this possibility seems unlikely (Quigley et al. 1980). The theory of defective synthesis and/or release of PIF suggests primary hypothalamic defect, as well as the decreased central conversion of L-Dopa to dopamine (Fine and Frohman 1978). The excessive release of PRL-releasing factor(s) is only speculative. Strong arguments against tumor autonomy are the excellent therapeutic results obtained with the dopamine-agonist ergot derivates, mainly with bromocriptine. Recent data (discussed later) give another explantation for this, though the problem of autonomy is still an open question. Based on the results of animal experiments, Malarkey (1979) suggested that prolactinoma cells are refractory to hypothalamic control as a result of the development of a defective receptor to PIF. The diminished effect of TRH or other stimulatory agents (metoclopramide, sulpiride, chlorpromazine), generally used in diagnostic tests to differentiate among prolactinomas and "functional" hyperprolactinemias seems to be in good agreement with this view. Moreover, in prolactinomas, an increased hypothalamic secretion of dopamine was suggested, implying reduced sensitivity of tumor lactotrophs (Quigley et al. 1979, 1980, Scanlon et al. 1981). Altered dopamine degradation and/or reduced receptor binding affinity are also possibilities.

If an adenoma is of primary pituitary origin, the selective total removal of the tumor should be followed by an immediate return of plasma PRL levels to normal, and the regulation of PRL secretion should retain its physiological characteristics. In fact, numerous studies prove that this happens (Hardy et al. 1978, Kirby et al. 1979, Edwards and Feer 1981, Pasztor et al. 1981, Tucker et al. 1981). When the adenoma was completely removed, ovulatory cycles resumed after 35 ± 4 days (Jacquet et al. 1978), galactorrhea ceased, the formerly impaired response to TRH and chlorpromazine became normal, suggesting the ab-

sence of all observable abnormalities in the remaining PRL-secreting pituitary cells. During normal pregnancies (where the PRL level is high) stimulation of the nipples provokes a further significant rise. In pregnant women with successfully operated prolactinomas similar results were found.

In rats, if the anterior pituitary is surgically separated from the hypothalamus, the PRL-releasing effect of TRH is blunted, reminding us of the situation observed in human prolactinomas. Moreover, in anencephalic infants, chlorpromazine directly stimulated PRL secretion (Taga et al. 1981).

Hyperprolactinemia is common in patients with chronic renal failure, the incidence reported between 20-37% rising nearly to 80% in patients on maintenance hemodialysis (Chirito et al. 1972, Nagel et al. 1973, Cowden et al. 1978, Gomez et al. 1980, Sieversten et al. 1980). Each hemodialysis patient receiving α-methyldopa (an antihypertensive, acting as a false transmitter in dopa-metabolism, thus with certain PRL-increasing effects) (Steiner et al. 1976, Arze et al. 1981) had even higher PRL levels (Gomez et al. 1980, Szucs et al. 1984b,c). In a young hypertensive woman with proliferative glomerulonephritis treated with α-methyldopa, hyperprolactinemia amenorrhea, and galactorrhea developed as renal function diminished (serum creatinine: 500 μmol/l). After discontinuing methyldopa, regular menses reappeared, galactorrhea stopped plasma PRL dropped, remaining only slightly elevated (Szucs and Csillag 1982).

A significant correlation exists between plasma creatinin and PRL in patients with various degrees of ranal failure (Cowden et al. 1978). A 16% decrease of PRL between the renal artery and the vein in healthy subjects proves the role of the kidney in PRL metabolism. The metabolic clearance rate of radiolabeled PRL decreases by 33% in hemodialysis patients (Sieversten et al. 1980). However, the frequency of hyperprolactinemia suggests impaired feedback regulation as well. Also the suppressibility with exogenous dopamine is blunted, suggesting a direct resistance at the dopamine-receptor level or in its postreceptor metabolism.

Hyperprolactinemia is common (30-40%) in patients with acromegaly. Hypersecretion of GH and PRL results from two histologically distinct cell types (Kirby et al. 1979). In hypothyroidism, probably TRH excess is included (Jackson 1982). In Cushing's disease the nyctohemeral rhythm, the nocturnal elevation is absent (Krieger et al. 1976). The basal morning levels and mean 24-hour levels are often elevated, but response to TRH or hypoglycemia seems to be normal. After selective adenectomy PRL secretion returns to normal (Caufriez et al. 1981). Suda et al. (1981) could not find these alterations. Hyperprolactinemia was found in 6 of 47 patients with the "empty sella syndrome", a syndrome with enlarged sella turcica due to intrasellar cysternal herniation without pituitary enlargement (Brismar 1981). In these patients the response to TRH or L-Dopa was normal.

It is of outmost importance to mention that various types of drugs elevate plasma PRL. The most important groups are the neuroleptics, antidepressants, antihypertensives, opiates, estrogens and antiestrogens. While examining a patient with hyperprolactinemia, drugs (or alcohol, Cicero 1981) as causative factors must be ruled out!

The clinical significance of hyperprolactinemia lies in the important but not fully clarified role of PRL in the human reproductive process.

Blood concentrations of PRL are slightly higher in healthy women due to the stimulatory effects of estrogens. Any further elevation can be the cause of female infertility. Basal plasma concentrations below 60 ng/ml indicate peripheral (ovarian) involvement (Pozo et al. 1978): regular menses may occur, but anovulation or defective progesterone secretion is common (luteal insufficiency). Above this limit hyperprolactinemia is usually associated with cessation of cyclic activity suggesting an interference at the level of the hypothalamus. Any type of menstrual abnormality (dysfunctional uterine bleeding, oligo-raromenorrhea, etc.) as well as decreased libido, hirsutism may occur. Galactorrhea is present in one-third of the cases (L'Hermite et al. 1978), while in cases of idiopathic galactorrhea without amenorrhea PRL remains normal. In women

with secondary amenorrhea the incidence of hyperprolactinemia is about 15% and a clear-cut prolactinoma is the cause in 5%.

After discontinuing oral contraception regular menses sometimes fails to appear. In the so-called "post-pill amenorrhea" hyperprolactinemia was found in 32% in patients with galactorrhea, while only in 2% without galactorrhea.

Hyperprolactinemia affects sexual behavior: each woman with prolactinoma had sexual dysfunction (Buvat et al. 1978). On the other hand, PRL was normal in 40 frigid women.

Most patients with mastopathia or benign breast diseases are normoprolactinemic, but in some of them hyperprolactinemia exists. However, if mastalgia occur, bromocriptine can give relief in both groups, implying a causative role for PRL (Mansel 1981, Editorial, Lancet 1982).

Bromocriptine seems to be effective in certain patients with premenstrual tension or periodic edema with or without hyperprolactinemia (Andersch et al. 1978).

Hirsutism is not uncommon in hyperprolactinemic women. δ-5-Androgens (androstendiol, dehydroepiandrosterone and its sulphate) are increased (Giusti et al. 1978). Dehydroepiandrosterone sulphate levels were higher in patients with prolactinomas than in those with secondary hyperprolactinemias (L'Hermite et al. 1978). In hemodialyzed uremic patients with elevated PRL, plasma dehydroepiandrosterone sulphate levels seem to be normal (Szucs et al. 1984b,c).

Several reports discuss the interrelation between hyperprolactinemia with or without pituitary tumors and male sexual disorders (Saidi et al. 1977, Carter et al. 1978, Roulier et al. 1978, Snyders et al. 1979, Schiavi 1981). The clinical spectrum involves impotence, hypogonadism, defects in spermatogenesis, occasionally galactorrhea and gynecomastia (Roulier et al. 1978, Goser and Schindler 1980). Trimmer (1981) suggests that drug-induced hyperprolactinemia causes impotence less frequently than primary PRL disorders. Of 8 men with prolactinoma, each had sexual dysfunction. Of 103 impotent men, plasma PRL was slightly elevated in 6.8% and 1 had macroprolactinoma (Buvat et al. 1978). In patients with anejaculation, the TRH test suggested an impaired PRL response. In another

study, hyperprolactinemia was found in 2 of 71 patients with infertility and none in 53 with impotence (Rjosk and Schill 1979). Although in some patients with oligospermia, hyperprolactinemia or prolactinoma were disclosed (Roulier et al. 1978), the effect of elevated plasma PRL on human spermatogenesis is poorly understood and should be interpreted with caution. Bromocriptine treatment improves sexual function only in hyperprolactinemic patients.

Hyperprolactinemia is common in uremic men on maintenance hemodialysis.. Decreased libido or impotence due to hyperprolactinemia was observed in about 25% (Gomez et al. 1980) to 78% (Cowden et al. 1978). Sexual dysfunction improves after bromocriptine (Bommer et al. 1979), even when serum testosterone remains unchanged (Szucs et al. 1984b,c). Although the acute PRL-lowering effect of bromocriptine is less pronounced in uremic patients, long-lasting treatment resulted in a significant reduction (Cowden et al. 1978, Szucs et al. 1984b,c).

In 1969 Hardy offered a special, safe and effective neurosurgical method to eradicate pituitary adenomas. The transphenodial approach, using magnification and direct illumination of the reduced operative field with the binocular surgical microscope has allowed the clear visualization of a microadenoma as small as 2 mm in diamater. Moreover, it has made it possible to achieve complete and selective microsurgical removal of the tumor with the preservation of normal pituitary gland and function (Hardy et al. 1978). The results are impressive, external irradiation to prevent or cure recidivas are seldom necessary (Edwards and Feer 1981). However, the results of surgical treatment in patients with macroprolactinomas are disappointing (Nabarro 1982).

Recently the therapeutic considerations have been reevaluated (Spark et al. 1982). While earlier neurosurgery seemed to be the first step, mainly in patients with the chiasma syndrome, or in infertile women to facilitate pregnancy, today dopamine-agonist treatment has to be taken into account (Krupp and Turkalj 1984). Bromocriptine, offered by Fluckiger and Wagner as early as 1968, in daily doses of 2.5-20 mg, effectively reduces plasma PRL levels. Clinical symptoms caused by

hyperprolactinemia disappear soon, and normal pregnancy occurs. Moreover, even the size of the tumor is reduced parallel with therapy. Using computed tomography, Thorner et al. (1981) observed rapid changes in prolactinoma volume after withdrawal and reinstitution of bromocriptine. The disappearing and reappearing visual field defect correlated with the therapy. Impressive improvement in perimetry was noticed as early as 2 days after initiating treatment (Chiodini et al. 1981). In a recent study (Chiodini et al. 1981), a clear-cut reduction in tumor size was shown in 18 of 29 patients. The reduction was rapid, but in some patients occurred only after 2-3 months.

The mechanism by which dopamine agonists can reduce tumor size is still open to question. An antimitotic effect on lactotrophs was suggested (MacLeod and Lehmayer 1973, Lloyd et al. 1975), and a specific antiproliferative effect om tumoral PRL-secreting cells with high proliferative rate in humans was also considered (Eversman et al. 1979). A reduction in the vascular bed of the tumor may be responsible for the rapid changes, however, dopamine agonists lack vasoconstrictive effects.

The possibility of reducing the size and stopping the secretory activity of a macroprolactinoma by treatment with bromocriptine should be emphasized because neurosurgery in this situation often fails both in eradicating the tumor and normalizing PRL levels. It should be mentioned that there is no strict correlation between pretreatment PRL levels and tumor shrinkage. Moreover, in some patients the PRL level fell without any decrease in tumor size (Chiodini et al. 1981).

Other drugs (Lisuride, pergolide mesylate, etc.) exist as well with similar effects on PRL levels and tumor size reduction.

Treatment with megavoltage radiotherapy alone or in combination with interim bromocriptine may result in definite tumor shrinkage and control of hyperprolactinemia (Grossman et al. 1984).

In the so-called secondary hyperprolactinemias, where possible, causal therapy, not medical treatment, is suggested.

3.2.3. ACTH

3.2.3.1. EXCESS SECRETION

(1) Cushing's disease

In 1932, Harvey Cushing described a syndrome characterized by abdominal obesity with thin extremities, cutaneous striae, hirsutism, amenorrhea, osteoporosis, hypertension and often diabetes mellitus. He called the syndrome "pituitary basophilism" now known as "Cushing's disease". Today it is well known that each clinical and metabolic abnormality observed in this disease results primarily from chronic excess of cortisol and androgens.

The pathophysiology of the syndrome is still debated but from a practical point of view we must distinguish between "primary adrenocortical neoplasm" (adenoma or carcinoma, almost always unilateral) which is beyond the scope of this review and "excessive ACTH production with bilateral adrenocortical hyperplasia".

Cushing's disease is a rare disease, occurring far more frequently (4:1) in females. The so-called "form fruste"-s (Glaz 1981) are found easier. In Zurich 30 cases were found among 30,000 patients in the Department of Medicine (Labhart 1974). In Budapest, at the Endocrinological Outpatients Clinic, 1st Department of Medicine, Semmelweis University Medical School we found 29 cases out of 8,000 patients with suspected endocrinological abnormalities, except diabetes mellitus, and thyroid diseases (25 pituitary-dependent, 2 ectopic ACTH syndrome, 1 adrenal adenoma, 1 alcohol-induced pseudo-Cushing's disease).

The excessive ACTH production, causing bilateral adrenocortical hyperplasia may originate from: (a) the pituitary (generally a microadenoma); (b) ectopic source (the ectopic ACTH syndrome); (c) alcohol induction (pseudo-Cushing's disease); (d) iatrogenic source (therapeutic use of ACTH).

It is no longer justified to differentiate hypercortisolism and Cushing's syndrome or Cushing's disease without specification of etiology, since the existence of an "autonomous" baso-

philic adenoma of the pituitary gland has become questionable. Basophilic, chromophobe or mixed-cell adenomas are all found (Rovit and Duane 1969). The multiplicity of pituitary changes are understandable when the transformational capacity of the adenohypophyseal cells are taken into account. Moreover, basophil adenomas are found in 15% of postmortem examinations without signs of adenocortical hyperfunction. Even the autonomy is questionable.

Pituitary tumors develop particularly often after total adrenalectomy (Nelson's syndrome), i.e., after removal of the suppressive influence of cortisol. Therefore the tumors have been considered hyperplasiogenic, developing under increased stimulation by the hypothalamic corticotropin-releasing factor (CRF).

The most constant histological finding in the pituitary in patients with Cushing's disease seems to be the so-called Crooke-cells (basophilic cells with a peculiar hyalinization of cytoplasma, with a tape-like hyalinized zone replacing the basophil granule). The hyalinization may be pronounced in animal experiments with cortisol, therefore, it is possible that it is not a sign of ACTH overproduction, but of glucocorticoids.

It is well known that ACTH is secreted episodically with a characteristic diurnal variation. A special two-phase negative feedback effect exists with cortisol. In Cushing's disease the diurnal variation is always absent and the absence of a nocturnal fall in ACTH secretion leads to a twofold increase in the daily output of cortisol (Retiene et al. 1965). Moreover, the hypothalamus is less sensitive to the negative feedback effect of cortisol, i.e., the regulatory cycle is set to a higher cortisol level (Liddle et al. 1962, Raux et al. 1975).

The ACTH feedback response to cortisol infusion has two (temporally and dynamically distinct) phases: an early, rate-dependent and a delayed, dose-dependent one. Patients with adrenalectomy for Cushing's disease have a normal second-phase feedback, but have an initial, paradoxical rise in plasma ACTH, suggesting that the negative first-phase feedback has been replaced by a positive feedback. In patients

with pituitary-dependent Cushing's disease whose pituitary adenoma was surgically removed in toto, the second phase feedback was intact, but the rate-sensing in the first phase remained abnormal (Lankford et al. 1981). This, too, suggests a site in the central nervous system for a feedback defect in the disease.

Other observations suggesting the role of higher centers in the pathogenesis of the ACTH-secreting pituitary tumors are: (a) Characteristic electroencephalographic changes remain present after cure (Krieger et al. 1975); (b) The pituitary tumor is not autonomous: different agents influence its ACTH secretion: high doses of glucocorticosteroids (Liddle 1960), TRH, LHRH, vasopressin, cyproheptadine (Krieger 1977, Krieger and Condon 1978, reactions not observed in healthy humans) and bromocriptine (Fluckiger and Wagner 1978).

The details of medical, surgical or radiotherapy are beyond the scope of this review. But in dealing with the pathophysiology of Cushing's disease, two important topics have to be mentioned: transsphenoidal microsurgery (Hardy 1969), and treatment with the serotonin-antagonist cyproheptadine (Krieger et al. 1975).

As mentioned above, the pathogenesis of Cushing's disease is still debated. Is the adrenal cortex or the pituitary the causal factor? Even now in monographs dealing with endocrine disorders, the syndrome is discussed under the heading "diseases of the adrenal cortex" (Hollo 1960, 1981, Labhart 1974). Recently, as a simple and safe method, transphenoidal surgery in certain centers has confirmed the presence of an ACTH-secreting pituitary tumor nearly in all cases (Bigos et al. 1980, Lamberts et al. 1980, Lankford et al. 1981). Based on this fact it seems likely that the pituitary tumor is the cause of Cushing's disease, however, long-term follow-ups are badly needed since it has not established whether the cure after the removal of the adenoma is permanent or not. The data suggesting a primary pathophysiologic role for CRF are also conclusive. For instance, there is histological evidence of ACT-secreting cell hyperplasia around the basophil adenoma: in the case of Lamberts et al. (1980) multiple ACTH-secreting

adenomatous cell nests and microadenomas were found throughout the pituitary. Electroencephalographic abnormalities remain unchanged, etc. It is possible that the suggested long-term follow-up of 21 patients with bilateral adrenalectomy must also be considered in patients with transsphenoidal microsurgery (Cohen et al. 1978). Ten pituitary tumors occurred among the patients: 8 bigger and 2 smaller ones. According to Cohen's opinion, treatment without preventive pituitary irradiation in patients with bilateral adrenalectomy is inadequate. We must emphasize again that long-term follow-up in patients with transsphenoidal microsurgery from this point of view is urgently needed.

In 1975, while studying the role of catecholaminergic and serotoninergic mechanisms in the regulation of CRF secretion, Krieger et al. (1975) established that cyproheptadine, a drug with antiserotoninergic activity induced remission in a patient with Cushing's disease. While other antiserotoninergic agents were ineffective, the use of cyproheptadine was beneficial in certain patients. The mechanism of action is unclear. The electroencephalographic abnormalities are corrected, and dexamethasone suppressibility returns in some patients (Krieger and Condon 1978). After removing the pituitary microadenoma, cyproheptadine changed the so-called "abnormal first-phase positive feedback" to cortisol infusion to a normal, negative feedback (Lankford et al. 1981). Even the site of action is unclear. In a patient with a carcinoid tumor of the foregut, cyproheptadine exerted a diminishing effect on the ectopic ACTH-producing cells, caused a remission in the symptoms of hypercorticolism, while leaving the highly elevated plasma serotonin level unchanged (Leveston et al. 1981).

(2) The ectopic ACTH syndrome

As mentioned above, ACTH is secreted by the cells of the pituitary. Recent data (Singer et al. 1978, Yalow 1979) disclosed that ACTH can be extracted from almost every human lung cancer tissue as well as from other APUD-cell tumors. While it is true that tumor-ACTH sometimes lacks biological activity ("big ACTH"), it is well known that in some patients with dif-

ferent malignomas a syndrome develops that is almost undistinguishable from pituitary-dependent Cushing's disease. The signs and symptoms of the "ectopic ACTH-syndrome" (Liddle et al. 1965, Yalow 1979) resemble those of Cushing's disease but hypokalemia and pigmentation are also common. After removing the primary tumor the signs due to adrenocortical overactivity disappear. Bronchogenic carcinoma is the most common (50%), but thymic carcinoma (25%), pancreatic carcinoma (15%), bronchial adenoma, cancer of the breast, colon, ovaries, testes, prostate, kidney, thyroid, salivatory glands (Labhart 1974) and carcinoid of the foregut (Leveston et al. 1981) may also occur.

(3) Ectopic secretion of CRF as a cause of Cushing's disease

In a case reported by Carey et al. (1984), the ectopic (prostatic) secretion of CRF resulted in excessive secretion of pituitary ACTH.

(4) Alcohol-induced pseudo-Cushing's syndrome

It is a rare condition induced by excessive alcohol consumption, which is clinically and biochemically undistinguishable from Cushing's syndrome and ceases after alcohol withdrawal (Smalls et al. 1976, Rees et al. 1977). The plasma level of ACTH is sometimes high, while in other cases it seems to be normal (Hasselbach et al. 1982). Moreover, ethanol had no acute effect on plasma cortisol in healthy male subjects (Davis and Jeffcoate 1983).

(5) Nelson's syndrome

In 1960 Nelson and coworkers described a syndrome of an ACTH-producing pituitary tumor which enlarges the sella turcica and develops after adrenalectomy for Cushing's disease. Melanin hyperpigmentation as well as visual field defects are usually present.

(6) Other syndroms with ACTH excess

These are usually secondary. Addison's disease (bilateral adrenocortical insufficiency), congenital adrenal hyperplasia (due to various enzymatic defects in cortisol production), some types of adrenogenital syndrome, and uremia (Aronin et al. 1981) must be mentioned.

3.2.3.2. ACTH DEFICIENCY

The syndrome of selective ACTH deficiency is rare (Nichols et al. 1978). The symptoms resemble the well known symptoms of Addison's disease, but the characteristic pigmentation is missing. Aldosterone secretion generally seems to be normal, therefore, severe hyponatremia is uncommon and develops only in patients on a sodium restricted diet.

The role of ACTH in the regulation of aldosterone secretion is dubious (Glaz 1981, Spat 1980). Some authors disclosed a correlation while other suggest that there is no need for ACTH in the regulation of aldosterone secretion (Merriam and Baer 1980).

Hypophysectomy, total or partial destruction of the pituitary due to various reasons causes ACTH deficiency.

3.2.4. PARATHORMONE (PTH)

In 1891 Von Recklinghausen distinghuished osteitis fibrosa from other diseases of the bone. The autopsy on a patient with this disease revealed a "lymph gland" above the left thyroid, now considered to be a parathyroid adenoma. In 1916, Schlagenhaufer suggested parathyroid tumor as a cause of osteitis fibrosa. In 1926, Mandl performed the first parathyroidectomy on a patient with severe bone disease and observed a dramatic improvement.

A close connection was suggested between chronic renal failure, bone lesions and the parathyroids by Bergstrand (1921). In 200 autopsies he found 10 cadavers with enlarged

parathyroids, out of which 9 had chronic renal disease as well. Castleman and Mallory (1937) suggested parathyroid hyperplasia to be a regular consequence of chronic renal failure.

Berson and Yalow worked out a parathormone (PTH) radioimmunoassay (RIA) in 1963.

3.2.4.1. EXCESS SECRETION: HYPERPARATHYROIDISM (HPT)

Primary hyperparathyroidism (pHPT) is a genuine disease, caused by a tumor or primary hyperplasia of parathyroid gland(s) without any recognized cause.

In secondary hyperparathyroidism (sHPT) the hyperfunction is compensatory and counteracts the decrease of blood-ionized calcium due to an underlying disease (mainly chronic renal failure or malabsorption).

Tertiary hyperparathyroidism is presumed when during the years of secondary HPT an autonomous adenoma originates from a hyperplasial parathyroid gland.

(1) Primary hyperparathyroidism(pHPT)

Recently pHPT has been considered a frequent disease with a prevalence of 1 in 1000 in the USA (Heath et al. 1980, Lafferty 1981) or 5.2 in Sweden (Christensson 1976). The disease is characterized by bone lesions (osteitis fibrosa sometimes with cysts: Morbus Recklinghausen) and renal calculi according to Albright and Reifenstein (1948). Nowadays we know that mild, asymptomatic cases are the most frequent (Coe and Favus 1980, Heath et al. 1980) and new symptoms (generalized calcipenic osteopathia, nephrocalcinosis, peptic ulcer, pancreatitis, neuromuscular and mental disorders, hypertension, anemia) have been added to the clinical spectrum.

The most common cause is a chief-cell adenoma, surrounded by a rim of normal tissue but oxyphil, clear cell or mixed-cell adenomas occur as well. Primary chief-cell hyperplasia (with characteristic disappearance of fat) is found

with increasing frequency, especially in familial cases or in the so-called multiple endocrine neoplasia syndrome (Eberle and Grun 1981). Sometimes it is almost impossible to differentiate between adenoma and hyperplasia using light or even electron microscopy. The best opportunity is during surgery: adenoma is suggested if one adenoma is enlarged while the others are atrophic. Adenomas are found in the thyroid, but may occur in the thymus behind the esophagus or other parts of the mediastinum. Parathyroid carcinomas are very rare.

Serum ionized calcium is the regulator of PTH secretion; neuroendocrine modulators (catecholamines, prostaglandins, Vora et al. 1981, Metz et al. 1978), 1,25-didydroxy-cholecalciferol (1,25-DHCC, Madsen et al. 1981) as well as serum Mg levels have only minor influence.

In the parathyroids a large polypeptide, pre-proparat hormone, is first synthesized then cleaved and stored in the gland as pro-PTH. The second cleavage also occurs in the glands, so the main secretory product in the circulation is the real hormone, 1-84 PTH (Habener 1981). The most important sites of degradation are the liver and the kidneys (Habener 1981, Segre et al. 1981). It is still debated whether the smaller peptide chains have any biological activity. The activity is connected to the terminal amino part of the molecule.

Serum PTH is determined by RIA. Since the first description by Berson and associates in 1963, various RIAs against C- or N-termini of the molecule have been used with varying results. It was disclosed that plasma PTH is immunoheterogeneous in hyperparathyroidism. The PTH(1-84) M_r 9,500 as well as the N-terminal fragments refer to the acute states of the disease, while the M_r 7,000 and C-terminal fragments reflect chronical states and have a better correlation with the clinical picture (Flueck et al. 1977). However, different PTH RIAs have not proved to be the ultimate discriminatory tests in the diagnosis of pPTH (Lafferty 1981). pPTH is considered to be an autonomous hyperfunction: despite elevated plasma Ca levels the parathyroids secrete PTH, which circulates in the blood in elevated or at least in high normal levels. This autonomy is

still debated: we know from the animal experiments of Gittes and Radde (1966) that the implantation of numerous parathyroid glands into intact rats causes long-standing hypercalcemia. In chronic renal failure sHPT is almost obligatory. After successful renal transplantation hypercalcemia sometimes develops and decreases only after months (David et al. 1973). According to Parfitt (1969) the secretion of the parathyroids depends on the mass of the glands and on the number of the secretory cells.

The action of PTH is mediated by cAMP. The most important target organs are the bone, the kidneys and the gut.

Thirty years ago pHPT seemed to be a rare condition, causing osteitis fibrosa cystica generalisata (M. Recklinghausen) or renal stone formation. Nowadays we know that osteitis fibrosa is a rare consequence of pHPT (about 6%) and renal calculi occur only in one out of five patients (Lafferty 1981) or even less frequently (4%, Heath et al. 1980). Recently mild asymptomatic forms discovered only by routine serum Ca analysis have been found in increasing frequency. But even these forms are potentially dangerous: in a 10-year follow-up study, 20% of such patients developed complications requiring parathyroid surgery (Frame and Rao 1980). Hypertension, unresponsive to angiotensin blockade (Zawada et al. 1980) is registered in 18-53% (Lafferty 1981). Emotional or mental disorders, diffuse calcipenic osteopathy ("osteoporosis") and diminished renal function are observed in every fith-sixth case, while peptic ulcers, pancreatitis and myopathy (Heath et al. 1980, Laferty 1981) are rare. Sometimes monoclonal gammopathy occurs (Zseli et al. 1980b). Anemia is uncommon (5%, Boxer et al. 1977) but sometimes removal of the adenoma leads to an improvemant. Severe anemia suggests the diagnosis of nonparathyroid neoplasma with hypercalcemia (Falko et al. 1976) Occasionally pHPT causes an acute syndrome, characterized by severe hypercalcemia, life-threatening cerebral, gastrointestinal, cardiac and renal disturbances ("parathyroid storm, Bayat-Mokhtari et al. 1980) leading to death without immediate surgery.

In typical cases with pHPT, plasma PTH and serum Ca (especially the ionized fraction) is elevated, plasma cAMP and urinary excretion of nephrogenous cAMP is increased, phosphorus as well as the tubular reabsorption of phosphate are decreased. Increased plasma alkaline phosphatase activity resembles compensatory osteoblastic activity. But in mild or atypical cases plasma Ca as well as immunoreactive PTH levels seem to be normal (Laferty 1981). Interestingly, the elevation of plasma chloride is the most common finding.

Surgery is the only possible therapy and is suggested even in mild, asymptomatic cases because the procedure is curative and easy to perform for experienced surgeons. When surgery is delayed, intensive follow-up is needed to prevent severe complications.

(2) Secondary hyperparathyroidism (sHPT)

sHPT is an obligatory complication of chronic renal failure. Phosphate retention and impaired renal degradation, skeletal resistance and calcium-malabsorption due to diminished 1,25-DHCC production are the main causes. Immunoreactive PTH levels are extremely elevated, mainly if C-terminal RIAs are performed, since C-terminal fragments are eliminated in the kidneys (Slatopolsky et al. 1980). Although these fragments are biologically inactive in physiological concentrations, in the observed high concentrations they may explain some of the alterations in uremic state. Massry (1977) and Massry and Goldstein (1979) suggested PTH as one of the most important uremic toxins.

The best-known consequence of secondary HPT in chronic renal failure is uremic bone disease, which develops almost at the beginning of renal disease and becomes more severe during the months of maintenance hemodialysis without adequate treatment (Szucs 1973). Treatment is very difficult, although vitamin D metabolites, frequent dialysis with elevated fluid-Ca content, calcitonin or other agents are often effective.

Soft tissue calcification occurs in the skin, joints, kidneys, lung, heart, blood vessels, brain, sometimes forming big palpable masses under the skin. Encephalopathy, peripheral

neuropathy, disturbances in the carbohydrate, lipid and mineral metabolism, sexual impotence, muscular alterations, gastrointestinal bleeding (Slatopolsky et al. 1980) and severe pruritus (BMJ Editorial 1980) were also observed.

Anemia is obligatory in chronic renal failure. The pathogenesis is not fully understood; diminished erythropoietin production, deficiency in iron, folic acid, protein or various amino acids, decreased life span of red blood cells, and myelofibrosis may be responsible. Toxic substances, for example, with markedly increased PTH levels have been suggested recently (Podjarny et al. 1981). Intact PTH or its C-terminal fragments exert an inhibitory effect on erythropoiesis, an effect which could be overcome by erythropoietin (Meytes et al. 1981). After subtotal parathyroidectomy (removal of 3 and 1/2 glands) many clinical signs (bone dystrophy, soft tissue calcification, neurological signs, general condition, anemia) may improve. We personally recommend subtotal parathyroidectomy to patients with an unusually high need for blood supply in the treatment of anemia (Mako et al. 1981, Szucs et al. 1983).

Another important disorder of calcium metabolism the hypercalciuria syndrome, has recently been divided into absorptive and renal forms, except the so-called normocalcemic pHPT with hypercalciuria. While in the absorptive form the parathyroid function is normal or even suppressed (Pak et al. 1974), sHPT develops in the renal form . It must be mentioned that Burckhardt and Jaeger (1981) failed to find an elevated immunoreactive PTH level in idiopathic renal hypercalciuria.

(3) Hypercalcemia in tumoral states

Hypercalcemia is a relatively common finding in patients with cancer, and develops also in patients without bone metastases. In certain cases plasma PTH is highly elevated, the tumor contains PTH-like activity, and after removal of the tumor (lung, breast, kidney) plasma PTH drops to normal.

A hypercalcemic peptide, the so-called osteoclast-activating or -stimulating factor, was isolated from leukocytes of patients with plasmocytoma or other lymphomas. A third peptide, osteolysin, has a direct osteolytic activity. (It

should be mentioned that hypercalcemia may develop due to prostaglandin overproduction in certain tumorous situations as well; Odell and Wolfsen 1978, Gennari 1981, Raisz and Kream 1981).

In primary (or sometimes in severe secondary) HPT, surgery is the only curative intervention. In tumorous hypercalcemias removal of the tumor or symptomatic treatment with calcitonin, mithramycin, corticosteroids and (if prostaglandins are involved) prostaglandin synthesis inhibitors are suggested.

(4) States with decreased PTH sensitivity

Active vitamin D (1,25-DHCC) is needed for PTH to exert its effect on the bony tissue. In chronic renal failure 1,25-DHCC production is blunted and skeletal resistance to PTH is involved in the enormous increase of immunoreactive PTH in the blood. But there are situations where the target cell resistance to PTH is hereditary. The syndrome is called pseudohypoparathyroidism (PHP), because clinical signs and laboratory parameters resemble those of hypoparathyroidism despite highly elevated immunoreactive PTH (iPTH) in the blood (Van Dop and Bourne 1983).

PHP Type I. Normal individuals respond to PTH administration with an elevated urinary cAMP excretion. In this disease serum Ca is decreased, P and iPTH are increased, and PTH fails to increase cAMP excretion, suggesting a defect in the PTH-receptor-adenyl cyclase mechanism.

In PHP Type II, after PTH administration cAMP excretion increases, but serum Ca, P or urinary P remain unchanged, suggesting presence of the defect after cAMP formation.

There are patients whose bones are sensitive to PTH but whose kidneys are not. In them hypocalcemia, hyperphosphatemia and elevated iPTH coexist with osteitis fibrosa and with lack of a phosphaturic effect of exogenous PTH. Urinary excretion of cAMP is various and resembles PHP Type I or II (Duck et al. 1981). In some cases a dissociation of PTH bioactivity and immunoreactivity was suggested (Deuxchaisnes et al. 1981).

The clinical spectrum involves short stature, round face, short neck, thick body, short metacarpal and metatarsal bones

(especially the fourth and fifth), short phalanges, abnormal tooth formation, fragile nails, dry skin, mental retardation and impaired glucose tolerance. Some relatives of PTH patients resemble them in outward appearance but characteristic laboratory findings are missing (pseudo-pseudohypoparathyroidism).

In human osteopetrosis or in the microphtalmic mouse, which is a good experimental model of this disease, osteoclasts fail to function normally and do not respond to PTH or calcitonin (Cohn et al. 1981).

3.2.4.2. DEFICIENT SECRETION: HYPOPARATHYROIDISM

The disease is characterized by hypocalcemia, hyperphosphatemia and consequent neuromuscular symptoms (manifest or latent tetany, convulsions). Soft tissue calcifications due to highly elevated plasma P may occur. The parathyroids are absent: most are unwillingly removed during surgery, but rare idiopathic forms occur as well. In the so-called diGeorge syndrome, parathyroids and the thymus (organs originating from the third and fourth branchial pouch) are absent.

Hypoparathyroidism or pseudohypoparathyroidism is treated with different forms of vitamin D or with its analogs.

3.2.5. CALCITONIN (CT)

Calcitonin (CT), a hormone with plasma calcium-lowering effect was recognized only in the sixties by Copp and his coworkers (Copp et al. 1961, Copp 1970). Its pathophysiological role in different diseases as well as its original function and place(s) of delivery are still debated.

In its phylogenesis CT is considered to be fundamentally a neuropeptide or neurotransmitter. The secretory cells migrate from the primitive nervous system and concentrate in various organs. In the human, mainly in the thyroid (parafollicular or C cells) but they can be found in the thymus, lung, gut (MacIntyre and Stevenson 1981), and pituitary (Samaan and Leavens 1981), resembling the gut hormones with their wide variation.

CT is now detected with radioimmunoassay (RIA) in the plasma and seems to be heterogenous in the human (Snider et al. 1975, Cecchetin et al. 1981). In pathological conditions, using gel filtration, different peaks suggested different forms with unknown biological activity. The heterogeneity and the differences in RIA methods explain why normal ranges vary between 30 and 500 pg/ml. Recently katacalcin, an other hypocalcemic hormone, synthesized in the CT-secreting cells as a part of a common precursor, was detected in the serum (Ali-Rachedi et al. 1983).

3.2.5.1. STATES WITH CALCITONIN EXCESS

Medullary carcinoma is a solid, but not anaplastic malignancy of the thyroid, composed of parafollicular (C) cells, i.e., it is a neuroectodermal cell disorder. CT is detected in the tumor and in the blood in high concentrations, but hypocalcemia is rare. Sometimes the tumor is familial, or occurs together with other hormone-producing adenomas (multiple endocrine neoplasia, Eberle and Grun 1981). Besides CT, the tumor may secrete other endopeptides, for instance ACTH, MSH, serotonin, etc. Diarrhea and flushings are common, perhaps due to prostaglandin overproduction (Milo-Keynes and Till 1971). Exaggerated CT response to calcium infusion is a valuable method in the early diagnosis (Jackson et al. 1973). Surgery seems to be the best therapy (Roka et al. 1982).

The plasma concentration of CT is sex-related: Hillyard et al. (1978) observed in women only 25% of the levels found in men, while Deftos and Parthemore (1980) suggested only a slight difference in the basal levels. But irrespective of age, the CT response to calcium infusion was less pronounced in women, indicating a decreased CT secretion (Deftos and Parthemore 1981) or perhaps a shortened biological half-life (Hollo 1978). During pregnancy or lactation (Hillyard et al. 1978, Munson et al. 1981) CT concentration increases, protecting maternal skeleton in case of excessive calcium need. Copp and Ma (1981) disclosed that the presence of C-cells are very

important in the periods of calcium stress: i.e., in neonatal period, pregnancy, lactation or prolonged exposure to low-calcium diet. During lactation, CT is responsible for calcium preservation. To avoid hypocalcemia, PTH secretion and 1,25-DHCC production is stimulated, thus increasing calcium absorption without osteolysis through the inhibition of osteoclastic bone resorption by CT (Munson et al. 1981).

Ectopic CT hypersecretion was demonstrated in different tumors, i.e., carcinoids, breast cancers, bronchial cancers, insulinomas and different APUD-omas (Milhaud 1981).

The occurrence of CT receptors in the normal breast is questionable. However, recently receptors have been demonstrated in breast cancer cell lines, implying the possibility that CT may influence certain breast cancers in vivo (MacIntyre and Stevenson 1981, Gennari 1981).

Compensatory CT elevation was reported in primary HPT, but CT reserve seemed to be limited (Becker et al. 1980). The increased level of CT in chronic renal failure is discussed later.

3.2.5.2. STATES WITH LOW PLASMA CALCITONIN LEVELS OR WITH DECREASED CALCITONIN SENSITIVITY

To study the consequences of the absence of a hormone is easiest when the secretory gland is missing. Since CT secretory cells are found in different places and organs, the existence of absolute CT deficiency is dubious. Immunoreactive CT was found in the urine and serum of thyroidectomized humans (Silva and Becker 1981), but after intravenous calcium load there is no CT elevation in the blood. The RIA methods for CT are not sensitive enough to measure subnormal values. Moreover, the compensatory effects of other Ca-regulator hormones makes the study of CT deficiency very difficult. Yet the long-standing and supernormal postprandial elevation in blood calcium after thyroidectomy suggests an important physiological role. Particular importance is attributed to states were CT reserve and/or effect is impaired.

The most important - if not the only - physiological effect of CT is the inhibition of bone resorption. When primary bone resorption exceeds bone formation without any other reason - and osteoporosis (OP) develops - it is evident to postulate CT deficiency or diminished effect.

As a part of the ageing process skeletal mass is lost at a rate of 0.3-0.7% per year. It is well known that basal levels of CT, as well as response to Ca infusions decrease with age in both sexes (Deftos et al. 1980). If bone loss exceeds the loss due to the ageing process, pathologically accelerated OP develops: 3-4% loss per year in postmenopausal osteoporosis (PM-OP). Basal and response CT levels are lower in women than in men (Hillyard et al. 1978, Deftos et al. 1980, MacIntyre and Stevenson 1981).

Moreover, the mineral content of the skeleton is 30% less in women, thus it is easy to understand why pathological crippling OP is far more common in the female.

In studying the endocrinological basis of pathological female OP, mainly the one developing after menopause, Albright and Reifenstein (1948) suggested a causative role for the imbalance between gonadal and adrenocortical hormones, implying estrogen deficiency as the most important factor. As estrogen deficiency occurs in each menopausal woman, the theory cannot explain the differences between physiological and pathological bone loss. Nordin (1961) in the sixties suggested deficient dietary calcium intake, and recently, decreased 1,25-dihydroxy-cholecalciferol levels were also taken into account.

As CT and its inhibitory action on bone resorption became well known, theories evolved about its pathological role. The first studies (Milhaud et al. 1978) reported on lower CT levels in women with PM-OP. Canniga et al. (1981) suggested CT treatment in PM-OP as a substitution therapy. According to them in OP-CT's action includes not only an inhibitory action on bone resorption, but also a stimulatory action on decreased 1-α-hydroxylase activity, which is mediated by secondary HPT. But the results of CT monotherapy in PM-OP are rather disap-

pointing (Jowsey et al. 1978, Chesnut et al. 1981, Agraval et al. 1981). In recent reports (Taggart et al. 1982, Zseli et al. 1982) basal CT levels in patients with PM-OP were found to be normal.

In experiments performed on rats deprived of gonads and adrenals but adequately substituted with cortisone, the size and duration of hypercalcemia after Ca load increased, while after exogenous CT administration the hypocalcemia was less pronounced (Hollo et al. 1975). In women with pathological PM-OP the induced hypercalcemia following intravenous Ca load persisted significantly longer (Hollo et al. 1978). All these observations can be explained by decreased CT sensitivity. After androgen, dehydroepiandrosterone or anabolic steroid administration, the above-mentioned alterations cease in OP women and in animal experiments, an effect which cannot be achieved with various estrogens, at least not to a full extent (Hollo et al. 1971, Hollo 1974). Moreover, in patients with pathological PM-OP, plasma levels and urinary excretion of dehydroepiandrosterone sulphate (Hollo et al. 1970), as well as the androstendione level were found to be significantly lower (Marshall et al. 1977). The results suggest that decreased CT sensitivity due to depressed androgen concentration is the most important pathogenic factor in the development of PM-OP (Hollo 1974).

Recently, normal basal serum levels, but limited CT reserve have been suggested, based on the finding that the increment in blood CT after Ca load is smaller in PM-OP than in age-matched healthy women, while basal levels are similar (Taggart et al. 1982, Zseli et al. 1982) or sometimes even elevated (Cecchettin et al. 1981).

Bone distrophy in chronic renal failure is another state wherein decreased CT sensitivity is implied. Plasma CT levels are elevated in uremia (Heyden and Franchimont 1974, Silva and Becker 1981, Cecchetin et al. 1981), but response to Ca load in animal experiments (Szucs et al. 1974) and in patients with moderate renal failure is blunted (Szucs 1977), just as is the effect of exogenous CT in uremic rats (Szucs et al. 1974) and

in patients on maintenance hemodialysis (Szucs and Horvath 1980). The ineffectiveness of CT therapy in uremic bone disease (Cundy et al. 1982) can be explained on the basis of decreased sensitivity.

Salmon and human CT seem to be novel drug candidates in the therapeutic arsenal. As mentioned above, in OP the results are contraversial (Jowsey et al. 1979, Agraval et al. 1981, Chesnut et al. 1981, Canigga et al. 1981). Further trials are needed, especially in combinations with different agents (Chesnut 1984, Szucs et al. 1984a).

Good results in arrest or temporary healing can be achieved in Paget's disease (Williams et al. 1978, Whythe et al. 1981). CT is widely used in the treatment of various hypercalcemias (Sjoberg et al. 1981). CT seems to be beneficial in different types of pancreatitis (Paul et al. 1979) and in the prevention of complications after retrograde pancreatography (Tulassay et al. 1981). Some effect is suggested in bone metastases of cancer (symptomatic relief, perhaps decrease in size and number, Gennari 1981) or in symptomatic treatment of different bone pains (Moricca et al. 1981).

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CHAPTER 4

ENDOGENOUS PEPTIDES AS INTEGRATORS OF REPRODUCTIVE FUNCTIONS

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4.1. PEPTIDE HORMONES IN MAMMALIAN REPRODUCTION

4.1.1. GONADOTROPIN-RELEASING HORMONE (GN-RH): GONADOLIBERIN, LUTEINIZING HORMONE-RELEASING HORMONE (LH-RH), FOLLICLE-STIMULATING HORMONE-RELEASING HORMONE (FSH-RH)

Harris (1955) clearly demonstrated the involvement of the hypothalamus in the control of gonadotropin secretion. The presence of a LH-releasing factor in rat hypothalamic extract was demonstrated by McCann et al. (1960). Hypothalamic extracts were also found to contain an FSH-releasing factor (Igarashi and McCann 1964).

For the successful isolation of the first 800 μg of pure LH-releasing factor, hypothalami of 165,000 pigs had to be processed. The observation that the LH and FSH activities were copurified in each step of purification led to the conclusion that the LH- and FSH-releasing activities originate from the same substance. The material carrier of both activities was subsequently sequenced and proved to be a decapeptide. The primary structure of the mammalian porcine peptide was shown to be as follows (Schally et al. 1971a,b):

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2.

Recently, the structure of the cDNAs encoding human and rat LH-RH-prohormones was determined and the corresponding peptide sequence deduced (Adelman et al. 1986). In addition to LH-RH, both cDNAs also encode a 56-amino acid peptide sequence, designated as gonadotropin-releasing hormone associated pep-

tide (GAP; Nikolics et al. 1985, Adelman et al. 1986, see also Chapter 3). The latter peptide has prolactin release-inhibiting properties.

An enormous number of LH-RH analogs were synthesized and biologically tested in the past few years. This has led to the elucidation of the role of individual amino acid residues in the biological activity of the molecule, which contributed to the laboratory synthesis of agonist analogs whose biological potency significantly surpasses that of the parent hormone. The His-Trp sequence appeared to constitute an "active center" in LH-RH, while pyroglutamic acid in position 1, and particularly glycine in position 6 and 10 proved to be the most critical residues for preserving conformation. All analogs with increased agonist activity have substitution(s) in position 6 and/or 10, which results in a greater receptor-binding affinity due to stabilization of the preferred conformation (Nikolics et al. 1977, Schally et al. 1979).

On the other hand, all inhibitory analogs have substitution(s) in the "active center". Such analogs have been designed to abolish the gonadotropin-releasing function with a simultaneous preservation of, or an increase in, the binding affinity of the molecule. Several potent inhibitory peptides (LH-RH antagonists) have been successfully used to inhibit gonadotropin release or ovulation in various species (Phelps et al. 1977) including humans (Gonzales-Barcena et al. 1977; for more information on the chemistry of Gn-RH see Reichlin 1985).

LH-RH releases LH and FSH from the pituitary gland. The FSH-releasing activity is about one fifth of the LH-releasing potency, and varies with the hormonal state of the organism and the frequency of LH-RH impulses.

Kinetic studies indicated that binding of tritiated LH-RH to pituitary plasma membrane receptors is a relatively rapid process at 37 °C. Maximal binding was observed within a period of 10-20 minutes with an association constant of 10^{-5} mol/sec (Theoleyre et al. 1976). For LH-RH, both tonic and phasic (pulsatile) secretion patterns were demonstrated, and both

seem to be necessary for a correct biological activity of the molecule.

In vitro studies revealed that cAMP or dibutyril cAMP release both LH and FSH from isolated pituitary cells. Sodium fluoride, which exerts a direct stimulatory effect on adenylate cyclase, stimulates LH release in vitro. Theophyllin or aminophyllin, the inhibitors of phosphodiesterase enzyme increase cAMP levels in the adenohypophysis. Theophyllin alone has no effect on LH release, but potentiates the effect of LH-RH. Imidazole, which stimulates phosphodiesterase activity, partially inhibits LH-RH activity (Jutisz et al. 1979, Labrie et al. 1979).

LH-RH, added to rat pituitary tissue in vitro, elicits a significant increase in cAMP. LH-RH analogs capable of releasing LH and FSH were found to exert a parallel stimulation of cAMP accumulation (Borgeat et al. 1974, Labrie et al. 1979). However, administration of LH-RH in vivo raises the serum level of LH within 15 minutes, without increasing pituitary cAMP accumulation. Others pointed out that cAMP-induced hormone release occurs only after a release mechanism had been initiated (Sundberg et al. 1976, Tang and Spies 1976).

Although it is often difficult to differentiate between hypothalamic and pituitary sites of action of the sex steroids, it was postulated that the effect of LH-RH on the secretion of LH and FSH is modulated by sex steroids (Sharp and Fraser 1978). The pulsatile release of LH-RH is also affected by sex steroids (Knobil 1980, Pohl and Knobil 1982, Dluzen and Ramirez 1987). Small doses of estrogen may increase pituitary response to LH-RH. Estradiol benzoate displays a biphasic effect on pitiutary responsiveness to LH-RH, depending on the concentration and duration of the action of estrogen (Vilchez-Martinez et al. 1974).

In ovariectomized rats, chronic treatment with testosterone had no effect on plasma LH levels in response to LH-RH. On the other hand, pituitary cells in culture preincubated in the presence of testosterone showed a decreased LH release to LH-RH (Jutisz et al. 1979).

In monolayer culture of female pituitary cells, progesterone in low doses did not inhibit basal LH-RH release. However, pretreatment of estrogen-primed ovariectomized rats with progesterone advanced and amplified the LH surge. Progesterone increases LH-RH accumulation in the median eminence too (Wise et al. 1981).

In addition to classical transmitters such as norepinephrine (Ramirez et al. 1986), neuropeptides, namely opioid peptides (for review see Grossman and Rees 1983, Ferin 1984) and neuropeptide Y (Khorram et al. 1987) also contribute to the regulation of LH-RH release. These neuropeptides may affect LH secretion either by acting directly on LH-RH neurons or indirectly, by modulating the activity of monoaminergic inputs (Kalra and Simpkins 1981, Wiesner et al. 1984, Petraglia et al. 1986, Khorram et al. 1987).

It was demonstrated that secretagogue-induced release of adenohypophyseal hormones in vitro requires Ca^{2+} in the medium (Jutisz et al. 1979). Prostaglandins were also suggested to participate in the regulation of anterior pituitary secretion (Hedge 1977).

Long-term administration of LH-RH in high doses, after a transient stimulation, inhibits gonadotropin release, and decreases gonadal function both in females and males. The mechanism of this paradoxical inhibitory effect is unclear, but down regulation of the pituitary receptors may be involved in the phenomenon. Apparently, the pituitary can only respond to pulsatile LH-RH stimuli, and becomes desensitized upon continous stimulation by LH-RH (Knobil 1980).

LH-RH analogs exert a number of direct effects on the reproductive organs. Most reports demonstrated inhibitory effects in females: termination of pregnancy (Bex and Corbin 1981), and a decreased ovarian steroidogenesis (Hsueh and Erickson 1979). However, more recent publications reported on an ovulation-inducing effect of LH-RH in hypophysectomized rats (Corbin and Bex 1981, Ekholm et al. 1982, McCann 1982). In males, LH-RH may also directly affect testosterone production (Sharpe et al. 1983).

Bioassays: Dispersed pituitary cells were used for measuring LH release following LH-RH administration in vitro (Nakano et al. 1976, Nikolics et al. 1977).

RIA: Being a relatively low molecular weight substance, LH-RH is a relatively poor antigen. In order to increase its antigenicity, LH-RH is usually coupled to various high molecular weight compounds, e.g. bovine (Nett et al. 1973) or human serum albumin (Jeffcoate and Holland 1973). LH-RH was successfully iodinated with ^{131}I or ^{125}I (Kerdelhue et al. 1973), using chloramine-T, or the lactoperoxydase method (Jutisz et al. 1979).

Radioreceptor assay was also developed for LH-RH determination. Pituitary cell membrane preparations were used as specific binding substrates, but suitable membrane preparations were obtained from rat liver, kidney, spleen, lung and testes, as well.

In order to stimulate gonadotrop hormone release in clinical conditions associated with an LH-RH deficit, the patients were treated with superactive LH-RH analogs. In such cases, the pituitary gland initially responded adequately, but became refractory after a certain period. The reduced responsiveness was ascribed to the exhaustion of the pituitary gonadotropin store due to the continuous exposure of the gland to the analogues (Rivier et al. 1979), or to the impairment of hormone release (Sandow et al. 1979). The so-called Kallman's syndrome may be due to a congenital lack of LH-RH. This is supported by specific morphological alterations in brain regions populated by LH-RH containing cells. (Crowley and McArthur 1980).

4.1.2. LUTEINIZING HORMONE (LH): LUTEOTROP HORMONE (LUTEOTROPIN), INTERSTITIAL CELL-STIMULATING HORMONE (ICSH)

LH belongs to the group of the pituitary glycopeptide hormones (FHS, LH, TSH) which, together with the placental HCG, display structural similarities. The α-chain of human LH comprise 96 amino acid residues and is identical with the α-chain of human FSH and TSH (see Chapter 3). On the other hand, the

β-chain contains 115 amino acids in a sequence as follows: Ser-Arg-Glu-Pro-Leu-Arg-Pro-Trp-Cys-His-Pro-Ile-Asn-Ala-Ile-Leu-Ala-Val-Glu-Lys-Glx-Gly-Cys-Pro-Val-Cys-Ile-Thr-Val-Asn-Thr-Thr-Ile-Cys-Ala-Gly-Tyr-Cys-Pro-Thr-Met-Arg-Val-Leu-Gln-Ala-Val-Leu-Pro-Pro-Leu-Pro-Gln-Val-Cys-Thr-Tyr-Arg-Asp-Val-Arg-Phe-Glu-Ser-Ile-Arg-Leu-Pro-Gly-Cys-Pro-Arg-Gly-Val-Asp-Pro-Val-Val-Ser-Phe-Pro-Val-Ala-Leu-Ser-Cys-Arg-Cys-Gly-Pro-Cys-Arg-Arg-Ser-Thr-Ser-Asp-Cys-Gly-Gly-Pro-Lys-Asx-His-Pro-Leu-Thr-Cys-Asx-Glx-Pro-His-Ser-Lys-Gly (underlined residues represent glycosylation sites). The complex carbohydrate moieties are linked to the asparagine residues at positions 13 and 30 in the β-chain (Papkoff et al, 1973, Shome and Parlow 1973). Human LH differs from HCG principally in the structure of the β-chain. LH circulates in the blood in a free form, which has a half-life of about 50 minutes.

In mammals, both LH and FSH are secreted throughout the entire estrous cycle, and both molecules seem to participate in all phases of ovarian activity. Recently, increasingly more attention is being paid to their combined effects. In general, FSH seems to act primarily as a growth factor, whereas LH seems much more to be a stimulator of secretion than of proliferation, and for its own action, the prior action of FSH is required.

LH is indispensable for ovulation. It stimulates steroid secretion of the follicular tissue, and the release of enzymes acting on collagen of the follicle wall. LH reaches a peak value at the time of the ovulation, a prerequisite of luteal development, but a low LH level satisfies the requirement of normal luteal function during the luteal phase. The corpus luteum does not function indefinitely, its lifespan is determined by several intra- and extra-ovarian factors.

LH increases ovarian blood flow in rats (Wurtman 1964, Varga et al. 1985) and sheep (Niswender et al. 1975), whereas it has no such an effect in hamster (Varga and Greenwald 1979) and dogs (Stark and Varga 1968). Specific LH-binding sites were demonstrated in thecal cells present in preantral and antral follicles (Midgley 1973), as well as in thecal and granulosa cells in large preovulatory follicles (Lee 1976). The in-

crease in the number of LH receptors in granulosa cells from both small and large antral follicles was shown to be associated with a more pronounced ability of these cells to produce cAMP and progesterone in response to the gonadotrop hormones (Channing and Tsafriri 1977, Richards 1978). In humans, specific LH receptors were found in corpora lutea during the follicular phase, indicating that regression of the corpus luteum is not primarily due to the loss of receptors. During early pregnancy receptor concentration in corpora lutea was found to be lower than in those during the menstrual cycle (Rajaniemi et al. 1981).

LH activates adenylate cyclase in membranes of the target cells and stimulates cAMP formation. cAMP is the intracellular messenger of the action of LH on progesterone secretion in luteal cells. Protein synthesis is required for stimulation of hormone secretion; protein synthesis inhibitors prevent the stimulatory effects of both LH and HCG on progesterone and estradiol secretion (Varga et al. 1973).

The Leydig cells respond to LH by an increased testosterone secretion, which requires a prior binding of the hormone to specific plasma membrane receptors. The average LH receptor content in adult rat testis is about 20,000 sites per Leydig cell. To invoke a full steroidogenic response only a minor fraction of the receptors is needed to be occupied by LH. The earliest effect of LH on Leydig cells is a receptor-mediated activation of adenylate cyclase and consecutive cAMP formation. In intact cells, cAMP levels rise within a few seconds, and increased testosterone production is detectable within 5-10 minutes. When testes are exposed to a high LH concentration, "utilization" of LH receptors exceeds the rate of replenishment, resulting in a net loss of receptors. This finding suggests that normal pulsatile elevation of circulating LH may lead to an intermittent loss of a small portion of the receptor population under physiological conditions. This mechanism would be consistent with the proposal that LH receptors in the testis may be used only once, and then they are processed or degraded rather than vacated and reutilized (Catt et al. 1980).

LH can be measured by the ovarian ascorbic acid depletion test, rat ventral prostate bioassay, or by testosterone production of dispersed Leydig cells. Most frequently, LH plasma levels are measured by RIA (Odell et al. 1967, Niswender et al. 1969). LH is secreted in pulses, thus it is necessary to obtain multiple samples from the same individual at appropriate intervals if a representative basal level is to be determined. LH antibodies usually cross-react with HCG, but specific assay has also been reported (Thorell et al. 1976).

LH (and HCG) can also be measured by radioreceptor assay, using rat interstitial cell plasma membrane fractions, or homogenates of rat ovary.

Disorders of the hypothalamo-pituitary-gonadal axis may originate from primary lesions at any of the following three levels: There may be an abnormality in LH-RH output, due to lesions in the hypothalamus or in higher neural areas; the anterior pituitary may also be unable to respond to LH-RH, and finally, the gonads may also lack the ability to respond to gonadotropins. Primary gonadal disorder can be diagnosed from high circulating gonadotropin levels but it may be difficult to distinguish between the hypothalamic and the pituitary forms of hypogonadotropic hypogonadism. In the latter form, no response can be elicited by exogenous or endogenous LH-RH. In the case of a hypothalamic defect, the pituitary gland readily responds to exogenous LH-RH. Although the LH-RH test does not distinguish between the types of secondary amenorrhea, it is frequently used to assist the setting of a correct diagnosis in such disorders.

4.1.3. FOLLICLE-STIMULATING HORMONE (FSH)

FSH also belongs to the family of the pituitary glycopeptide hormones. It also consists of two polypeptide chains, α and β. The 96 amino acid long α-chain is identical to the α-chain of TSH, LH and HCG (for sequence data see Chapter 3). The characteristic β-chain comprises 115 amino acids ordered in the following sequence:

(Asx-Ser)-Cys-Glu-Leu-Thr-Asn-Ile-Thr-Ile-Ala-Ile-Glu-Lys-Glu-Glu-Glu-Cys-Arg-Phe-Cys-Ile-Ser-Ile-Asn-Thr-Thr-(Thr,Asx,Trp)-Glu-Thr-Cys-Ala-Gly-Tyr-Cys-Tyr-Thr-Arg-Asp-Leu-Val-Tyr-Lys-Asp-Pro-Ala-Lys-Pro-Arg-Ile-Gln-Lys-Thr-Cys-Thr-Phe-Lys-Glu-Leu-Val-Tyr-Glu-Thr-Val-Arg-Val-Pro-Gly-Cys-Ala-His-His-Ala-Asp-Ser-Leu-Tyr-Thr-Tyr-Pro-Val-Ala-Thr-Gln-Cys-His-Cys-Gly-Lys-Cys-Asp-Ser-Asp-Ser-Thr-Asp-Cys-Thr-Val-Arg-Gly-Leu-Gly-Pro-Ser-Tyr-Cys-Ser-Phe-Gly-Glu-Met-(Glx,Lys) (underlined residues indicate glycosylation sites and amino acids in parentheses represent compositional data only; Shome and Parlow 1974).

The gonadotropin-secreting cells of the pituitary secrete FSH in response to LH-RH. The response may be modified by estrogens, progestagens, or by both, and also by androgens and inhibin, a peptide present in both the follicular fluid and the testicular tissue. FSH circulates in a free form in the blood where its metabolic clearance rate (t1/2) is about 4 hours.

Early development of the follicle may not depend on gonadotropic stimulation. The follicle becomes increasingly sensitive to gonadotropin during formation of the granulosa cell layers. Both proliferation and organization of the granulosa cells, as well as the development of the thecal cells are under FSH control. FSH blood levels are elevated around the time of the ovulation with peak values in proestrus and estrus. The prolonged estrous portion of the FSH peak may be responsible for selecting follicles for ovulation in the subsequent cycle (Hirschfield 1981). FSH stimulates hormone secretion in hamster ovary throughout the whole length of the cycle (Varga and Greenwald 1979).

As the first event of the action of FSH, its binding to membrane receptors on the granulosa cells (Richards and Midgly 1976), and a subsequent stimulation of the adenylate cyclase system should be mentioned. FSH receptors of the granulosa cells are distinct from their LH receptors. The effect of saturating doses of LH and FSH on the adenylate cyclase system is not additive (Kolena and Channing 1971), indicating that LH and FSH might act on the same adenylate cyclase but through separate receptors.

In humans, ordinarily only one follicle matures during the menstrual cycle. However, when ovulation is induced in fertile women by exogenous LH and FSH, maturation of several follicles and polyovulation may occur. In animals, large doses of FSH increase the number of ripening follicles. Antiestrogens prevent the FSH-induced follicle maturation and estrogen treatment increases the number of developing follicles. The effect of FSH on follicle maturation appears to be mediated via estrogen secretion.

FSH specifically binds to cells of the seminiferous tubules, but not to Leydig cells, or other tissues. After birth, FSH binding capacity of rat testis increases during the first 15 days and remains constant thereafter. This time schedule corresponds to the period in which the number of Sertoli cells increases in the testis. FSH binding is unaffected by hypophysectomy, which results in a reduction of the number of germ cells but not in that of the Sertoli cells. All these suggest that Sertoli cells might be responsible for binding of FSH. Following binding, FSH stimulates adenylate cyclase as well as cAMP formation.

FSH controls the growth and the hypertrophy of testis in prepubertal animals, and may act synergistically with LH, thereby allowing a higher rate of testosterone synthesis and an increased responsiveness to LH (Setchell 1978).

As a not very sensitive bioassay, FSH and LH can be measured by assessing the weight-gain in rat ovaries. Serum levels may be exactly measured by available RIAs. Radioreceptor assay was also developed for measuring these hormones using the rat testicular tubular system for FSH binding.

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4.1.4. PLACENTAL PEPTIDE HORMONES

One of the most remarkable physiologic phenomena one encounters is the adaptation of both the fetus and the mother to the unusual endocrine milieu accompanying pregnancy, in whose formation the placenta plays a major role. The placenta is a unique organ provided with the capability of producing both

steroidal and nonsteroidal hormones. Peptide hormones produced by the placenta include: human chorionic gonadotropin (HCG), human placentál lactogen (HPL) or human chorionic somatomammotropin (HCS), human chorionic thyrotropin (HCT), ACTH, TRH, LH-RH and somatostatin. Each of these hormones is produced in the syncytiotrophoblastic layer of the placenta and can be measured by available RIA techniques.

4.1.4.1. HUMAN CHORIONIC GONADOTROPIN (HCG, hCG)

In 1927, Aschheim and Zondek reported that urine of pregnant women displayed gonadotropic activities. The urine component responsible for such activities was subsequently identified as a glycoprotein, termed HCG. HCG was shown to consist of two dissimilar subunits, designated as α and β. The α-subunit of HCG has a molecular weight of 10,200 (without the carbohydrate moieties) and comprises 92 amino acids (Fig.4.1).

a.

Ala-Pro-Asp-Val-Gln-Asp-Cys-Pro-Glu-Cys-Thr-Leu-Gln-Glu-Asp-Pro-Phe-Phe-Ser-Gln-
Pro-Gly-Ala-Pro-Ile-Leu-Gln-Cys-Met-Gly-Cys-Cys-Phe-Ser-Arg-Ala-Tyr-Pro-Thr-Pro-
Leu-Arg-Ser-Lys-Lys-Thr-Met-Leu-Val-Gln-Lys-Asn-Val-Thr-Ser-Glu-Ser-Thr-Cys-Cys-
Val-Ala-Lys-Ser-Tyr-Asn-Arg-Val-Thr-Val-Met-Gly-Gly-Phe-Lys-Val-Glu-Asn-His-Thr-
Ala-Cys-His-Cys-Ser-Thr-Cys-Tyr-Tyr-His-Lys-Ser

b.

Ser-Lys-Glu-Pro-Leu-Arg-Pro-Arg-Cys-Arg-Pro-Ile-Asn-Ala-Thr-Leu-Ala-Val-Glu-Lys-
Glu-Gly-Cys-Pro-Val-Cys-Ile-Thr-Val-Asn-Thr-Thr-Ile-Cys-Ala-Gly-Tyr-Cys-Pro-Thr-
Met-Thr-Arg-Val-Leu-Gln-Gly-Val-Leu-Pro-Ala-Leu-Pro-Gln-Val-Val-Cys-Asn-Tyr-Arg-
Asp-Val-Arg-Phe-Glu-Ser-Ile-Arg-Leu-Pro-Gly-Cys-Pro-Arg-Gly-Val-Asn-Pro-Val-Val-
Ser-Tyr-Ala-Val-Ala-Leu-Ser-Cys-Gln-Cys-Ala-Leu-Cys-Arg-Arg-Ser-Thr-Thr-Asp-Cys-
Gly-Gly-Pro-Lys-Asp-His-Pro-Leu-Thr-Cys-Asp-Asp-Pro-Arg-Phe-Gln-Asp-Ser-Ser-Ser-
Ser-Lys-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Ser-Asp-Thr-
Pro-Ile-Leu-Pro-Gln

Fig. 4.1. Amino acid sequence of the α(a)-and β(b)subunits of human chorionic gonadotropin. Underlined residues represent potential glycosylation sites

Its amino sequence is almost identical to those of the pituitary glycopeptide hormones: FSH, LH and TSH (Bellisario et al. 1973, Carlsen et al. 1973).

The molecular weight of the β-chain is 15,500 (without the carbohydrate moieties) and comprises 145 amino acids. There are sequence homologies between the β-subunit of LH and HCG; out of the 115 amino acid residues of the β-chain of LH, 80 proved to be identical to those of the β-chain of HCG (Closset et al. 1973, Fig.4.1). Molecular weight of the carbohydrate moieties comprises about 30% of the total. The major function of the carbohydrate residues may be the prolongation of the half-life of HCG. The separated subunits of HCG are biologically inactive. The biological activity of the hormone appears to be bound largely to the β subunit; the β-subunit proved to be interchangeable.

HCG is secreted and stored in the syncytiotrophoblasts. Its synthesis may be under the control of locally produced LH-RH, also a product of the syncytiotrophoblasts. Increased levels of HCG are found in pregnants with multiple fetuses and in women with hydatiform mole and choriocarcinoma. Similarly to LH, the major biological function of HCG is to stimulate progesterone synthesis in the maternal corpus luteum and to stimulate ovarian as well as testicular steroid secretion in the fetus. The role of HCG in human pregnancy is not fully understood. HCG may stimulate progesterone secretion in trophoblasts as well as in fetal testis to produce testosterone prior to LH secretion by the fetal pituitary. HCG inhibits lymphocyte response to phytohemaglutinin, an effect compatible with the observation that survival of skin allografts are prolonged during pregnancy. It is supposed that HCG may also have an immunologic role preventing the rejection of implanted blastocyst by the maternal organism (Marrs and Mishell 1980).

Like other protein hormones, HCG is synthesized on membrane-bound ribosomes. The α- and β-subunits are translated from separate mRNAs, rather than synthesized in tandem on the same mRNA. Synthesis of the β-subunit may be rate-limiting in the synthesis of intact HCG.

In placental explants, or in cultured choriocarcinoma cells, LH-RH, cAMP and epidermal growth factor stimulate the release of HCG. Dopamine inhibits HCG secretion, whereas pimozide, a dopaminergic antagonist, augments HCG secretion. However, the physiological factors controlling HCG synthesis and release are still far from being satisfactorily known (Simpson and McDonald 1981).

4.1.4.2. HUMAN PLACENTAL LACTOGEN (HPL)

HPL, also called human chroionic somatomammotropin (HCS) is composed of 191 amino acids (M_r 22,300) and contains two intramolecular disulfide bridges spanning between cysteine residues at positions 53 and 165 as well as 182 and 189, respectively. HPL has the following primary structure:
Val-Gln-Thr-Val-Pro-Leu-Ser-Arg-Leu-Phe-Asp-His-Ala-Met-Leu-Gln-Ala-His-Arg-Ala-His-Gln-Leu-Ala-Ile-Asp-Thr-Tyr-Gln-Glu-Phe-Glu-Glu-Thr-Tyr-Ile-Pro-Lys-Asp-Gln-Lys-Tyr-Ser-Phe-Leu-His-Asp-Ser-Glu-Thr-Ser-Phe-Cys-Phe-Ser-Asp-Ser-Ile-Pro-Thr-Pro-Ser-Asn-Met-Glu-Glu-Thr-Gln-Gln-Lys-Ser-Asn-Leu-Glu-Leu-Leu-Arg-Ile-Ser-Leu-Leu-Leu-Ile-Glu-Ser-Trp-Leu-Glu-Pro-Val-Arg-Phe-Leu-Arg-Ser-Met-Phe-Ala-Asn-Asn-Leu-Val-Tyr-Asp-Thr-Ser-Asp-Ser-Asp-Asp-Tyr-His-Leu-Leu-Lys-Asp-Leu-Glu-Glu-Gly-Ile-Gln-Thr-Leu-Met-Gly-Arg-Leu-Glu-Asp-Gly-Ser-Arg-Arg-Thr-Gly-Gln-Ile-Leu-Lys-Gln-Thr-Tyr-Ser-Lys-Phe-Asp-Thr-Asn-Ser-His-Asn-His-Asp-Ala-Leu-Leu-Lys-Asn-Tyr-Gly-Leu-Leu-Tyr-Cys-Phe-Arg-Lys-Asp-Met-Asp-Lys-Val-Glu-Thr-Phe-Leu-Arg-Met-Val-Gln-Cys-Arg-Ser-Val-Glu-Gly-Ser-Cys-Gly-Phe.

Out of the 191 amino acid residues, 162 are identical with those in human pituitary growth hormone. The amino acid sequence of HPL displays similarities to that of human prolactin as well. In these polypeptides, there are four regions containing internally homologous sequences. This suggests that each of these peptides is formed in two duplications of a single primordial peptide of smaller molecular weight (Niall et al. 1971). Thus, it is not surprising that HPL has both lactogenic and somatotropic properties.

HPL in human pregnancy is believed to act as an insulin antagonist, and to be responsible for maternal insulin resistance in the second half of pregnancy. The growth-promoting activity of HPL is only 1% of that of pituitary GH. Even so, however, this hormone, because it is produced in huge quantities, may elicit considerable physiological effects in pregnant women. In rodents, this peptide appears to be luteotropic, and regulation of progesterone secretion might be its primary function in these species. However, there is little evidence for luteotropic activity of HPL in human (Simpson and McDonald 1981).

HPL is synthesized in the syncytiotrophoblasts. Its serum levels increase parallel with advancement of pregnancy, and a close correlation was observed between the mass of the placenta and the serum levels of HPL. The rate of its production (1g/day) is the greatest among all protein hormones in man (Friesen et al. 1969). In intact perfused placental lobules, dibutyryl cAMP and Ca^{2+} stimulate HPL release. Intraamniotically administered $PGF_{2\alpha}$ causes a rapid reduction in maternal serum HPL levels (Yliokorkala and Pennanen 1973). Glucose seems to be essential for HPL secretion, but high concentration of glucose in the medium inhibits HPL release (Belleville et al. 1979).

4.1.4.3. HUMAN CHORIONIC THYROTROPIN (HCT)

HCT was isolated from placental and hydatiform mole tissues. It does not appear to be identical with pituitary TSH. Contrary to its pituitary counterpart, HCT antibodies do not cross-react with HCG, but cross-react with TSH from various animal species. TRH causes an increase in the serum level of pituitary TSH, but HCT levels remain unchanged (Kanazawa et al. 1976). During pregnancy, HCT plasma levels continuously increase, reaching the highest concentrations in the third trimester. Its physiological role remains still unclear.

4.1.4.4. PLACENTAL ACTH

The presence of ACTH-like materials in human term placentas were reported in the fifties (Opsahl and Long 1951, Assali and Hamermesz 1954). The ACTH-like material from the human placenta reacts with antisera raised against N-terminal (1-24), mid-portion (13-18) and C-terminal regions of the ACTH molecule (Rees et al. 1975). Bioassays measuring placental ACTH are 3-5-fold less sensitive than RIAs. Dexamethasone does not affect ACTH content of the placenta (Liotta et al. 1977). Like pituitary ACTH, placental ACTH is produced as a part of a larger precursor molecule, together with α-MSH, β-lipotropin and β-endorphin. By prolonging incubation time, the radiolabel progressively disappears from the large molecular weight fraction and simultaneously appears in smaller peptides, including ACTH and β-endorphin (Krieger et al. 1980).

The physiological role of the placental ACTH is unclear at present. ACTH concentrations in the umbilical cord plasma and the amniotic fluid are high, but there is no evidence indicating that either the placental or the fetal immunoreactive ACTH penetrate the placenta in humans.

4.1.4.5. PLACENTAL LH-RH

Human placenta contains a substance which is similar to the hypothalamic LH-RH by both immunological and biological criteria. Placental LH-RH is produced in cytotrophoblasts, and is supposed to stimulate HCG synthesis in this tissue (Siler-Khodr and Khodr 1978, Simpson and McDonald 1981).

4.1.4.6. PLACENTAL TRH

Human placenta also contains a substance with immunological and biological properties similar to those of TRH. This placental TRH does not release chorionic TSH (HCT), HPL or HCG. It is presently unclear whether placental TRH has any role in placental hormone secretion or any relevance to pregnancy (Shambough et al. 1979, Simpson and McDonald 1981).

4.2. OVARIN PEPTIDES IN THE MAMMALIAN REPRODUCTIVE FUNCTIONS

Since 1965, an immense number of published studies have provided relevant data on the hormonal events associated with amenorrhea and female infertility, which led to a better understanding of the molecular mechanism underlying these conditions and also to a qualitative improvement in their therapy. Even so, however, a considerable number of important physiopathological problems remained unsolved.

It is hoped that recently collected information on the existence and the mode of action of ovarian peptides affecting gonadal functions and the reproductive system in general will contribute to the solution of at least some of these problems. A number of ovarian peptides functions as true hormones, whilst others act as paracrine agents only on neighboring cells. The name cybernins was introduced by Guillemin (1981) to designate such locally acting gonadal peptides.

A rather compelling set of evidence is now available on the physiological role of nonsteroidal ovarian regulatory substances, mostly peptides. However, an overhelming majority of these peptides have not yet been purified to homogeneity. Thus, quantitative measurement of their plasma levels under various physiological and pathological conditions cannot be carried out, due to the lack of appropriately sensitive RIA methods. Consequently, studies concerned with their physiological roles and chemical structures are very much dependent on presently available bioassays.

4.2.1. INHIBIN

Inhibin, an agent believed to have an important, if not exclusive, role in suppressing both the synthesis and the secretion of FSH was first described by Franchimont and associates (1981) in human seminal fluid, in aqueous extracts of spermatozoa, testes and ovaries, in the follicular fluid of cows, pigs, humans, as well as in media conditioned by Sertoli and

granulosa cells. Some of the physicochemical properties of the inhibin preparations purified by various techniques in a number of laboratories differed significantly (de Jong et al. 1981, Sairam et al. 1981, Ramasharma et al. 1982).

Inhibin proved to be a moderately thermolabile substance, whose biological activity could be abolished by digestion with trypsin, papain and pepsin (de Jong et al. 1981), although inhibin activity in bovine seminal plasma and follicular fluid proved to be trypsin resistant yet pronase-sensitive in other experiments (Hermans et al. 1982).

One of the most intriguing aspects of inhibin research is the unusual molecular heterogeneity and wide spectrum of biological activity of the molecule. The more than hundredfold difference between reported molecular sizes of inhibin-like peptides from various sources may be due to a number of factors such as differences in the employed purification schemes, aggregation, polymer formation, enzymic degradation and binding proteins, but the existence of a whole array of inhibin-like peptides cannot be ruled out either.

Although inhibin was recently described to be present in human placenta (McLachlan et al. 1986) and seminal α-inhibin-like immunoreactivities were found in human pituitary, hypothalamus and serum (Ramasharma and Li 1986), structural data are available in relative abundancy only of inhibins derived from seminal plasma and ovarian follicular fluid, the main sources of inhibin-like peptides.

As to the human seminal plasma inhibin, multiple forms have been sequenced in a number of laboratories (reviewed by Sheth and Arbatti 1985). Of these, the partial sequencing of a "small" inhibin having an approximate M_r of 5,000 has been reported by Seidah and associated (1984b). Amino acid compositional data suggested 35 residues for this peptide. Of these, the first N-terminal 31 amino acids have been sequenced leaving the C-terminal 3-4 residues undetermined. However, a synthetic replicate of this 31 residue fragment, termed inhibin-like peptide(1-31) and abbreviated as ILP(1-31), displayed full biological activity both in vitro and in vivo (Yamashiro et al. 1984). Subsequently, two additional peptides

having inhibin-like activity have been isolated from human seminal plasma: one proved to be a 52, and the other a 92 amino acid peptide, respectively (Li et al. 1985). The former was designated α-inhibin-52 (α-IB-52) and the latter α-IB-92. Sequence comparison revealed that the C-terminal 52 residues of α-IB-92 are identical to α-IB-52, whereas the N-terminal 31 residues of α-IB-52 corresponds to the structure of ILP(1-31), now designated α-IB-31. Amino acid sequences of the three α-IBs are demonstrated in Fig.4.2.

```
                                   10                                  20
Thr-Tyr-His-Val-Asp-Ala-Asn-Asp-His-Asp-Gln-Ser-Arg-Lys-Ser-Gln-Gln-Tyr-Asp-Leu-
                                   30                                  40
Asn-Ala-Leu-His-Lys-Thr-Thr-Lys-Ser-Gln-Arg-His-Leu-Gly-Gly-Ser-Gln-Gln-Leu-Leu-
                                   50                                  60
His-Asn-Lys-Gln-Glu-Gly-Arg-Asp-His-Asp-Lys-Ser-Lys-Gly-His-Phe-His-Arg-Val-Val-
                                   70                                  80
Ile-His-His-Lys-Gly-Gly-Lys-Ala-His-Arg-Gly-Thr-Gln-Asn-Pro-Ser-Gln-Asp-Gln-Gly-
                                   90      92
Asn-Ser-Pro-Ser-Gly-Lys-Gly-Ile-Ser-Ser-Gln-Tyr
```

Fig. 4.2. Amino acid sequences of human seminal plasma α-inhibin-92 (full sequence); α-IB-31 (residues at positions 41 through 71) and α-IB-52 (residues at positions 41 through 92)

Almost simultaneously, the complete amino acid sequence of a 94 amino acid inhibin-like peptide, termed human seminal plasma β-inhibin, has also been reported (Seidah et al. 1984a). Its primary structure was established to be: Ser-Cys-Tyr-Phe-Ile-Pro-Asn-Glu-Gly-Val-Pro-Gly-Asp-Ser-Thr-Arg-Lys-Cys-Met-Asp-Leu-Lys-Gly-Asn-Lys-His-Pro-Ile-Asn-Ser-Glu-Trp-Gln-Thr-Asp-Asn-Cys-Glu-Thr-Cys-Thr-Cys-Tyr-Glu-Glu-Glu-Ile-Ser-Cys-Cys-Thr-Leu-Val-Ser-Thr-Pro-Val-Gly-Tyr-Asp-Lys-Asp-Asn-Cys-Gln-Arg-Ile-Phe-Lys-Lys-Glu-Asp-Cys-Lys-Tyr-Ile-Val-Val-Glu-Lys-Lys-Asp-Pro-Lys-Lys-Thr-Cys-Ser-Val-Ser-Glu-Trp-Gly-Ile. This rather acidic peptide selectively and specifically suppressed the release of pituitary FSH, but left the LH secretion unaffected. β-inhibin has a strongly basic C-terminal fragment which is easily cleaved by mild triptic digestion. Of this observation it was postulated that the FSH-inhibiting activity may reside within this region of the molecule. This assumption was subsequently confirmed when a

synthetic replicate of the C-terminal 28 residues showed a biological activity similar to that of the native hormone in in vitro assays (Arbatti et al. 1985) indicating that this fragment represents the active core of the molecule (see, however, Kohan et al. 1986 for contradictory results).

It may be surprising that no significant sequence homology exists between seminal plasma α- and β-inhibins. On the other hand, approximately 40% homology was found between the primary structures of "small" α-IB-31 and bacterial enolase (Seidah et al. 1984b).

Follicular fluid inhibin has been purified and partially characterized mainly from porcine and bovine sources (reviewed by Channing et al. 1985, de Jong and Robertson 1985). Two forms (A and B) of inhibin have been isolated from porcine follicular fluid (Ling et al. 1985). Each form comprises two dissimilar subunits (α and β) of M_r 18,000 and 14,000, respectively, that are crosslinked by one or more disulfide bridge(s). The two forms differ in the N-terminal sequence of the β-subunits. Preliminary structural comparison of porcine and bovine ovarian inhibins revealed that they have similar properties (Hasegawa et al. 1985, Robertson et al. 1985). Subsequently, Mason and associates (1985) have identified and sequenced cloned cDNAs encoding inhibin precursor molecules and deduced the amino acid sequences of the precursor and the mature α- and β-subunits for both the A and B forms of inhibin by using the N-terminal amino acid sequence data of the mature subunits. In this study it was established that inhibins A and B are glycoproteins whose α-subunits are common to both forms, while each form has its own, distinct (variable) β-subunit. Predicted amino acid sequence of the common α-subunit and those of the variable β-subunits are shown in Fig.4.3. While there is only insignificant sequence homology between the mature α- and β-subunits (mostly around the similarly distributed cysteine residues), β_A- and β_B-subunits are 70% homologous. It should be reminded that this type of structural organization of porcine ovarian inhibins (constant α-subunit and variable β-subunits) resembles that of the pituitary and placental glycoprotein hormones: FSH, LH, TSH and of chorionic gonado-

$\alpha_{A,B}$-subunit

```
                                                  10                  20
Ser-Thr-Ala-Pro-Leu-Pro-Trp-Pro-Trp-Ser-Pro-Ala-Ala-Leu-Arg-Leu-Leu-Gln-Arg-Pro-
                                                  30                  40
Pro-Glu-Glu-Pro-Ala-Val-His-Ala-Asp-Cys-His-Arg-Ala-Ser-Leu-Asn-Ile-Ser-Phe-Gln-
                                                  50                  60
Glu-Leu-Gly-Trp-Asp-Arg-Trp-Ile-Val-His-Pro-Pro-Ser-Phe-Ile-Phe-His-Tyr-Cys-His-
                                                  70                  80
Gly-Gly-Cys-Gly-Leu-Pro-Thr-Leu-Pro-Asn-Leu-Pro-Leu-Ser-Val-Pro-Gly-Ala-Pro-Pro-
                                                  90                  100
Thr-Pro-Ser-Phe-Ile-Phe-His-Tyr-Cys-His-Gly-Gly-Cys-Gly-Leu-Pro-Thr-Leu-Pro-Asn-
                                                  110                 120
Leu-Pro-Leu-Ser-Val-Pro-Gly-Ala-Pro-Pro-Thr-Pro-Val-Gln-Pro-Leu-Leu-Leu-Val-Pro-
                                                  130                 140
Gly-Ala-Gln-Pro-Cys-Cys-Ala-Ala-Leu-Pro-Gly-Thr-Met-Arg-Ser-Leu-Arg-Val-Arg-Thr-
                                                  150                 160
Thr-Ser-Asp-Gly-Gly-Tyr-Ser-Phe-Lys-Tyr-Glu-Thr-Val-Pro-Asn-Leu-Leu-Thr-Gln-His-
                164
Cys-Ala-Cys-Ile
```

β_A and β_B subunits

```
                                                  10                  20
A:Gly-Leu-Glu-Cys-Asp-Gly-Lys-Val-Asn-Ile-Cys-Cys-Lys-Lys-Gln-Phe-Phe-Val-Ser-Phe-
B: -  -  -  -  -  -  - Arg-Thr  - Leu  -  - Arg-Gln  -  -  - Ile-Asp  -
                                                  30                  40
A:Lys-Asp-Ile-Gly-Trp-Asn-Asp-Trp-Ile-Ile-Ala-Pro-Ser-Gly-Tyr-His-Ala-Asn-Tyr-Cys-
B:Arg-Leu  -  -  - Ser  -  -  -  -  -  - Thr  -  - Tyr-Gly  -  -  -
                                                  50                  60
A:Glu-Gly-Glu-Cys-Pro-Ser-His-Ile-Ala-Gly-Thr-Ser-Gly-Ser-Ser-Leu-Ser-Phe-His-Ser-
B: -  - Ser  -  - Ala-Tyr-Leu  -  - Val-Pro  -  - Ala-Ser  -  -  - Thr
                                                  70                  80
A:Thr-Val-Ile-Asn-His-Tyr-Arg-Met-Arg-Gly-His-Ser-Pro-Phe-Ala-Asn-Leu-Lys-Ser-Cys-
B:Ala  - Val  - Gln  -  -  -  -  - Leu-Asn  -  Gly-Thr-Val-Asn  -  -
                                                  90                  100
A:Cys-Val-Pro-Thr-Lys-Leu-Arg-Pro-Met-Ser-Met-Leu-Tyr-Tyr-Asp-Asp-Gly-Gln-Asn-Ile-
B: - Ile  -  -  -  - Ser-Thr  -  -  -  -  - Phe  -  - Glu-Tyr  -  -
                                                  110  116
A:Ile-Lys-Lys-Asp-Ile-Gln-Asn-Met-Ile-Val-Glu-Glu-Cys-Gly-Cys-Ser
B:Val  - Arg  - Val-Pro  -  -  -  -  -  -  -  -  - Ala
```

Fig 4.3. Amino acid sequence of the mature α-subunit common to both forms (A and B) of porcine ovarian inhibin ($\alpha_{A,B}$) and those of the variable β-subunits (β_A and β_B) as were deduced from nucleotide sequences of cloned cDNAS. Underlined residues represent possible glycosylation sites. Dashes indicate identical residues. Empty space (residue at position 74 in β_B sequence) indicates deletion introduced for maximizing homology

tropin. In these hormones, the variable (β) subunits confer the different biological specificities of each molecule, whilst the constant (α) subunit presumably has a role in overall conformation of the complex. There is no evidence for differences in the biological properties of inhibins A and B. Unlike the known glycoprotein hormones, the two chains of both inhibins A and B are linked by disulfide bonds, a feature which is more characteristic of immunoglobulins. Recent data indicate that follicular fluid inhibin exists in multiple molecular forms including both high and low molecular weight species (Miyamoto et al. 1986).

As to their biosynthesis, each inhibin chain is released through proteolytic processing of a larger precursor, the two types of subunits representing the C-terminal region of each polypeptide precursor (for more recent data on the genes encoding rat, bovine and human inhibins see Davis et al. 1986, Forage et al. 1986, Stewart et al. 1986).

Surprisingly, a significant structural homology was found between inhibin β-subunit sequences and the primary structure of human transforming growth factor-β (TGF-β) (see Chapter 8). These molecules are of nearly equal length (inhibin β_A subunit: 116 residues; β_B subunit: 115; TGF-β: 112) and show a strinkingly similar distribution of their nine cysteine residues. However, the almost identical amino acid sequences of human and murine TGFs-β (see Mason et al. 1985 for references) render it unlikely that the inhibin β-subunit would be the porcine equivalent of human TGF-β (see also Ying et al. 1986).

Inhibin bioassays in general use are based on the selective FSH suppressing effect of the molecule. For assessing biological activity of inhibin both in vivo and in vitro assays are available.The in vivo assays can be performed in various animals, occasionally in parabiotic experiments, whilst cultured pituitary cells are most frequently used as in vitro assays. RIA and radioreceptor assays have also been elaborated for quantitation of inhibin. Unfortunately, however, none of the methods presently available are sensitive enough to allow the determination of the serum levels of inhibin (Baker et al. 1981).

In the ovaries, inhibin is thought to be produced by the granulosa cells (Channing et al. 1981). Inhibin and progesterone production change inversely in vitro. Androstenedion, testosterone, and dihydrotestosterone equally stimulate inhibin production in vitro, whereas progesterone proved to be inhibitory under identical conditions. On the other hand, pregnant mare serum gonadotropin and estrogens do not have any detectable effect on the inhibin secretion in vitro (Franchimont et al. 1981).

Inhibin inhibits both the synthesis and the secretion of FSH in a species-nonspecific manner. The relatively small in-

hibin molecule is assumed to be associated with a large protein characteristic of the producing gonadal tissues. This gonad-specific protein can be liberated by proteolysis and this might also happen during the isolation procedures (Franchimont et al. 1981).

Inhibin acts at the hypothalamic, pituitary and gonadal levels. Centrally administered inhibin containing extract depresses FSH secretion by a hypothalamic mechanism in rats (Lumpkin et al. 1981, Moodbidri et al. 1981). Inhibin blocks LH-RH synthesis in the hypothalamus with a consecutive reduction in the FSH, but not LH levels in the serum of rats. Inhibin was shown to block basal FSH secretion of isolated pituitary cells despite the presence of LH-RH in the culture medium, indicating a highly selective action on the FSH secretion. Inhibin markedly inhibits FSH synthesis with a consecutive reduction in the FSH content of the medium, but also in the cultured pituitary cells themselves, the effect being more pronounced in the cells than in the medium. In LH-RH containing medium, inhibin was shown to exhibit only a moderate inhibitory action on LH secretion but a more expressed inhibiton on FSH secretion. TSH, prolactin and GH secretion, however, remained unaffected under identical conditions (Franchimont et al. 1981).

Inhibin was shown to prevent the incorporation of tritiated thymidine into testicular DNA and to decrease progesterone secretion in cultured granulosa cells (Demoulin et al. 1981). Studies concerned with the effect of inhibin on FSH uptake by rat testis indicates that inhibin might modulate the effect of FSH at the gonadal level (Vanage et al. 1981). Inhibin was shown to affect transcriptional events (Sarvamangala et al. 1984).

Inhibin is present in highest concentration in the small follicles of the ovary. During follicular maturation the concentration of 17-β estradiol and of inhibin changes inversely in the follicular fluid (Channing et al. 1981). This phenomenon as well as the effect of androgens on follicular inhibin production might play a role in the development of ovarian follicular atresia in vivo, a process of unknown mechanism at present (Franchimont et al. 1981).

Proteins structurally distinct from inhibins but displaying inhibin-like activities on FSH secretion have also been isolated and partially characterized from bovine (three proteins with a M_r of 31,000, 35,000 and 39,000, respectively) and porcine follicular fluids (M_r 35,000). The bovine and porcine proteins, whose interrelationship is unclear at present, were separately named FSH-suppressing proteins (FSPs; Robertson et al. 1987) and follistatin (Ueno et al. 1987) respectively. On the other hand, activin (a homodimeric structure consisting of inhibin β-subunits) and the structurally related transforming growth factor-β (see Chapter 8) were shown to stimulate FSH secretion (for a recent review on inhibins and activins see Ying 1987).

4.2.2. OOCYTE MATURATION INHIBITOR

Under in vivo conditions the oocytes are arrested in the dictyotene phase of meiosis in the follicles, while in vitro the absence of follicular fluid allows the cells to undergo spontaneous maturation (Shemesh, 1979). This is interpreted as an indication that the follicular fluid might contain some unidentified substance(s) with the capability of arresting oocyte maturation (Channing and Pomerantz 1981). The postulated oocyte maturation inhibitor (OMI) was partially purified from sow follicular extract (Stone et al. 1978). Amongst the two active fractions identified in the extract, one had a molecular weight of less than 2,000 while the other less than 1,000. The latter was purified 15,000-fold and its peptide character was demonstrated by Channing et al (1981).

For detecting biological activity of the purified extract, cultured cumulus-enclosed oocytes have been used as an in vitro assay (Stone et al. 1978, Hillensjo et al. 1980).

Changes in the concentrations of OMI and ihibin were shown to parallel in the follicular fluid, indicating that both inhibin and OMI might be produced by the granulosa cells (Channing et al. 1980). OMI acts by inhibiting oocyte maturation as

well as progesterone secretion in the cumulus cells (Channing et al. 1980). OMI actions do not seem to be species-specific. FSH was reported to stimulate OMI-like activity, an effect prevented by both testosterone and androstendione. Estradiol or LH had no effect on OMI activity (Channing et al. 1981).

4.2.3. FOLLICLE-STIMULATING HORMONE RECEPTOR-BINDING INHIBITOR, AND LUTEINIZING HORMONE RECEPTOR-BINDING STIMULATOR

Fractions separated from aqueous extracts of rat and calf testes, bovine follicular fluid, and from human serum all have been described to inhibit the binding of FSH to testes or ovaries in various species (Reichert et al. 1981). The postulated active agent was called follicle-stimulating hormone receptor-binding inhibitor (FSH-BI), referring to its assumed mode of action. On the other hand, another fraction from the same sources stimulated the binding of LH to its receptors, that is why it was named LH-binding stimulator (LH-BS). The exact chemical structure of these agents are unknown at present, but intensive efforts are being made in several laboratories to elucidate their chemical properties and possible biological functions (Reichert et al. 1979).

One of the highly purified fractions with a molecular weight of about 700 displayed a pronounced FSH-BI activity, and the material carrier of the activity was shown to be partially thermolabile and composed of amino acids in 45% by weight. Two additional fractions with only a moderate FSH-BI activity were shown to be thermostable and contained only a negligible amount of amino acids.

The finding that 70% of the FSH-BI activity in the mentioned fraction was lost after treatment with N-bromosuccinimide, or after incubation in the presence of immobilized carboxipeptidase Y, or aminopeptidase-M, as well as the fact that a highly purified active fraction displayed ninhydrin positivity, indicate the peptide character of the active agent(s). Attempts to demonstrate the presence of sugar, lipid, steroid or nucleotide components in the active

fraction remained essentially unsuccessful (Reichert et al. 1981).

By employing a separation scheme distinct from that used for the purification of FSH-BI activity, a fraction with a pronounced LH-BS activity but without a detectable FSH-BI activity could be separated. The two fractions behaved differently on anion exchange columns, with the FSH-BI but not the LH-BS, activity being retained on the anion exchanger (Reichert et. al. 1981).

FSH-BI and LH-BS were also studied in the hemodialysates of kidney patients. From the retentate obtained following ultrafiltration of hemodialysates through an UM 2 membrane an active fraction could be prepared by a multistep separation procedure, which markedly stimulated the binding of FSH to rat testis receptors but inhibited LH binding without the slightest FSH-BI activity. Available evidence supports the assumption that the observed effects are mediated by specific substances and are not due to the effects of aspecific contaminants (Reichert et al. 1981).

The quantitation of FSH-BI and LH-BS in biological fluids presently encounters serious difficulties, mainly due to the lack of suitable reference materials. Consequently, the binding inhibitory activity is usually assessed by estimating the ID_{50} value of the material in a radioligand binding assay, while the stimulatory activity is estimated by measuring the binding of specific radioligands both in the presence and the absence of the stimulatory material (Reichert et al. 1981).

The lack of highly purified substances also presents substantial difficulties in studies concerned with the mechanism of action of the binding inhibitors and stimulators. Data obtained with partially purified materials indicate a mixed (competitive and non-competitive) type effect on the binding of ^{125}I-labeled human FSH to testicular receptors (Reichert et al. 1981).

Establishing the physiological relevance of binding stimulator(s) and inhibitro(s) and also their possible pharmacological utilization is seriously hampered by our rudimentary knowledge concerning their biological activities in vivo, by

their apparently low concentrations in the body fluids, and by the lack of satisfactorily purified materials. Large-scale purification of the substances is now in progress in several laboratories, and will probably make them shortly available in quantities which will allow their characterization and structural identification.

4.2.4. LH RECEPTOR-BINDING INHIBITOR

Corpus luteum extracts from a variety of pseudopregnant or pregnant animal species were reported to contain a component(s) with an appearent LH receptor-binding inhibitory capacity (Yang et al. 1976, Sakai et al. 1977). The activity of the responsible agent, called LH receptor-binding inhibitor (LH-BI) seems to be bound to an agent(s) acting species-specifically on the corpus luteum in various animals (Yang et al. 1981). In extracts from rat ovaries in the luteal phase, both nondialyzable (large) and dialyzable (small) LH-BI activities were detected, whereas in extracts from porcine corpus luteum only the nondialyzable material was present. By ion exchange chromatography of rat ovarian extracts the LH-BI activity could be localized in a single fraction displaying dose-dependent bioactivity (Yang et al. 1981). The dialyzable LH-BI fraction had a molecular weight of about 3,800. By using a multistep separation procedure the nondialyzable LH-BI activity could be resolved into a minor and two major fractions with unchanged biological activities and with a molecular weight of around 20,000 (Yang et al. 1981). The finding that the charcoal-treated extract of corpus luteum inhibited coitus-induced ovulation in rabbits suggests that the LH-BI effect is not due to steroids. However, the polypeptide character of the responsible biologically active agent is uncertain at present (Yang et al. 1981).

LH-BI activity can be detected with ^{125}I-labeled LH in the homogenate of rat or porcine ovaries in the luteal phase. Extracts with LH-BI activity inhibit the binding of labeled LH to their ovarian receptors in a dose-dependent manner (Yang et al. 1976). Available data indicate that LH-BI activity is due

neither to a damage on the LH molecule caused by LH-BI, nor to the binding of the latter to LH (Yang et al. 1981). Storage at -20°C increased the yield of the extractable LH-BI susbtance. This may be explained by the disappearance of an LH-BI antagonist during storage, or by the action of a specific enzyme converting the inactive precursor into an active compound.

Both LH- and HCG-stimulated progesterone synthesis can be inhibited by extracts with LH-BI activity in vitro. The reported correlation between the LH-BI-induced inhibition of receptor binding and progesterone synthesis indicates that LH-BI may act as an LH antagonist at the receptor level in vitro (Yang et al. 1981). LH-BI does not compete with ^{125}I-LH for binding sites in receptor preparations. Further studies are needed to understand exactly the mechanism of action of LH-BI as well as its role in the regulation of reproductive functions.

4.2.5. GONADOCRININ

Ying and Guillemin (1979) described a number of gonadal substances with peptidic character which may participate in the control of the hypothalamo-pituitary axis. A small peptide partially purified from rat follicular fluid was shown to stimulate the release of pituitary LH and FSH, both in vitro and in vivo. The peptide with such activities was termed gonadocrinin (Ying and Guillemin 1979).

Follicular fluid of immature rats pretreated with pregnant mare serum gonadotropin was fractionated to purify gonadocrinin activity. Employing a multistep separation procedure, the biological activity was recovered in a separate homogenous peak. Digestion with trypsin completely abolished biological activity of this fraction, indicating the peptidic character of the active agent which, according to preliminary data, might be a 32 amino acid peptide (Ying and Guillemin 1981).

Gonadocrinin-like substances are usually assayed by determining gonadotropin secretion of cultured pituitary cells where, similarly to LH-RH, they stimulate gonadotrop hormone

secretion without affecting prolactin, growth hormone and TSH production to the slightest degree (Ying and Guillemin 1981).

Gonadocrinin activity has been detected in porcine and bovine ovaries (in the follicular fluid) and testes. Preliminary studies indicate that mature follicles contain more gonadocrinins than immature ones. Gonadocrinins are possibly produced in the granulosa cells, and affect LH and FSH secretion both in vivo and in vitro. Some of the gonadocrinins display actions similar to those of LH-RH, however, their affinity to the ovarian and hypophyseal LH-RH receptors is higher than is the affinity of LH-RH to the same structures (Ying et Guillemin 1981). This phenomenon raises the question of whether this hormone is an authentic ligand for the gonadal receptors. Gonadocrinin with LH and FSH secretion-augmenting activity may influence ovarian steroidogenesis by modulating gonadal LH receptor activity in vivo (Ying and Guillemin 1981). This might be the point of attack through which this peptide exerts its physiological action in the regulation of reproductive processes.

4.2.6. GRANULOSA CELL MATURATION STIMULATOR AND GRANULOSA CELL LUTEINIZATION INHIBITOR

In studies by Ledwitz-Rigby and Rigby (1981) evidence was presented on distinct biological activities present in the ovarian follicular fluid. One of them was shown to promote the morphological maturation of immature granulosa cells and to potentiate the effect of LH and FSH on such cells in the immature antral follicles. In addition, it also stimulates follicular secretion of both progesterone and estrogens. The postulated agent was termed granulosa cell maturation stimulator. Its effect is most prominent on granulosa cells originating from the smallest (immature) antral follicles, but is only moderate on those present in larger and more mature follicles. Another activity detected in the follicular fluid had an effect opposite to that of the maturation stimulator and was referred to as granulosa cell luteinization inhibitor. It acts

predominantly on granulosa cells of the large follicles, which undergo luteinization in the absence of LH and FSH in vitro. The luteinization inhibitor presumably interfers with the process of morphological luteinization, progesterone secretion, adenyl-cyclase activity and with the responsiveness of cells to a variety of hormones (Ledwitz-Rigby and Rigby 1981).

The luteinization inhibitory activity is thought to be due to a group of molecules rather than to a single hormone-like substance in the follicular fluid. Ultrafiltration experiments indicated a molecular weight between 10,000 and 100,000 for the agent, although a fraction with a similar inhibitory activity but with a molecular weight of about 2,000 could also be separated from the follicular fluid (Ledwitz-Rigby et al. 1977). Steroid removal by charcoal extraction did not affect the activity of the extract, indicating a nonsteroid origin of the observed effect. The activity resists freezing and thawing, and also persists following long-term storage.

Both the inhibitory and the stimulatory activities are assayed in cultured granulosa cells emitted from small-, medium- and large-sized follicles. By using this assay system, the process of luteinization, progesterone secretion, as well as the cAMP content of the cells may be assessed individually or simultaneously.

The inhibition of FSH binding to the granulosa cells by follicular fluid may explain why follicular fluid suppresses progesterone secretion. Manifestation of the inhibitory activity is not accompanied by a decreased cell viability. By increasing progesterone secretion, the stimulatory agent displays an opposite effect. In follicular fluid from smaller follicles the inhibitory activity is dominatory, whereas the stimulatory activity prevails in that from the larger follicles (Ledwitz-Rigby and Rigby 1981).

The activity of follicular fluid proved to be nonspecies-specific. The majority of mammalian ovarian follicles undergo atresia instead of ovulation. Little is known of the processes determining which follicles proceed towards atresia and which ones towards ovulation. However, it is generally agreed that the process is controlled by gonadotropins, steroids and perhaps by other hormones, including ovarian ones.

4.2.7. RELAXIN

Hisaw (1926) first described the capability of a watery extract from pregnant sow ovaries to inhibit uterine contractions and to soften the interpubic ligaments in guinea pigs. The observed effects were attributed to an agent named relax in. Subsequently, methods more or less suitable for the purification of relaxin have been described in a remarkably large number in the pertinent literature (Griss et al. 1967, Schwabe et al. 1978, Sherwood 1979, Anderson 1982). The activity of the final (pure) relaxin preparation was described to be 200-3000 guinea pig units (GPU), the latter being defined as the quantity of extract producing a manually detectable relaxation of the pubic symphysis in 8 out of 12 estrogen primed guinea pigs in 6 hours (see Schwabe et al 1978).

Due to its easy availability the porcine material was used for initial studies, although relaxin was subsequently purified from a number of other species as well (Bigazzi et al. 1980). Primary structures of porcine (James et al. 1977, Schwabe et al 1976, 1977) and rat relaxin (John et al. 1981) were determined and shown to be composed of two distinct polypeptide chains (A and B) with structures shown in Fig.4.4. Relaxin resembles insulin, both having A and B chain polypeptides with two interlinking disulfide bridges, and a third intrachain bridge. Their sequence homology is also striking, although insulin molecules from different species are far more homologous than are relaxin molecules from different species: porcine and rat insulins show sequence homologies over 90% while sequence homologies between relaxin molecules from these two species is less than 50%. Many of the charged residues in contact with the aqueous environment are likely to be situated on the surface of the relaxin molecule. The distinct number of such residues may be responsible for differences observed in the antigenicity of the rat and porcine molecules (Sherwood et al. 1979, Sherwood and Crnecovic 1979). The many similarities between insulin and relaxin suggest that both may have evolved from a common ancestral gene (Schwabe et al. 1978). As a matter of fact, the sequence homology extends not only to in-

sulin but to a much broader group of molecules, a feature providing a basis for listing relaxin among the insulin-like growth factors. Apart from insulin and relaxin, the group includes nerve growth factor, somatomedins, multiplication-stimulating activity and several forms of nonsuppressible insulin-like activities, all discussed in Chapter 8. Recent studies have also indicated homologies in the secondary and tertiary structure of relaxin and insulin, but the structural homologies are not associated with functional ones: purified relaxin preparations do not display insulin-like activites (Rawitch and Moore 1980). Shortening of the A chain of relaxin molecule at the N-terminus results in a loss of activity, whereas the C-terminal region of the B chain seems to be functionally unimportant. On the other hand, the methionyl and arginyl residues appear to be essential for biological activity (Schwabe et al. 1978).

Two human genes for relaxin were assigned to chromosome 9 (Crawford et al. 1984). mRNS species coding for the porcine relaxin sequence were isolated by Gast and associates (1980) from sow ovarian extract. The isolated mRNS directed the synthesis of an approximately 23,000 molecular weight protein displaying relaxin immunoreactivity. It had a 2,500 molecular weight signal sequence and a stable intermediate with a molecular weight of about 20,000. Subsequently, a cDNA encoding porcine relaxin has also been isolated and its structure determined (Haley et al. 1982). Similar studies on the structure of rat preprorelaxin revealed that the molecule has a 22 residue long signal sequence, which is followed by the B chain (35 residues), the connecting C-peptide (105 residues), and finally by the A chain (24 residues) (Hudson et al. 198la,b).

Besides chemically determined and/or cDNA-derived porcine and rat sequences, the primary structures of sand tiger shark (Gowan et al. 1981) and human relaxins (Hudson et al. 1983) have also been deduced from cloned cDNAs. All these relaxin sequences are shown in Fig.4.4.

Hudson and coworkers (1984) have reported the expression of a relaxin gene in human ovaries. The predicted structure of

A chains

```
                                                  10                                  20
h:  Arg-Pro-Tyr-Val-Ala-Leu-Phe-Glu-Lys-Cys-Cys-Leu-Ile-Gly-Cys-Thr-Lys-Arg-Ser-Leu-

r: pGlu-Ser-Gly-Ala-Leu  -  Ser  -  Gln  -  -  His  -  -  -  -  Arg  -  -  Ile-

s:  Ala-Thr-Ser-Pro   - Met-Ser-Ile  -  -  -  Ile-Tyr  -  -  -  -  Lys-Asp-Ile-

                                                  10
p:          Arg-Met-Thr  -  Ser  -  -  -  -  Gln-Val  -  -  Ile-Arg-Lys-Asp-Ile-

                  24
h:  Ala-Lys-Tyr-Cys
                  24
r:   -  -  Leu  -
                  24
s:  Ser-Val-Leu  -
                  22
p:   -  Arg-Leu  -
```

B chains

```
                                                  10
h:                  Lys-Trp-Lys-Asp-Asp-Val-Ile-Lys-Leu-Cys-Gly-Arg-Glu-Leu-Val-Arg-
                                        10                                          20
r: Arg-Val-Ser-Glu-Glu  -  Met  -  Gln  -  -  Gln-Val  -  -  -  Gly-Tyr-Ala  -

s:                  Gln-+++-+++-+++-+++-+++-+++-+++  -  -  -  -  Gly-Phe-Ile-  -

p:                  Gln-Ser-Thr-Asn  -  Phe  -  -  Ala  -  -  -  -  -  -  -

                   20                                                30   32
h: Ala-Gln-Ile-Ala-Ile-Cys-Gly-Met-Ser-Thr-Trp-Ser-Lys-Arg-Ser-Leu
                                                  30                        35
r:  -  Trp  -  Glu-Val  -  -  Ala     Ser-Val-Gly-Arg-Leu-Ala  -
                                        26
s:  -  Ile  -  Phe-Ala  -  -  Gly  -  Arg
                                                  29
p: Leu-Trp-Val-Glu  -  -  -  Ser-Val-Ser  -  Gly-Arg
```

Fig. 4.4. Primary structures of the A and B chains of human (h), rat (r), sand tiger shark (s) and porcine (p) relaxins. Full sequence is given for the human peptide. In the remaining sequences only the amino acid substitutions are indicated. Dashes represent residues identical to those in the human peptide. Aligment was made for maximizing homology. Cross mark triplets indicate undetermined residues

human relaxin obtained by analysing cDNA clones somewhat differed from that previously reported (Hudson et al. 1983).

Kwok and Bryant-Greenwood (1977) identified several forms of biologically active relaxin molecules in ovarian extracts that slightly differed both in net charge and amino acid composition from the peptides shown in Fig.4.4. Sow ovarian extract was also shown to contain several polypeptides of different molecular weight displaying relaxin immunoreactivity (Kwok et al. 1978). The one having a molecular weight of about

19,000 might be identical with the stable intermediate peptide isolated by Gast and associates (1980). A fraction purified from porcine ovarian extract had a molecular weight somewhat larger than 6,000 and displayed relaxin activities only after trypsine digestion (Frieden and Yeh 1977). Kwok and associates (1980) observed intensive proteolysis during relaxin purification and warned that this should be carefully considered when structure determination of the final product is aimed (see also Gordon et al. 1986 and Miyamoto et al. 1986).

Bioassays in general use are mostly based on the effects of relaxin on the pubic symphysis and the uterine contractions. The first assay described by Abramowitz and associates (1944) was the guinea pig symphysis palpation assay. This is based on manual palpation of the relaxin-induced softening of the interpubic ligaments. The uterine contraction-inhibiting effect of relaxin was utilized in the uterine motility-inhibition assays, of which both in vivo and in vitro varieties were developed. The latter assays proved to be more sensitive but less specific than the symphysis assay, and the extraordinarily rapidly developing tachyphylaxis makes the use of such assays rather time consuming (Eltze 1979, Schwabe et al. 1978, St-Louis 1981).

A RIA method has also been developed for porcine relaxin and a homologous RIA for rat relaxin (Sherwood et al. 1975a,b 1980, Sherwood and Crnekovic 1979). By using ^{125}I-labeled polytyrosyl relaxin as an antigen and porcine relaxin antiserum raised in rabbit, the peptide could be quantitated also in humans (O'Byrne and Steinetz 1976, Weiss et al. 1978, Weiss and Goldsmith 1981).

Serum levels of relaxin have been measured in different species under various conditions. Pre-delivery serum levels were found to be 5.5 ng/ml in pigs. This rose to 10.7 ng/ml during parturition and to a 3-8 times higher value postpartum (Afele et al. 1979). In this species, a pre-delivery relaxin surge could be induced by prostaglandin $F_{2\alpha}$ An extensive literature exists on changes in relaxin concentrations during the reproductive and gestation cycles (Weiss et al. 1976,

1978, 1981, Boyd et al 1981, Stewart and Stabenfeldt 1981). Human values measured in early normal pregnancies as well as in the state of imminent abortions are also available (Quargriarello et al. 1979a,b,c). In contrast to pigs, a pre-delivery relaxin surge could not be detected in humans (Quargriarello et al. 1980).

Relaxin is widely distributed in the phylogenetic scale. Apart from mammals, it was detected both in avian and elasmobranch species, but the physiological role that relaxin might play in extramammalian species remains unclear at present. By using both immunohistochemical and bioassay techniques (Anderson and Long 1978), relaxin was detected in the cytoplasmic granules of porcine corpora lutea (Belt et al. 1971), in corpora lutea of cows in late pregnancy (Fields et al. 1980), in human decidua (Bigazzi and Nardi 1981), in pre-delivery human placenta (Fields and Larkin 1981), in human gestational corpora lutea (Mathieu et al. 1981), and in human placental basal plates (Yamamoto et al. 1981). The role what immunoreactive relaxin-like substances might play in milk secretion and in human seminal plasma (Loumaye et al. 1980, Lippert et al. 1981) is unclear at present.

Relaxin is synthesized in the corpora lutea, mainly during pregnancy, but relaxin production could also be detected in corpora lutea of nonpregnant women following HCG stimulation (Quagriarello et al. 1980, Thomas et al. 1980). Serum levels of relaxin and HCG display parallelism during the first gestational trimester, and also in spontaneous abortions (Quagriarello et al. 1981). Relaxin secretion and consecutive cervical softening were shown to be inhibited by amino-glutethimide in pregnant rats, an effect which could not be counteracted by estrogen or progesterone, but cervical dilatability was restored by relaxin administration under such conditions (Schwabe et el. 1978).

Relaxin displays a rather wide spectrum of biological activity in the reproductive system. Its plasminogen activator, collagenase- and proteoglycan-releasing effects (Too et al. 1984) explain the action of this hormone on the pubic symphysis, a specific effect of relaxin, whose mechanism of ac-

tion is not completely understood. Relaxin elicits the release of lysosomal enzymes, which can be counteracted by glycocorticoids or progesterone (Schwabe et al. 1978). The cAMP system may be involved in the relaxin effects (Braddon 1978, Cheah and Sherwood 1980).

Relaxin inhibits myometrial contractions both in vitro and in vivo, and this effect can be prevented by both oxytocin and prostaglandin $F_{2\alpha}$ (Chamley et al. 1977). Downing and associates (1980) regard relaxin not simply as an inhibitor, but also as an important coordinator of myometrial contractions. In this capacity, relaxin is estrogen-dependent (Porter et al. 1979, 1981a,b). The antagonistic effect of prolactin and relaxin on isolated uterus strips (Bigazzi and Nardi 1981) suggests that relaxin might play an important role in coordinating uterine contractions during parturition. Extracts from human corpora lutea were also shown to be inhibitory on the contractions of human myometrium in vitro (Szlachter et al. 1980). Progesterone and relaxin act synergistically on this preparation (Beck et al. 1982).

Additional activities of relaxin include its contribution to the decidual changes in the endometrium (Schwabe et al. 1978), as well as to the increased water and glycogen content and size of the uterus (Zarrow and Brennan 1957, Vasilenko et al. 1980, 1981). Relaxin stimulates rat uterine collagen synthesis (Frieden and Adams 1985), and affects the shape of rat myometrial cells in culture (Hsu and Sanborn 1986) as well as the activities of several enzymes in the myometrium (Schwabe et al. 1978), and elicits cervical dilatation both in pigs and humans (Kertiles and Anderson 1979, MacLennan et al. 1980, 1981). It also exercises a moderate dilating effect in the vagina. Relaxin, estrogen and progesterone act synergistically upon lactation (Schwabe et al. 1978, Sherwood et al. 1981).

There are wide-scale speculations on the physiological roles which relaxin might play. In mice and guinea pigs, relaxin is supposed to take part in the preparation of the birth canal to the event of parturition by softening the interpubic ligaments and the uterine cervix. This effect, however, does not seem to be of primary importance in primates. This peptide

may have a role in the induction of parturition in beef (Musah et al. 1986). By inhibiting myometrial contractions in many species, relaxin may have its share in ensuring myometrial quiescence in the course of gestation, probably by playing some role in the central control of oxytocin release (Summerlee et al. 1984). There is evidence that the maternal pituitary suppresses the secretion of relaxin in pregnant rats (Golos and Sherwood 1984). The finding that relaxin precursor mRNA levels undergo characteristic changes in the rat ovary during pregnancy also indicates the involvement of this peptide in some presently unspecified gestation-related events (Crish et al. 1986). Since available data do not fully explain the exact role of relaxin in parturition, especially in mammals, further studies are needed on this field.

4.3. ENDOGENOUS PEPTIDES IN THE INTEGRATION OF THE CONTRACTILE ACTIVITY OF THE MYOMETRIUM

Our knowledge on the mechanism of labor onset is still incomplete. Evidence collected in lower mammalian species suggests that progesterone is the major factor keeping the uterus in a quiescent state during pregnancy (Csapo 1956). The findings that synthetic progesterone injected directly into the uterine wall (Bengtsson 1962), or intravenous infusion of the immediate precursor of progesterone (pregnenolone) temporarily or partially inhibited human labor (Scommegna et al. 1970), suggest that endogenous progesterone promotes relaxation of the uterus also in humans. However, administration of large doses of progesterone failed to suppress uterine activity in humans (e.g., Brenner and Hendricks 1962) in contrast to other mammalian species.

The action of progesterone seems to be mediated through stabilization of lysosomal phospholipase A_2, the enzyme by which the obligatory precursor of prostaglandins (PG) (arachidonic acid) is released. Thus the decreasing production of progesterone in late pregnancy results in promoting PG production (Csapo and Csapo 1974). PGs released from various intrau-

terine sources are considered to play a significant part in the mechanisms controlling the onset and progression of labor (Mitchell 1981).

In contrast to some animals, peripheral and amniotic fluid concentrations of progesterone and estradiol do not consistently change before parturition in humans, but the concentrations of estrone sulphate in peripheral plasma and amniotic fluid rise before term (Flint 1979). These data suggest that progesterone, and estrogens may have a permissive role in the onset of labor in humans, but are probably not involved in the acute control of the timing of parturition. The quiescent state of the human uterus during pregnancy is attributed to a balance between the biological activities of estrogen and progesterone (see e.g., Willcourt 1983). Once this balance is altered, uterine contractions commence.

Release of PGs within the uterus is believed to be one of the final events preceding onset of labor. Initiation of labor is associated with some alterations in the fetal membranes, the decidua and the myometrium (for review see e.g., Thorburn and Challis 1979). The primary event that triggers the mechanisms resulting in these biochemical changes has yet to be elucidated.

In the forthcoming discussion it is attempted to review the endogenous peptides that may play a part in the mechanisms controlling uterine activity during pregnancy. It should be stressed, however, that these peptides do not specifically affect uterine contractility, but they do have other physiologically defined roles as well.

4.3.1. NEUROHYPOPHYSEAL HORMONES

Oxytocin (OT) and arginine-vasopressin (AVP) are hormones of the neurohypophysis (see Chapter 3). They are the most potent endogenous stimulators of uterine musculature (for review see Milenov 1976, Whalley 1978, Akerlund et al. 1983, Fuchs et al. 1983). OT was shown to initiate uterine contractions by a direct action on the myometrium and also indirectly via PG

production in the decidua and/or amnion (Whalley 1978, Husslein et al. 1981, Fuchs et al. 1982, Kimura 1983). OT was demonstrated to bind to the microsomal fraction of the myometrial cells and to inhibit ATP-induced Ca^{2+} accumulation (Carsten and Milleri 1977). Similarly to OT, AVP has a direct stimulatory effect on the myometrium and it can also induce PG release, primarily in the endometrium (Lundstrom and Green 1978). OT and PGs (Brummer 1971, 1972), furthermore, AVP and $PGF_{2\alpha}$ have synergistic effects on human myometrium during pregnancy (Laudanski and Akerlund 1980). AVP can potentiate OT effects on uterine muscle (Fuchs 1973). Observations on human deliveries yielding anencephalic fetuses indicate that fetal OT, AVP and ACTH are not obligatory factors in initiating labor. However, in the absence of these hormones the course of labor is protracted (Swaab et al. 1977, Swaab and Oosterbaan 1983). All these data suggest that both fetal OT and AVP may be involved in the control of labor.

It has also been suggested that in some fetal mammals, a third neurohypophyseal hormone, arginine-vasotocin, may play a role in controlling labor (for review see Swaab and Boer 1979, Chard and Silman 1981).

The precise mechanism responsible for the release of OT is still unknown. However, available data indicate that estrogen, $PGF_{2\alpha}$, relaxin, opioid peptides in the central nervous system, as well as aminergic and cholinergic mechanisms stimulate the release of OT from the neurohypophysis (Gillespie et al. 1972, Amico et al. 1981, Dawood 1983, Summerlee et al. 1984). In women, stimulation of the lower genital tract elicits OT release (Chard and Gibbens 1983). OT is also released into the circulation in episodic spurts (Dawood et al. 1979b), although the validity of this observation was questioned by Leake et al. (1981). In the human fetus, OT is present in quantities measurable by radioimmunoassay (RIA) as early as the 14th weeks and possibly even earlier. OT content of fetal pituitary gland increases with gestational age, the mean OT content being 544 ng per gland near term (Khan-Dawood and Dawood 1984). Immuno- and bioreactive OT was also found in human ovaries (Khan-Dawood and Dawood 1983, Guoth et al. 1984) as well as in the human placenta (Makino et al. 1983).

There is a considerable controversy in regard of the immunoreactive OT levels of maternal blood in pregnancy, and the timing of OT secretion during labor. In primates, some authors registered fluctuatingly increasing OT levels in plasma and amniotic fluid throughout pregnancy (Vasicka et al. 1978, Dawood et al. 1979a,b Kimura 1983). Others failed to detect elevated OT levels in human plasma and amniotic fluid in the second half of pregnancy until stage 1 (Gibbens et al. 1976, Mitchell et al. 1980, Fuchs et al. 1983) or stage 2 of labor (Forsling et al. 1979, Leake et al. 1981, Glatz et al. 1981). In contrast to OT, immunoreactive AVP levels of maternal plasma are normal even at delivery (Pohjavuori and Fyhrquist 1983).

Both human and animal fetuses were shown to release OT and AVP during spontaneous labor (Chard et al. 1971, Fuchs and Saito 1971). OT and AVP levels of human umbilical cord plasma were found to be 68 and 76 pg/ml, respectively, at delivery (Swaab et al. 1978). In primates, OT levels were consistently higher in umbilical arterial blood than in umbilical venous blood. OT levels in umbilical arterial blood were higher in the neonates spontaneously delivered than in those delivered by elective Caesarean section. OT levels in amniotic fluid were higher during labor than prior to its onset (Dawood et al. 1978, 1979a,b, Kimura 1983). OT in the amniotic fluid seems to originate mainly from the fetus through urinary output (Seppala et al. 1972) or by direct efflux from the cord vessels. This is confirmed by serial measurements in ewes that showed higher OT levels in fetal than in maternal blood (Dawood et al. 1983). Data published by Swaab and Oosterbaan (1983) indicate that amniotic fluid OT might be produced by fetal organs other than the brain. AVP levels in human umbilical arterial blood were also found to be higher following spontaneous delivery than after an elective Caesarean section (Polin et al. 1977, Pohjavouri and Fyhrquist 1983). Asphyxia induces a high increase in plasma levels of AVP in lamb and human fetuses (Daniel et al. 1978, Parboosingh et al. 1983). The marked elevation of AVP levels in fetal plasma seems to be

due to delivery stress rather than to a generalized increase in the activity of the fetal endocrine system resulting in the onset of labor. In human fetuses both OT and AVP levels increase progressively from late pregnancy and throughout the first and second stages of labor reaching a peak value during delivery (for review see Chard and Silman 1981).

It is well known that uterine responsiveness to OT increases with advancing gestational age, especially during labor. OT sensitivity in pregnant rat is directly related to the concentration of OT receptors in the myometrium (Fuchs et al. 1983). Concentrations of OT receptors in rat, guinea-pig, sheep (for review see Liggins 1981) and in the human uterus (Fuchs et al. 1982) increase significantly in late pregnancy, reaching a maximum during spontaneous labor. OT receptors were found not only in the myometrium but also in the decidua and the amnion (Husslein et al. 1982, Kimura 1983), and their concentrations correlate with those of estrogen receptors (for review see Liggins 1981). OT was shown to stimulate PG production in decidual tissue, presumably via a receptor-mediated process et al. 1981). There is evidence that OT receptor concentrations are controlled by the estrogen/progesterone ratio (Soloff et al. 1977, Fuchs et al. 1983), and also the sensitivity of human uterus to AVP appears to be under the control of sex steroids (Bengtsson 1970). Since the concentrations of OT receptors in the myometrium and decidua increase parallel at term, circulating OT levels do not need to rise significantly to elicit PG production in the uterus, which can potentiate the action of subthreshold levels of OT which is required for initiating uterus contractions. $PGF_{2\alpha}$ appears to contribute to the progress of labor by releasing OT from the posterior pituitary gland (Gillespie et al. 1972). Husslein et al. (1983) suggested that both OT and $PGF_{2\alpha}$ are required, and must act synergistically on the human uterus, for the initiation and maintenance of labor contractions, but OT may be the major stimulus for initiating labor (Fuchs et al. 1983). This OT is most likely of fetal origin. Studies on baboon indicated namely that OT can be rapidly transferred from fetal circulation to amniotic fluid, to the uterine venous blood, and to

the maternal circulation (Dawood et al. 1979). On the other hand, in hypertonic saline-induced abortions the increase in $PGF_{2\alpha}$ production is preceded by a transient but significant OT secretion detectable in the maternal plasma prior to onset of labor, and by an increase in uterine response to OT (Fuchs et al. 1984). The spurts of OT in the fetus are accompanied by a still greater release of AVP (for review see Chard and Silman 1981). The cysteine amino peptidase responsible for OT degradation in the pregnant plasma is released preferentially into the maternal plasma from trophoblast cells and does not cross the placental barrier (Leake et al. 1980). These data suggest that the OT of fetal origin acting on the amnion and/or decidua may have a major part in initiating and accelerating labor, and that fetal AVP may also participate in the process.

4.3.2. ADENOHYPOPHYSEAL HORMONES

4.3.2.1. FETAL ACTH AND ACTH-RELATED PEPTIDES

In monkeys, fetal hypophysectomy leads to adrenal atrophy and may prolong pregnancy (for review see Novy 1977). In humans, anencephaly is associated with adrenal atrophy and a high percentage of both pre- and post-term labors (Honnebier and Swaab 1973). The finding that infusion of ACTH into sheep fetuses resulted in an increased cortisol production and premature delivery indicates that the fetal hypothalamo-hypophyseal-adrenal system plays a triggering role in the onset of parturition in this species (for review see Liggins et al. 1977). However, in contrast to sheep, the primate pituitary does not seem to play a decisive role in initiating labor, instead it is rather involved in the precise control of the mechanisms underlying labor initiation. In primate a number of peptides such as ACTH, α-MSH and CLIP (see Chapters 3 and 5) are found in the pituitary that are derived from a large common precursor peptide which may control the fetal adrenal gland (for review see Chard and Silman 1981, and Chapter 5).

The finding that ACTH or corticosteroid administration into human fetuses initiates labor in postterm pregnancies (Mati et al. 1973, Nwosu et al. 1976) points to the existence of a fetal hypothalamo-hypophyseal-adrenal mechanism which is concerned with the timing of labor also in man. Fetal cortisol, whose production is controlled by the pituitary gland, acts on the rate limiting steps in estrogen biosynthesis and may be regarded as the trigger for parturition (for review see Mitchell 1979, Thorburn and Challis 1979). AVP may be one of the hypothalamic stimuli that releases ACTH in the fetus (for review see Challis and Thorburn 1976).

In humans, ACTH levels in the umbilical cord and maternal blood were found to increase during labor (Rust et al. 1980, Pohjavuori and Fyhrquist 1983). Since ACTH does not cross the placenta (Miyakawa et al. 1974) these changes are likely due to the stress caused by the uterine contractions both in the fetus and the mother. In human amniotic fluid, ACTH levels were higher between the 26th to 30th week of gestation than in early and late pregnancy, and it was unaffected by uterine contractions (Tuimala et al. 1976).

Uterine contractions could be induced in postterm pregnant women by ACTH infusion (Miyakawa et al. 1981). In human pregnancies, ACTH infusion elicits an increase in the plasma levels of $PGF_{2\alpha}$ metabolite (13,14-dihydro-15-keto-$PGF_{2\alpha}$) (Zahradnik et al. 1982). In addition, ACTH can stimulate the contractions of human myometrial strips obtained at term (Pajor et al. 1984c). These observations suggest that ACTH may play a part in the stimulation of human uterus at term by a mechanism that is different from that operating in the adrenal gland target. However, the physiological significance of ACTH and ACTH-related peptides secreted by the human placenta in the control of labor remains elusive for the present time.

4.3.2.2. PROLACTIN

Prolactin (PRL) is a polypeptide hormone of pituitary origin (see Chapter 3). In vitro experiments with ovine PRL demonstrated that PRL has the capability of modifying contraction pattern seen in nonpregnant rat myometrium in response to OT (Horrobin et al. 1973). PRL reduces the contractile activity of myometrial strips prepared from late pregnant guinea-pig uterus, while similar preparations from less advanced pregnancy were less sensitive to PRL. In appropriate concentrations, PRL could stop OT-stimulated contractions of human myometrium preparations obtained from uterus undergoing Caesarean section (Horrobin 1973). PRL in higher concentration exerts a slight inhibitory effect on the contractility of pregnant rabbit myometrium in vitro (Mati et al. 1974).

During pregnancy, a five- to twenty-fold increase in maternal plasma levels of PRL can be observed, reaching a maximum shortly before delivery. There is a marked fall in maternal blood PRL during spontaneous and induced labor with values remaining low for 20 minutes postpartum (for review see Anderson 1982, Haddad and Morris 1984). Elevated PRL secretion observed during pregnancy is mediated by estrogen (for review see Baumann 1979).

PRL levels in umbilical cord blood are higher than in maternal blood, but these levels do not correlate directly (Haddad and Morris 1984). The fetal pituitary gland starts to synthesize PRL from around the 10th gestational week onward, but a rapid rise in the PRL production occurs in the third trimester. Surprisingly, anencephalic infants have normal pituitary PRL content (for review see Baumann 1979). PRL seems to be specifically concentrated in amniotic fluid. Bromocriptine administered throughout pregnancy suppresses both maternal and fetal blood PRL but has little or no effect on amniotic fluid PRL (Del Pozo et al. 1977), indicating that the regulation of amniotic fluid PRL concentration is different from that of blood PRL levels. PRL concentrations in human amniotic fluid are low at the initial stage of gestation. Hereafter, however, the levels increase tremendously reaching a peak

value at the 20th week of gestation, and declining thereafter, (Tyson et al. 1972, Anderson 1982, Freeman et al. 1984). Amniotic fluid PRL may originate from the fetal pituitary gland (Clements et al. 1977), but may be released by the placenta as well (Zheng et al. 1982). Human decidual (Golander et al. 1978, Riddick et al. 1978) and myometrial tissues (Walters et al. 1983) were shown to synthesize and secrete a PRL species similar to pituitary PRL. Decidual and myometrial PRL seem to be under the local control of the placental fetal membrane-uterine unit, operating through mediators such as estrogen and progesterone (Markoff et al. 1983, Handwerger et al. 1983).

Available data suggest that PRL within the concentration range found in human amniotic fluid can inhibit myometrial activity. This assumption is supported by the observation that women requiring OT stimulation during labor have higher serum PRL levels at the onset of labor, compared to those without OT stimulation (Haddad and Morris 1984).

In addition it was reported (Chatterjee 1976) that PRL could preserve pregnancy in 10-day pregnant rats injected with $PGF_{2\alpha}$, which alone produces luteolysis with subsequent termination of pregnancy. Thorburn and Challis (1979) suggested that PRL could be responsible for maintaining the quiescence of the uterus in early pregnancy, presumably by inhibiting PG synthesis in the decidua and amnion.

In contrast to the results obtained with PRL in human uterus, both the frequency and the amplitude of the contractions can be increased by PRL in spontaneously contracting isolated uterus strip prepared from estrogen-primed animals. Furthermore, PRL and relaxin exert antagonistic effects on the spontaneous motility of rat uterus (Bigazzi and Nardi 1981). All these together raise the possibility that PRL in concentrations present in the maternal circulation may modify the contractile activity of human uterus and may also reflect a different sensitivity of the uterus to PRL during pregnancy. However, it is unknown at present whether the in vitro observations can, or cannot, be applied to uterus functioning in vivo.

4.3.3. RELAXIN

Relaxin is a polypeptide hormone (for structural data see section 4.2.7.) - that is formed in largest quantity in the ovarian gland and the placenta, but is also produced in the decidua and uterine cervical tissue (Dallenbach and Dallenbach-Hellweg 1964, Maillot et al. 1977, Bigazzi et al. 1980, Fields and Larkin 1981).

It has long been known that in isolated uterus preparations from laboratory rodents, the contractions are inhibited by relaxin (Kroc et al. 1959). According to a more recent report, human ovarial (corpus luteum) extract also exerts an inhibitory effect on the spontaneous motility of human myometrium in vitro (Szlachter et al. 1980). Relaxin also inhibits both spontaneous and $PGF_{2\alpha}$ induced contractions of mammalian myometrium in vivo, without affecting the sensitivity of myometrium to OT (Porter 1979). Since estradiol had a similar effect in some species, the possibility was raised that relaxin effect on the myometrium may be mediated by estrogen (Porter 1979). In rats, relaxin was demonstrated to improve the coordination of myometrial contractile activity (Downing et al. 1980). Relaxin-induced inhibition of uterine contractions were shown to be potentiated by progesterone in rats (Brenner et al. 1984). Relaxin counteracts the effect of PRL on rat uterine strips (Bigazzi and Nardi 1981).

Relaxin binds to the uterus (Cheah and Sherwood 1980) and is capable of elevating intracellular cAMP by a mechanism which probably does not require β-adrenergic mediation (Sanborn et al. 1980). It is presently not clear, however, whether the effects of relaxin on cAMP and the contractile activity are causally related. In rats, myometrial relaxin receptor concentration appears to be related to plasma estrogen and relaxin levels. The number of relaxin receptors in the myometrium decreases in late pregnancy (Mercado-Simmen et al. 1982), which is probably due to the high levels of circulating relaxin.

In the course of pregnancy of laboratory rodents and pigs, relaxin concentrations increase both in the plasma and the ovary as the term approaches, reaching an especially high value immediately before parturition (Anderson et al. 1973, Sherwood et al. 1975, O'Byrne et al. 1976). At the end of human pregnancy, an elevated relaxin-like activity was observed in the extracts of uterine cervix followed by a further increase during labor (Maillot et al. 1977). In human plasma, the relaxin level detected by bioassay rose parallel with the gestational age (Zarrow et al. 1955), while plasma levels of heterologous immunoreactive relaxin decreased as pregnancy advanced (O'Byrne et al. 1978).

The data presented above suggest that relaxin might have a role in maintaining myometrial quiescence during pregnancy and in the coordination of myometrial contractions during labor.

4.3.4. VASOACTIVE INTESTINAL POLYPEPTIDE

Vasoactive intestinal polypeptide (VIP) essentially is a neuropeptide (see Chapters 5 and 6). At the same time, VIP is a potent smooth muscle relaxing agent with an inhibitory effect on non-pregnant human uterus. Although VIP inhibits myometrial activity during the first trimester of human gestation, it is without any effects on in vitro test systems prepared from uterus at term (for review see Kachelhoffer et al. 1979, Ottesen 1983). VIP-containing nerves were shown to be associated with uterine smooth muscle in several species (Larsson et al. 1977) including humans (Polak and Bloom 1979). The human placenta proved to be an extraordinarily rich source of VIP (Attia et al. 1976) and is also capable of synthesizing this peptide (Humphrey et al. 1979).

RIA-assessed umbilical cord arterial and venous VIP levels were shown to be higher than VIP concentrations in maternal peripheral blood. During labor, a significant increase occurs in VIP concentrations of maternal plasma (Ottesen et al. 1982a). Specific and high-affinity receptors for VIP were described in the uterus (Ottesen et al. 1982b). Receptor-mediated

VIP effects are likely realized through mechanisms involving activation of the adenylate cyclase system, but not β-receptor-mediated events (Bolton et al. 1981). In rabbits, estrogen and progesterone treatment renders the myometrium more sensitive to the relaxing effect of VIP (for review see Ottesen 1983). Since both OT and $PGF_{2\alpha}$ seem to be involved in the initiation of human labor (Fuchs et al. 1983, 1984), the reported abolishment of OT- and $PGF_{2\alpha}$-induced myometrial contractions (Ottesen et al. 1979, 1980) may be interpreted to mean that VIP may have a role in maintaining the quiescent state of uterus during gestation.

4.3.5. KININS

In general, the bradykinin (BK) family of peptides are potent vasoactive susbtances (see Chapter 13). However, besides vascular smooth mucles, BKs also act on other types of smooth muscles including uterine musculature (for review see Walaszek 1975). BK stimulates the myometrium via a direct mechanism and by releasing PG both in the endometrium and the pregnant uterus, (Whalley 1978, for review see Terragno and Terragno 1979). PG-releasing activity of BK appears to be controlled by sex steroids (Phaily and Senior 1978). BK-evoked contractile responses of myometrium is potentiated by PGF_2 (Chivers and Whalley 1977). The effect of BK on the uterus is also potentiated by some endogenous peptides (Hamberg et al. 1968), such as human fibrinopeptide A (FA) and bovine fibrinopeptide B (FB) (Gladner 1966, Febar an Van der Meer 1973).

Studies on the kallikrein-kinin-kininase system provided circumstantial evidence for the involvement of kinins in the control of myometrial activity during pregnancy and parturition. In some species, plasma kininogen levels increase parallel with gestational age (Martinez et al. 1962, Weigerhausen et al. 1968, Porter et al. 1972, McCormick and Senior 1974). Changes in the concentration of circulating kininogen appears to be related to estrogen/progesterone ratio in the blood (Senior and Whalley 1974). Depletion of plasma kininogen in

pregnant rats by treatment with cellulose sulphate at the 19-22 days of pregnancy results in a prolongation of the gestation period, an increase in the duration of preparturient behavior (labor), and a delay in the onset of delivery (McCormick et al. 1974). The treatment of pregnant rats with aprotinin, a kallikrein, plasmin and tissue kininogenase inhibitor, at days 19-22 of the gestation had a similar effect (Senior and Whalley 1976). Malofiejew (1973) reported an increased plasma kallikrein activity in women during stage 2 of labor. Up to the 20th day of pregnancy the kininase activity in rat plasma decreased parallel with advancing gestation (Erdos and Yang 1970). Kininogen content and kininase activity of the amniotic fluid are lower at the onset of labor than in the second trimester of gestation (Delhaye et al. 1967, 1972). Kininogenase and kininase activities were demonstrated both in the human myometrium and the placenta (Uszynski 1970, Starostina 1972). All these data indicate that intrauterine kinin formation increases, and kinin degradation decreases at term of delivery.

In the human uterus, kininogenase and kininase activities are lower in cases of uterine inertia than in those of physiological labor (Starostina 1972). Uterine contractile activity in vivo is reversibly inhibited by aprotinin administered during labor (Konopka et al. 1973b), although similar effect could not be elicited in uterus preparation in vitro (Whalley and Riley 1979). There is a known relationship between the function of kallikrein-kinin and hemostatic systems (for review see Movat 1979). On the one hand, kallikrein or plasmin can activate factor XII (Hageman factor) and, through activation of this factor, the thromboplastic, fibronilytic and the kinin-forming systems are also set into motion. Accordingly, kinins may also be involved in facilitation of the contractile activity of uterus also in humans.

4.3.6. ANGIOTENSIN II

Angiotensin II (AGII) is a vasoactive octapeptide (see Chapters 3 and 5) which is formed from an inactive decapeptide (angiotensin I) by a converting enzyme identical to kininase. The latter is produced in the lung, placenta and the myometrium (Uszynski 1970, Erdos 1979). Angiotensin I is enzymatically released from plasma α-globulin by the action of renin.

It was repeatedly shown that AGII produces myometrial contractions (Paiva and Paiva 1960, Dubin and Ghodgaonkar 1980). At least two types of AGII receptors were found in rat uterus musculature (Moore 1980) that are distinct from the OT receptors (Kwok and Moore 1980). Uterine AGII receptors seem to be under the regulatory influence of estrogen and progesterone. The levels of AGII receptors are low in rat uterus at the final stage of gestation (Schirar et al. 1980a,b).

Interactions between AGII and its receptor induces changes in ion permeability of cell membranes that ultimately results in an increased cytosolic concentration of free Ca^{2+} which, on the other hand, is responsible for the ensuing contraction of actomyosin in the smooth muscle (Freer and Smith 1976, Devynck and Meyer 1978). In addition to this direct action, AGII also indirectly contributes to myometrial contraction through its sex steroid-dependent PG releasing action (Baudouin-Legros et al. 1974, Dubin and Ghodgaonkar 1980).

Both renin activity and renin substrate are raised in plasma of pregnant women due to an increase in progesterone levels during gestation. Plasma AGII is approximately doubled in the course of pregnancy, and this is associated with a characteristic decrease in vascular sensitivity to AGII (Symonds et al. 1976, for review see Mott 1979). Both the myometrium and the chorion-decidua are capable of producing renin (Symonds et al. 1968, Johnson 1980, Craven et al. 1983). Changes in activity of the renin-angiotensin system in the endometrium are independent of the activity of the plasma renin system (Johnson 1980). Renin activity in the amniotic fluid (Brown et al. 1964) can be attributed to local synthesis. Neither renin nor AGII cross the placental barrier, but placenta may be the main source of both maternal and fetal AGII (Symonds 1979).

Following the onset of labor, plasma renin activity increases further, particularly in stage 2 (Symonds et al. 1976, Symonds and Pipkin 1980, for review see Mott 1979). At delivery, renin activity and AGII levels are usually higher in cord blood plasma than are in maternal plasma (Pipkin and Symonds 1977). Renin activity in umbilical venous plasma is lower in long-lasting labors than in those with normal duration, yet AGII concentrations are higher in the former cases (see review by Mott 1979). This indicates that the onset of labor is accompanied by renin release in the uteroplacental tissues resulting in an increased local formation of AGII.

Data presented above allow us to conclude that AGII produced in the uterus at the terminal stage of gestation, especially during labor, may facilitate the contractility of human pregnant uterus.

4.3.7. ADDITIONAL PEPTIDES

4.3.7.1. SUBSTANCE P AND NEUROTENSIN

In regard of their activity on smooth muscles, substance P (SP) and neurotensin (NT) (see Chapters 5 and 6) are similar to the kinins, but they contract rat uterus less actively in vitro than do BKs (e.g., Horton 1959, Carraway and Leeman 1973 and Chapter 13). Immunoreactive SP was detected both in maternal and umbilical cord blood (Skrabanek et al. 1980) and also in the uterus (Alm et al. 1978). Despite some confusing data (Kenneth et al. 1981), their potential regulatory role in uterine contractility during gestation cannot be ruled out.

4.3.7.2. PEPTIDES ASSOCIATED WITH HEMOSTASIS

The ultimate result of the activation of the coagulation cascade is thrombin formation. Owing to the action of thrombin on fibrinogen, two polypeptides, fibrinopeptide A (FA) and fi-

brinopeptide B (FB) are formed, whose amino acid sequences have been established (Fig.4.5). Bovine FB and human FA were found to potentiate the BK-induced contraction of the isolated uterus (Gladner 1966, Febar and Van der Meer 1973). Human FA was reported to elicit the contraction of rat uterus in vitro (Febar and Van der Meer 1973). A combination of human FA and FB exerts a dose-dependent inhibitory effect on human myometrial strips prepared from term uterus (Pajor et al. 1984a).

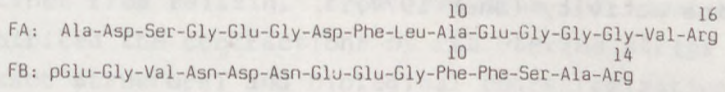


Fig. 4.5. Primary structures of human fibrinopeptide A (FA) and fibrinopeptide B (FB)

Fibrin degradation product (FDP) consists of a number of polypeptides with various molecular weight. FDP was shown to potentiate the effect of OT, BK and AGII on the rat uterine strips and to exert a direct stimulatory action on these strips (Malofiejew 1967). FDP produced by the addition of plasmin to human fibrinogen or plasma in vitro stimulates the contractions of human myometrial strips prepared from uterus removed prior to the onset of labor, but inhibits the contractions of similar test system prepared from uterus performing labor contractions (Basu 1969). A partially purified human plasma fraction with M_r ranging between 500 and 20,000, that was previously exposed to fibrinolysis, was shown to inhibit the contractions of human myometrial strips prepared from laboring uterus (Pajor et al. 1984b).

Significant changes occur in the hemostatic activity in the course of pregnancy, especially during labor (Konopka et al. 1973a, Gilabert et al. 1978, Howie 1979), which is reflected in increased clotting and fibrinolytic activities of the uteroplacental circulation (Basu and Jeffcoate 1970, Hahn 1974, Foley et al. 1977). Since the activation of hemostasis is more pronounced in pathological pregnancies than in normal ones (Wallmo et al. 1984), peptides released within the uterus in association with elevated hemostatic activity characteris-

tic of such cases may be capable of influencing contractility of the pregnant uterus. This hypothesis is supported by several observations. Physiological labor can be reversibly inhibited by a protease inhibitor (aprotinin) and the inhibition can be prolonged by synthetic anti-fibrinolytic agents (Konopka et al. 1973b). However, intravenous infusion of a fibrinolysis inhibitor in patients with abruptio placentae and with consecutive uterine inertia results in a rapid recoordination of uterine activity (Sher 1977).

4.3.7.3. PARATHYROID HORMONE FRAGMENT

The synthetic parathyroid hormone (PTH) fragment containing the amino terminal 34 amino acids was reported to inhibit OT-, $PGF_{2\alpha}$-, acetylcholine- and electrical stimulation-induced myometrial contractions in vitro (Pang et al. 1981). This suggests that the PTH(1-34) fragment, which also occurs in the circulation (see Chapter 3) has a non-specific depressing effect on the contractile mechanism of the uterus. Since maternal PTH levels increase during late gestation (for review see Pipkin 1979), the potential regulatory role of PTH in the control of uterus contractions should also be considered.

4.3.7.4. UNIDENTIFIED POLYPEPTIDES

Some peptides with unidentified structure may also be involved in the control of contractile activity of myometrium during pregnancy. A polypeptide with OT-like activity in vitro has been partially purified and characterized from the serum (Peterlik 1968). Hunter and Howard (1960) described a factor with peptidic character (hysterotonin) in the decidua and amniotic fluid of toxemic patients which induced uterine contractions. A polypeptide with contracting activity on rat uterus in vitro has been partially purified from hemodyalysates of pregnant women with toxemia (Vorne et al. 1974).

Jung and Klock (1969) detected a dialyzable factor of peptidic nature which was released upon stretching of human myometrium strips. This factor was inhibitory on OT-induced uterus contractions in vitro. Pajor and associates (1982) have partially purified a peptide factor from human amniotic fluid with an inhibitory activity on the contractions of human pregnant uterus strip preparations (uteroinhibin). Guoth and coworkers (1984) have succeeded in the isolation of a polypeptide distinct from relaxin, from extracts of sow ovaries, which inhibited the contractions of rat uterine strips.

The exact structural and biological characterization of the numerous uterus-active agents reported as various "factors" in the pertinent literature may contribute to our better understanding of the mechanisms concerned with the regulation of uterus contractions both in normal and pathological conditions.

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CHAPTER 5

ENDOPEPTIDES IN THE INTEGRATION OF NEURAL FUNCTIONS. THE NEUROPEPTIDES

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INTRODUCTION

The "neuropeptide" term is self-explanatory: it serves to denote biologically active peptides which are generated, stored and released by neural elements and may function as neurotransmitters, neuromodulators or neurohormones. The number of presently known neuropeptide sequences is well over 50 (Palkovits 1984, 1986, Krieger 1985; Table 5.1).

Introduction of new techniques and research strategies greatly accelerated the discovery of novel peptide sequences with a potential neuropeptide function. Cloning and sequencing of cDNAs encoding the precursors of biologically active end-products, pioneered by Nakanishi and his coworkers (1979, 1980, see also Noda et al. 1982, Nawa et al. 1983), and the analysis of transcription events (for review see Schwartz and Costa 1986), led, among other discoveries, to establishing the complete amino acid sequence of precursors of various opioid peptide families, those of mammalian tachykinin group (Kotani et al. 1986, Nakanishi 1986) and to the description of calcitonin gene-related peptides (Amara et al. 1982, 1985). Furthermore, when the complete amino acid sequence of a large-molecular weight precursor becomes known, it might reveal several hitherto unidentified sequences which, considering the probable routes of processing, can be suspected to appear regularly among the end-products and having neuropeptide function on their own right. The work based on the suggestion, put forward by Tatemoto and Mutt (1980, 1981), that the selective isolation of peptide amides from tissue extracts might

Table 5.1. The list of neuropeptides[1]

neuropeptide[2]	synonym(s)	abbreviation	reference
1. "Hypothalamic" neuropeptides			
1.1. Corticotropin releasing factor		CRF	Vale et al.(1981), Bloom et al.(1982), Fischman and Moldow (1982)
1.2. Growth hormone release inhibiting hormone	Somatostatin, Somatotrop hormone release inhibiting hormone	SRIF	Vale et al.(1974), Brownstein et al. (1975), Elde and Parsons (1975)
1.3. Growth hormone releasing hormone	Somatoliberin, Somatocrinin	GRF	Guillemin et al. (1982), Spiess et al.(1983), Sawchenko et al. (1985),
1.4. Luteinizing hormone releasing hormone	Gonadoliberin, Luliberin, Gonadotropin-releasing hormone	LH-RH, LRF, GnRH	McKann (1962), Barry et al.(1974), Palkovits et al. (1974)
1.5. Thyrotropin releasing hormone	Thyroliberin	TRH	Brownstein et al. (1974), Olivier et al. (1974)
2. "Hypothalamic-neurohypophyseal" peptides			
2.1. Oxytocin		OT	Swanson (1977), Buijs et al. (1978), Dogterom et al. (1978)
2.2. Vasopressin		AVP	Zimmerman et al. (1975), Swanson (1977), Buijs et al. (1978), Dogterom et al. (1978)
3. Brain-born "pituitary" peptides			
3.1. Corticotropin	Adrenocorticotrop hormone	ACTH	Krieger et al. (1977), Larsson (1977), Watson et al. (1978b)
3.2. Corticotropin-like intermediate lobe peptide		CLIP	Watson (1980), Akil et al. (1984)
3.3. Growth hormone	Somatotrop hormone Somatotropin	GH, STH	Pacold et al. (1978)
3.4. Luteinizing hormone	Luteotrop hormone, Luteotropin, Interstitial cell stimulating hormone	LH, ICSH	Hyyppa and Liira (1979)
3.5. Melanocyte stimulating hormones (α, β, γ)		MSH	Guillemin et al. (1962), Jacobowitz and O'Donohue (1978), Nakanishi et al. (1979, 1980)

3.6. Prolactin	Lactotrop hormone	PRL	Fuxe et al. (1977a), Hokfelt et al. (1978a), Pacold et al. (1978), Toubeau et al. (1979)
3.7. Thyroid stimulating hormone	Thyrotropin	TSH	Pacold et al. (1978)
4. "Brain" peptides			
4.1. Carnosine			Margolis (1974)
4.2. Neurotensin		NT	Carraway and Leeman (1973, 1975)
4.3. δ-sleep inducing peptide		DSIP	Schonenberg and Monnier (1977)
5. Brain-born "gastrointestinal" peptides			
5.1. Cholecystokinin		CCK	Vanderhaeghen et al. (1975), see Dockray and Gregory (1980)
5.2. Insulin			Havrankova et al. (1978), Herschman (1986)
5.3. Galanin			
5.4. Glucagon/glicentin			Conlon et al. (1979), but see Martinez and Potier (1986), Palkovits (1986)
5.5. Motilin			Chey et al. (1980), Chan-Palay et al. (1981), Nilaever et al. (1981)
5.6. Pancreatic polypeptides[3] (avian, bovine, neuropeptide Y peptide YY)		APP, BPP, NPY, PYY,	Loren et al. (1979a), Tatemoto and Mutt (1980), Tatemoto (1982)
5.7. Peptide histidine-isoleucine		PHI-27	Tatemoto and Mutt (1980, 1981)
5.8. Secretin			Mutt et al. (1979)
5.9. Vasoactive intestinal polypeptide		VIP	Larsson et al. (1976), Fuxe et al. (1977b)
6. Brain-born "peripheral hormone" peptides			
6.1. Angiotensin II.		AT-II.	Fischer-Ferraro et al. (1971), Fuxe et al. (1976), Phillips et al. (1979)
6.2. Atrial natriuretic polypeptides	Atriopeptides	ANP	Jacobowitz et al. (1985), Saper et al. (1985)
6.3. Bradykinin			Hori (1968), Correa et al. (1979),
6.4. Calcitonin-gene-releated peptides (α and β)		CGRP	Amara et al. (1982, 1985)

Table 5.1. cont.

7. "Non-mammalian" peptides			
7.1. Bombesin[4]/Gastrin releasing peptide		BOM/GRP	Erspamer and Melchiori (1973), Brown et al. (1978), Erspamer et al. (1978)
7.2. Hydra head activator			Schaller and Bodenmuller (1981)
7.3. Molluscan cardioexcitatory neuropeptide		FMRF-amide	Price and Greenberg (1977), Greenberg et al. (1983)
7.4. Proctolin			Brown (1975), Bishop et al. (1981)
8. Other peptides			
8.1. Opioid peptides			
8.1.1. β-endorphin and related peptides	C fragment	β-EP or β-end	Bradbury et al. (1976) Li and Chung (1976)
8.1.1.1. β-EP(1-27)	C' fragment		Bradbury et al. (1976), Smyth and Zakarian (1980),
8.1.1.2. β-EP(1-26)			Bradbury et al. (1976), Smyth and Zakarian (1980)
8.1.1.3. β-EP(1-17)	γ-endorphin	γ-EP	Ling et al. (1976) Guillemin et al. (1976)
8.1.1.4. β-EP(1-16)	α-endorphin	α-EP	Ling et al. (1976) Guillemin et al. (1976)
8.1.2. Enkephalins and releated peptides			
8.1.2.1. Leu-enkephalin		Leu-enk or Leu-E or LE	Hughes et al. (1975)
8.1.2.2. Met-enkephalin		Met-enk or Met-E or ME	Hughes et al. (1975)
8.1.2.3. ME-Arg[6], Phe[7]			Stern et al. (1979)
8.1.2.4. ME-Arg[6], Gly[7], Leu[8]			Kilpatrick et al. (1981a) Ikeda et al. (1982), Jones et al. (1982)
8.1.2.5. "Bovine adrenal medulla" peptides		BAM-peptides	Mizuno et al. (1980a,b), Evans et al. (1985)
8.1.2.6. Peptide E			Kilpatrick et al. (1981b),
8.1.2.7. Peptide F			Jones et al. (1980)
8.1.2.8. Metorphamide			Weber et al. (1983a)
8.1.3. Neoendorphins and dynorphins			
8.1.3.1. α-neoendorphin			Kangawa et al. (1979, 1981), Minamino et al. (1981)
8.1.3.2. β-neoendorphin			Kangawa et al. (1979, 1981), Minamino et al. (1981)

8.1.3.3. Dynorphin A(1-17)			Goldstein et al. (1981)
8.1.3.4. Dynorphin A(1-8)			Seizinger et al. (1981), Weber et al. (1982)
8.1.3.5. Dynorphin B(1-29)	Leumorphin		Nakao et al (1983)
8.1.3.6. Dynorphin B(1-13)	Rimorphin		Fischli et al. (1982), Kilpatrick et al. (1982)
8.1.4. Anodynin			Pert et al. (1976)
8.1.5. Kyotorphin			Takagi et al. (1979a,b)
8.1.6. (Exorphins)[5] (casomorphins, wheat gluten opioid peptides)			Zioudrou and Klee (1978), Henschen et al. (1980)
8.1.7. (Dermorphins)[5]			Erspamer and Melchiori (1980)
8.2. Tachykinins			
8.2.1. Substance P		SP	Chang et al (1971)
8.2.2. Substance K	neurokinin A, neurokinin α, neuromedin L	SK	Maggio et al. (1983), Nawa et al. (1983)
8.2.3. Neuromedin K	neurokinin B neurokinin β	NK	Kangawa et al. (1983) Kimura et al (1983) Kotani et al. (1986)

Footnotes to Table 5.1.

1: For recent lists see Brownstein 1980, Iversen 1983, Krieger 1985, Palkovits 1986

2: The designation within the quotation marks serves to indicate the "previously known status"

3: The APP- and BPP-like materials reported previously to occur in the brain are , by all probabilities, identical with NPY. In fact, NPY is likely to be present in brain and peripheral neural elements but not in non-neural elements of the gastrointestinal tract and the reverse holds for PYY (Tatemoto 1982).

4: Bombesin may have been listed also among the "gastrointestinal" peptides

5: None of these peptides are neuropeptides; i.e. their occurrence (or of related structures) in neural elements has not been demonstrated as yet. They are listed to make the list of natural opioid peptides as complete as possible.

reveal new biologically active peptides, resulted in the discovery of previously unknown peptide amides, such as the pancreatic polypeptide-related neuropeptide Y and peptide YY (Tatemoto and Mutt 1980, Tatemoto 1982) and the so-called "peptide histidine isoleucine" (PHI-27) which has structural similarities to secretin, glucagon, gastric inhibitory peptide and vasoactive intestinal polypeptide.

The wealth of information accumulated on the functional and evolutionary aspects of neuropeptides and their receptors has changed our view drastically at several points. To mention just the most important ones, when the early reports came to indicate a possible brake of long-held Dale s principle (Dale 1935) substantiating that a neuron should contain and release only one transmitter substance, the existence of a neuropeptide and a "classical" neurotransmitter within the very same neuron (Hokfelt et al. 1980a,b) was regarded as an exception rather than a rule; presently the opposite view appears to be favoured (Lundberg and Hokfelt 1983, Hokfelt et al. 1986, see also books edited by Cuello 1982 and Chan-Palay and Palay 1984). Thus, the functional interplay between the "classical" neurotransmitters and neuropeptides should be analysed at more numerous levels than it was previously thought of. As to the evolutionary aspects, information-carrying peptide molecules, considered previously unique to vertebrates, have been shown to be present also in unicellular organisms and plants (for discussion see LeRoith and Roth, 1984, Krieger 1985). Although the possibility that the genes coding for these peptides were transferred from vertebrates to microbes by a plasmide-type recombinant DNA event cannot be ruled out with certainty, it is more likely that they arose evolutionarily in unicellular organisms and progressed phylogenetically in a Darwinian fashion (LeRoith and Roth 1984). The evolutionary conservations of biologically important sequence cores within the peptide molecules is remarkable; in several instances, the functional analogy between the actions of an information-carrying peptide in two species of distant location on the phylogenetic scale, can also be recognized.

5.1. CHEMISTRY OF NEUROPEPTIDES

Only those peptides are discussed here which rank primarily among the neuropeptide group. The discussion is mainly limited to listing the chemical structures; in certain cases the precursors, the peculiarities of processing and some relevant aspects of possible mode of action, are also touched. The neuropeptides, as is a rule for peptides of similar size, are produced from large-molecular weight precursors by sequential, limited proteolysis ("processing") by endo- and exopeptidases (for review see Loh et al. 1984). The endopeptidases involved in the generation of neuropeptides are often referred to as trypsin-like (e.g. Lindberg et al. 1984, see also Loh et al. 1984). Although the site-selectivity of splitting quite often resembles that of trypsin, the criteria for a real trypsin-like classification are seldom met. Thus, instead, the "proprotein converting enzyme" (Docherty and Steiner 1982) designation should be preferred (Loh et al. 1984).

The chemical structural-biological activity relationships are not treated; they are to be found in the recent reviews (Sheppard 1977, 1978, 1979, Morley 1983, Veber and Freidinger 1985).

(1) Bombesin, a 14 amino acid peptide
pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-MetNH_2
Erspamer and Melchiorri 1973, Brown et al. 1978, Erspamer et al. 1978) was originally isolated from amphibian skin, later, bombesin-like substance(s) were found to be present in mammalian tissues including brain (Polak et al. 1978). The bombesin-like substance(s) detected in mammals is assumed to be closely related to mammalian gastrin releasing peptide (GRP). The sequence of the C-terminal 10 amino acids in the 27-amino acid GRP molecule is highly homologous to that of the amphibian bombesin. Bombesin is present in mammalian tissues in more than one molecular form (Walsh et al. 1981).

(2) Calcitonine gene-related peptide (CGRP) occurs in two molecular forms, α and β . β-CGRP
(Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-

Asn-Val-Gly-Ser-Lys-Ala-PheNH_2) differs from α-CGRP in one amino acid: it contains Lys instead of Glu in position 35 (Amara et al. 1982, 1985).

α-CGRP is a putative novel neuropeptide predicted on the basis of alternative RNA processing events of primary transcripts of the calcitonin gene (Amara et al. 1982). An mRNA product of a gene related to the one encoding calcitonin/α-CGRP was also identified in rat brain and thyroid gland and was designated as β-CGRP (Amara et al. 1985). α-CGRP mRNA appears to be the predominant product of calcitonin/α-CGRP gene expression in the brain. Both α- and β-CGRP mRNAs were shown to occur at several brain sites.

(3) Carnosine is a dipeptide: Ala-His (Margolis 1974)

(4) Cholecystokinin (CCK).

In context with the occurrence of CCK in the brain it should be pointed out that the gastrin-like substance isolated from brain by Vanderhaeghen et al. (1975) proved to be CCK-8 sulfate (Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-PheNH_2) rather than gastrin proper (Dockray and Gregory 1980). The C-terminal 5 amino acids are common to both peptides, thus, C-terminal-specific antisera to gastrin crossreact with CCK. The predominant form of CCK-like material in mammalian brain, including man, is CCK-8 sulfate (Rehfeld 1978, 1981, Dockray and Gregory 1980, Frey 1983), which refers to sequence positions 26-33 of CCK-33, which is the major form at the periphery.

Some authors have claimed, however, that CCK-33 is as abundant as CCK-8 in rat and bovine brain (Simon-Assman et al. 1983); the occurrence of CCK-4 (Rehfeld and Golterman 1979), CCK-12 (Rehfeld 1981), CCK-39 (Jansen and Lamers 1983), CCK-58 (Ichihara et al. 1984) and some "desoctapeptide" fragments of CCK-33, CCK-39 and CCK-58 (Eng et al. 1983) in the brain, has also been reported. All characterized forms of CCK appear to have a sulfated tyrosine (Dockray 1982); recently, however, desulfated CCK-octapeptide has also been detected in brain (Frey 1983).

(5) Neuropeptide Y (NPY), a 36 residue peptide: Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-TyrNH_2 (Tatemoto 1982). It has a high degree of sequence homology with peptide YY isolated from porcine intestine, and the pancreatic polypeptides of various species (avian, bovine and porcine, respectively: Tatemoto 1982).

(6) Neurotensin consists of 13 residues: pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu (Carraway and Leeman 1973, 1976).

(7) Opioid peptides and nonopioid peptides derived from opioid peptide precursors.

In the light of the most recent findings, the family of endogenous opioid peptides, possessing direct opiate agonist activity, can be divided into three groups. Each group has a common precursor molecule; in each line, the posttranslational processing results in several sequences having opiate-like biological properties on their own.

Since there is an unhealthy confusion in the nomenclature of both the propeptides and the end-products, the nomenclature used most frequently in the literature (reviewed by Hollt 1983, Smyth 1983, Weber et al. 1983b, Akil et al. 1984) and also the amendments recommended at the INRC Meeting held in 1983 are considered. The schematic representation of prepropeptides and the putative or proven products of posttranslational processing is given in Figs 5.1-5.3.

Preproopiomelanocortin (bovine: 265 amino acids, Fig.5.1).

The existence of a common, large molecular weight precursor for β-lipotropin (LPH)- and ACTH-related peptides was proposed first by Mains and his coworkers (Mains et al. 1977) and Rubinstein and his coworkers (Rubinstein et al. 1978). The terminology proposed by the latter group was approved at the 83 INRC Meeting. The complete amino acid sequence of prepropeptide was determined by Nakanishi et al. (1979, 1980).

Preproenkephalin A (bovine: 263 amino acids, human: 267 amino acids, Fig.5.2). The precursor, the complete amino acid sequence of which has been established by Comb and

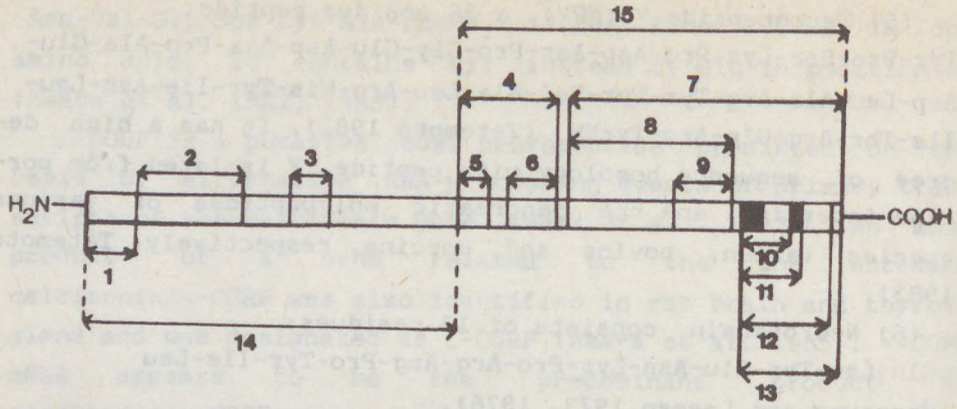


Fig. 5.1. Schematic drawing of the structure of preproopiomelanocortin. 1) signal peptide; 2) calcitonin-like structure (CLS); 3) γ-MSH; 4) ACTH; 5) α-MSH; 6) CLIP; 7) β-LPH; 8) γ-LPH; 9) β-MSH; 10) α-EP; 11) γ-EP; 12) β-EP(1-27); 13) β-EP(1-31); 14) 16K-fragment corresponds to the non-opiomelanocortin portion of the precursor; 15) the opiomelanocortin portion of the precursor. (The abbreviations are explained in the text)

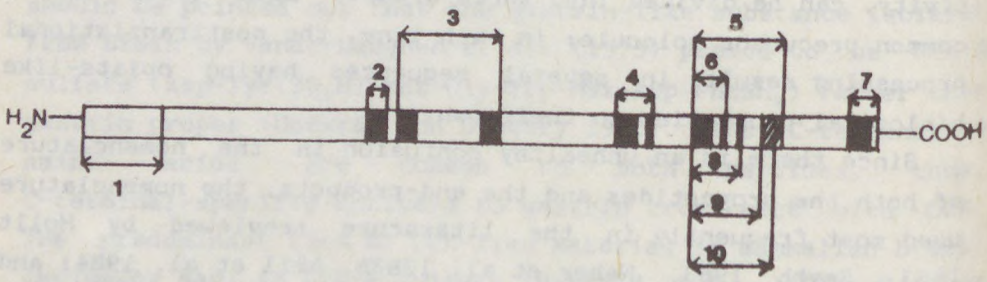


Fig. 5.2. Schematic drawing of the structure of preproenkephalin A. 1) signal peptide; 2) Met-enkephalin (ME); 3) peptide F; 4) ME-Arg^6-Gly^7-Leu^8 octapeptide; 5) peptide E; 6) metorphamide; 7) ME-Arg^6-Phe^7 heptapeptide; 8) BAM-12; 9) BAM-20; 10) BAM-22. (The abbreviations are explained in the text)

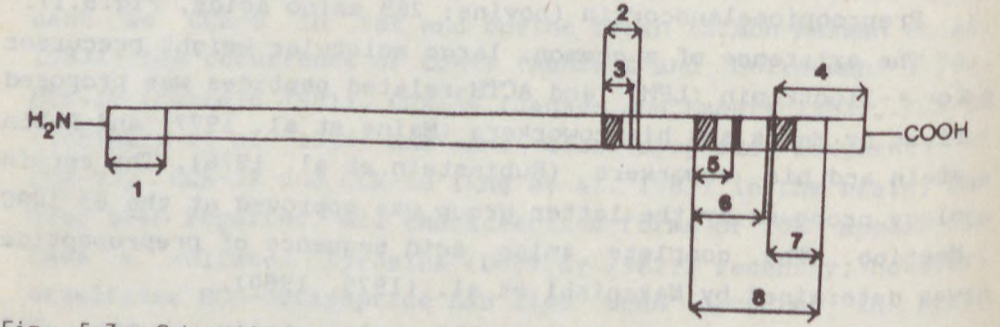


Fig. 5.3. Schematic figure of the structure of preproenkephalin B. 1) signal peptide; 2) α-neoendorphin; 3) β-neoendorphin; 4) leumorphin; 5) dynorphin(1-8); 6) dynorphin(1-17); 7) rimorphin; 8) 4K dynorphin. (For alternative terms see the text)

his coworkers (1982), gives rise to various combinations of opioid end-products containing one or two of six possible methionin-enkephalin sequences, and the single leucine-enkephalin sequence. The "A" suffix was proposed originally by Kakidani et al. (1982) to differentiate from the precursor of another family of leucine-enkephalin-containing peptides (see Fig.5.3). Considering the fact that members of the latter group have been designated as neoendorphins (Kangawa and Matsuo 1979, Kangawa et al. 1981, Minamino et al. 1981) and dynorphins (Goldstein et al. 1979, 1981), respectively, the opinion expressed at the 83 INRC Meeting was to preserve the preproenkephalin term (without any suffix) to designate the precursor of the present peptide family, and to apply another terminology to the neoendorphin/dynorphin precursor (designated originally as preproenkephalin B by Kakidani et al. 1982). No reasonable solution could be proposed to create a uniform nomenclature among the end-products of processing.

Preproenkephalin B (porcine: 256 amino acids, Kakidani et al. 1982, Fig.5.3). Since this prepropeptide gives rise to neoendorphin- and dynorphin-related opioid peptides, the more logical, although rather sophisticated term recommended by the INRC is preproneoendorphin/dynorphin.

To avoid confusion and the usage of multiple phantasy names the following simplification was proposed at the INRC Meeting: (i) Neoendorphin(1-10) and (1-9) instead of α- and β-neoendorphins, respectively; (ii) The term dynorphin A for the indicated 17-amino acid peptide was approved; (iii) The dynorphin B term was proposed to designate the whole, 29-amino acid C terminal sequence (i.e., the one originally designated as leumorphin by Nakao et al. 1983); thus the tridecapeptide hitherto designated as dynorphin B (rimorphin) would be dynorphin B(1-13).

To avoid misunderstanding, for each processed opioid peptide in the three groups, in addition to the structures and phantasy names created by the discoverers, the proposed INRC terminology and the sequence position occupied in the original prepropeptide are listed below.

(A) Preproopiomelanocortin-derived peptides

All the sequences possessing opioid activity are segments of β-lipotropin (Li et al. 1965, Pankov 1973) located at the C terminus of preproopiomelanocortin (sequence positions 173-365 in bovine precursor).

(a) β-endorphin (β-EP) also termed C fragment (Li and Chung 1976, Bradbury et al. 1976) contains 31 amino acid residues: Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Val-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln. The untriakontapeptide β-endorphin [β-EP(1-31)] is located at the C-terminus of β-LPH. β-endorphin-related opioid peptides are β-EP(1-27) also termed C'-fragment, β-EP(1-26) (Smyth and Zakarian 1980, Zakarian and Smyth 1982), β-EP(1-17) (γ-endorphin, Ling et al. 1977) and β-EP(1-16) (α-endorphin, Guillemin et al. 1977). Peptides lacking opioid activity, but closely related to the above listed ones are N_α acetyl-β-EP(1-31), N_α acetyl-β-EP(1-27), $N_\alpha$acetyl-β-EP(1-26) (Smyth et al. 1979, Seizinger and Hollt 1980, Smyth and Zakarian 1980, Zakarian and Smyth 1982) and destyrosine α- and γ-endorphins referring to β-EP(2-16) and (2-17), respectively (Van Ree et al. 1978b, De Wied 1978, De Wied et al. 1978b).

The endogenous, brain region-specific processing and thus the possible physiological role of β-EP(1-31), β-EP(1-27), β-EP(1-26) and the N-acetyl derivatives thereof is rather firmly established (Seizinger and Hollt 1980, Smyth and Zakarian 1980, Zakarian and Smyth 1982). Although the appearance of α - and γ -endorphins and related peptides among the end-products of preproopiomelanocortin processing was widely debated (see Hughes 1979), the endogenous formation (Loeber et al. 1979, Burbach et al. 1980, Verhoef et al. 1980) and functional significance (De Wied and Jolles 1982, Van Ree et al. 1986) of this peptide line is abundantly documented by the followers of the Utrecht pharmacological school.

(b) β-melanocyte stimulating hormone (β-MSH), β-LPH(41-58), an additional nonopioid peptide with 18 amino acid residues:

Arg-Glu-Gly-Pro-Tyr-Lys-Met-Gly-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp is also contained within the nonopioid portion of β-lipotropin.

(c) ACTH-related peptides. ACTH (1-39) (sequence positions 132-170 in the preproopiocortin molecule; for structural data see Chapter 3) contains the 22 amino acid corticotropin-like intermediate-lobe peptide [CLIP, ACTH(18-39)]: Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe and the 13 residue long α-melanocyte stimulating hormone [α-MSH, ACTH(1-13)]: AcSer-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-ValNH$_2$ where AcSer indicates an acetylated seryl residue.

(d) γ-melanocyte-stimulating hormone (γ-MSH) is the third melanotropin sequence occurring in the preproopiocortin at positions 77-88 of bovine precursor (Nakanishi et al. 1979). The peptide was ranked among the probable end-products of processing (Nakanishi et al. 1979, 1980).

(B) Preproenkephalin A-derived peptides

The precursor was isolated both from peripheral (adrenal medulla) and CNS sources (Lewis et al. 1980, Kojima et al. 1982). The complete amino acid sequence was determined by Comb et al. (1982), and Noda et al. (1982). The processing pathways are different in the central nervous system and at the periphery (Liston et al. 1984). A region-specific processing of proenkephalin-A in the CNS was also suggested (White et al. 1986). In a study carried out in three different projection systems in rat brain, the major end-products appeared to be Met-enkephalin, Met-enkephalin-Arg^6-Phe^7 and Met-enkephalin-Arg^6,Gly^7,Leu^8, varying amounts of BAM-18 peptide and minor quantities of metorphamide and peptide E (see the structures below) were also detected. The possible end-products of processing are discussed below.

(a) Enkephalins. There are two enkephalins, termed Met- and Leu-enkephalin, respectively, differing only in their C-terminal residues: Tyr-Gly-Gly-Phe-Met/Leu (Hughes et al. 1975).

These pentapeptides were the first identified members of the endogenous opioid peptide family.

(b) Met-enkephalin-Arg^6-Phe^7 heptapeptide. The amino acid sequence of this heptapeptide (sequence positions 261-267, i.e., the C terminus of human precursor) is as follows: Tyr-Gly-Gly-Phe-Met-Arg-Phe (Stern et al. 1979).

(c) Met-enkephalin-Arg^6-Gly^7-Leu^8 octapeptide. The octapeptide (sequence positions 186-193 in the human precursor) was sequenced by Jones et al. (1982): Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu.

(d) Met-enkephalin-Arg^6-Arg^7-Val^8-NH_2 octapeptide amide. This peptide (sequence positions 210-217 in the human precursor) is also termed methorphamide (Weber et al. 1983a). The octapeptide amide (Tyr-Gly-Gly-Phe-Met-Arg-Arg-ValNH_2) is a result of an atypical processing pattern demonstrated also for the dynorphin group: the cleavage occurs at a single arginine residue instead of paired basic amino acids.

(e) BAM-12 peptide. This dodecapeptide (sequence positions 210-221 in the human precursor) was isolated originally from the adrenal medulla as is reflected by the abbreviation (BAM= bovine adrenal medulla; Mizuno et al. 1980a). Its sequence is shown in Fig.5.4.

(f) BAM-18 peptide. The sequence of BAM-18 peptide (sequence positions 210-227 in the human precursor) corresponds to a C-terminal six amino acid extended form of BAM-12 (see Fig.5.4) and is contained by the "peptide E" molecule (Evans et al. 1985).

(g) BAM-20 peptide. The 20 amino acid peptide (sequence positions 210-229 in the human precursor, see Fig.5.4) was first described by Mizuno et al. (1980b). It is a C-terminal two amino acid extended form of BAM-18.

(h) BAM-22 peptide. This is the largest BAM peptide (sequence positions 210-231 in the human precursor, see Fig.5.4) hitherto described (Mizuno et al. 1980b).

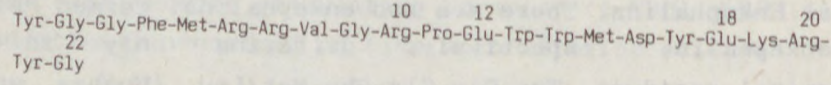


Fig. 5.4. Primary structures of BAM-peptides. Numbers represent the C terminus of BAM-12, BAM-18, BAM-20 and BAM-22 peptide, respectively

BAM-(12,20,22) peptides were not found in bovine adrenal medulla extracts by Kilpatrick et al. (198la,b); in their opinion at least two of these BAM peptides may be generated by enzymes not generally involved in precursor processing. Thus, the artifactual nature of this group cannot be excluded.

(i) Peptide E. The 25-amino acid peptide (sequence positions 210-234 in the human precursor) is a three amino acid (Gly-Phe-Leu) extended form of BAM-22 peptide (see Fig.5.4) and is flanked by a Met- and Leu-enkephalin sequence at its N and C terminus, respectively (Kilpatrick et al. 198la,b).

(j) Peptide F. The 34-amino acid peptide (Tyr-Gly-Gly-Phe-Met-Lys-Lys-Met-Asp-Glu-Leu-Tyr-Pro-Leu-Glu-Val-Glu-Glu-Glu-Ala-Asn-Gly-Gly-Glu-Val-Leu-Gly-Lys-Arg-Tyr-Gly-Gly-Phe-Met) is bordered by two Met-enkephalin sequences (Jones et al. 1980).

(C) Preproenkephalin B-derived peptides
(preproneoendorphin/dynorphin-derived peptides)

The processing of precursor into its end-products is much less delineated than it can be said about the above-discussed opioid peptide families; this especially holds true for the routes of dynorphin B processing. One difference, however, can be pointed out as compared to the proopiomelanocortin-derived end-products, notably that hitherto there is no evidence of possible N-acetylation of proneoendorphin/dynorphin-derived products (Seizinger et al. 1982; for review see Akil et al. 1984, Arendt et al. 1985).

(a) α-neoendorphin/neoendorphin(1-10). The decapeptide (sequence positions 175-184 in the porcine precursor: Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys) was detected and characterized by Kangawa and Matsuo (1979) and Kangawa and his coworkers (1981).

(b) β-neoendorphin/neoendorphin(1-9). The amino acid sequence of this nonapeptide (sequence positions 175-183 in the porcine precursor) is identical to that of α-neoendorphin, but it lacks the C-terminal lysine residue (Minamino et al. 1981).

(c) Dynorphin A(1-17). This is a heptadecapeptide (sequence positions 209-225 in the porcine precursor: Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Glu) described by Goldstein et al. (1981). The amino acid sequence of N-terminal tridecapeptide was determined first by Goldstein and his group in 1979 (Goldstein et al. 1979) followed by the elucidation of the structure of the complete heptadecapeptide in 1981.

(d) Dynorphin A(1-8). This octapeptide (sequence positions 209-216 in the porcine precursor) corresponds to the N-terminal eight amino acids of dynorphin A(1-17) Seizinger et al. (1981).

(e) Leumorphin. The designation proposed at the 83 INRC is dynorphin B(1-29). The 29-amino acid peptide, also termed leumorphin (sequence positions 228-256 in the porcine precursor: Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Glu-Phe-Lys-Val-Val-Thr-Arg-Ser-Glu-Glu-Asp-Pro-Asn-Ala-Tyr-Tyr-Glu-Glu-Leu-Phe-Asp-Val) constitutes the C terminus of preproneoendorphin/dynorphin (Nakao et al. 1983).

(f) Rimorphin. The designation proposed by the INRC is dynorphin B(1-13); the term dynorphin B initially coined for this sequence is still widely used. The tridecapeptide, also termed rimorphin (sequence positions 228-240 in the porcine precursor) is the result of atypical processing. It corresponds to the N-terminal 13 amino acid fragment of leumorphin. Interestingly it can occur in two larger active opioid peptide sequences, namely at the N terminus of leumorphin (see above) and at the C terminus of 4K dynorphin (see below) (Fischli et al. 1982, Kilpatrick et al. 1982).

(g) 4K dynorphin. The 32 amino acid, 4000 dalton dynorphin (sequence positions 209-240 in the porcine precursor) is flanked N-terminally by dynorphin A(1-17) whilst C-terminally by dynorphin B(1-13) (rimorphin) and the two are linked by a Lys-Arg dipeptide sequence (Fischli et al. 1982).

(h) Anodynin is a pronase-resistant peptide present in blood. Its structure is still unknown (Pert et al. 1976).

(i) Kyotorphin. The endogenous dipeptide Tyr-Arg described by Takagi et al. (1979a,b) does not act directly on the opioid

receptors; it is a possible enkephalin releaser, and thus an indirectly acting opioid peptide (Shiomi et al. 1981).

(j) Exorphins. As it is indicated by the term "exorphin" (coined by Zioudrou and Klee 1978) the members of this group, present in milk and cereals, respectively, cannot be regarded strictly as endogenous ones, although milk peptides may fall into this category. None of these peptides - or related ones - has been suggested as yet to occur in neural elements; the compulsion to put them on record in this chapter is manyfold. The first is - and this holds also for the dermorphins listed below - to make the list of natural opioid peptides as complete as possible. This way none of these fascinating substances would escape mentioning. A further reason is that these peptides either as nutritional factors (Wunderink et al. 1986) or as endogenous ones (milk-derived peptides) may have a role in the control of complex behavioral functions and also in the pathomechanism of certain psychiatric disorders (Terenius and Nyberg 1986, Wunderink et al. 1986; for further details see section 5.6).

Casomorphins, peptides with opioid activity have been isolated from pepsin hydrolysate of β-casein (Zioudrou and Klee 1978) and chloroform-methanol extract of casein (Henschen et al. 1980). Of these several opioid components were separated. A heptapeptide, designated as β-casomorphin (Henschen et al. 1980) was identified and found to be identical with a segment of bovine β-casein (sequence positions 60-66): Tyr-Pro-Phe-Pro-Gly-Pro-Ile. The pentapeptide product obtained by carboxypeptidase Y digestion, is an even stronger opioid than the heptapeptide (Henschen et al. 1980). Casomorphin-related opioid peptides were found also in human milk (Terenius and Nyberg 1986).

The Gluten-exorphins are pronase-sensitive substances with opioid activity that were isolated from pepsin hydrolysate of wheat gluten (Zioudrou and Klee 1978).

(k) Dermorphin. The heptapeptide-amide: Tyr-D-Ala-Phe-Gly-Tyr-Pro-SerNH_2 (Erspamer and Melchiorri 1980) is the prototype of a new class of opiate-like peptides extracted from the skin of frogs belonging to the genus Phyllomedusa.

(l) δ-sleep-inducing peptide (DSIP). The nonapeptide (Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu) was described by Schonenberger and Monnier (1977). The listing of the substance among neuropeptides (Barchas et al. 1978) awaits further clarification.

(m) Mammalian tachykinins. Until quite recently, substance P (Chang et al. 1971) was the only member of tachykinin family (for review see Erspamer 1981) identified also in mammals. Other related substances thought to exist in mammals have been described in terms of putative non-mammalian relatives (kassinin-like, physalemin-like, etc. substances; for review see Harmar 1984). In 1983 the structures of two new mammalian members of this family, neuromedin K or neurokinin α (Kangawa et al. 1983, Kimura et al. 1983) and substance K (to indicate its apparent immunochemical similarity to kassinin) or neurokinin β (Maggio et al. 1983) as well as the large molecular-weight precursors of substance P and substance K (Nawa et al. 1983) were identified (for review see Harmar 1984, Nakanishi 1986). The biosynthetic origin of neuromedin K in the brain became known quite recently (Kotani et al. 1986). To compare the structural homologies, the amino acid sequences of non-mammalian tachykinins are also presented (Fig.5.5).

In bovine species, two precursors were demonstrated for substance P, designated as α- and β-preprotachykinin A (Nawa et al. 1983). β-preprotachykinin A gives rise also to sub-

Mammalian

Neuromedin K : Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-MetNH_2^{10}

Substance K$^+$: (Arg)His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-MetNH_2^{11}

Substance P : Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH_2^{11}

Non-mammalian

Physalemin : pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-MetNH_2^{11}

Eledoisin : pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-MetNH_2^{11}

Kassinin : Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-MetNH_2^{12}

Fig. 5.5. Amino acid sequences of mammalian and non-mammalian tachykinins. *Note: the deletion of the N-terminal arginyl residue and the C-terminal amidation in the course of post-translational processing of substance K are highly probable but are not yet unequivocally proven (Nawa et al. 1983)

stance K whereas α-preprotachykinin A liberates only a single identified tachykinin sequence i.e., the one corresponding to substance P. The precursor for neuromedin K was designated as preprotachykinin B (Kotani et al. 1986).

5.2. ENDOPEPTIDES COMMON TO BRAIN AND INTESTINE

It is now widely known that there is a vast overlap between the arsenal of peptides used as chemical messengers by neurons on the one hand, and by endocrine cells, on the other (Pearse 1976, Dockray and Gregory 1980, Acher 1981). This recognition has prompted Pearse (1969, 1976) to suggest that all these peptide-manufacturing and -handling cells - united under the APUD acronym - share not only some cytochemical and ultrastructural characteristics, but are also derived from a common, neuroectodermal ancestor. However, experimental embryologic studies did not favour the concept of common origin (Andrew 1974, Pictet et al. 1976, Fontaine and De Douraun 1977).

When one is bound to discuss peptides common to the brain and gastrointestinal tract (including pancreas), one must clearly distinguish the different levels of "identity" (Dockray and Gregory 1980, Acher 1981). Thus, substance P (Chang et al. 1971, Studer et al. 1973), neurotensin (Carraway and Leeman 1973, Kitabgi et al. 1976), cholecystokinins (Dockray and Gregory 1980), somatostatin (Brazeau et al. 1973, Spiess et al. 1979) and dynorphin (Goldstein et al. 1981, Tachibana et al. 1982) were isolated and chemically characterized from both brain (or pituitary) and gut. The gastrin-like immunoreactivity that was shown to be present in the brain (Vanderhaeghen et al. 1975) could not be accounted for by authentic gastrin, but a C-terminal fragment of CCK, most probably CCK-8 (Dockray 1976, Dockray et al. 1978, Rehfeld 1978; for details see section 5.1).

VIP (Said and Mutt 1972) and bombesin (Dockray et al. 1979) isolated and characterized from the gut, were detected in the brain by immunohistochemistry, radioimmunoassay and bioassay (Said and Rosenberg 1976, Dockray et al. 1978, Besson et al. 1979).

Met- and Leu-enkephalins (Hughes et al. 1975) and TRH (Brownstein et al. 1974, Olivier et al. 1975) discovered and characterized from the brain, could be identified in the gut by radioimmunoassay, bioassay and immunohistochemistry (Smith et al. 1976, Hughes et al. 1977, Leppaluoto et al. 1978, Schultzberg et al. 1978, 1980). In the light of additional experimental data (e.g., McKnight et al. 1978, Tang et al. 1982) the chemical identity of brain and intestinal enkephalins could be suggested.

β-endorphin and its shorter fragments (α- and γ-endorphins and the des-tyrosine-derivatives thereof) were reported to occur in the gut, as detected by the combination of high pressure liquid chromatography and radioimmunoassay (Verhoef et al. 1980). The finding awaits confirmation and the origin of these peptides needs further clarification.

Of these peptides in the gut (and pancreas), neurotensin, insulin, and CCK occur primarily in endocrine cells, although there are probably small populations of neurotensin and CCK-containing neurons. It is of interest that whilst CCK-33 occurs mostly, if not exclusively, in endocrine cells, CCK-8 can be detected both in nerves and endocrine cells (Dockray and Gregory 1980, Dockray and Hutchinson 1980). Somatostatin and substance P (Schultzberg et al. 1978, 1980) whilst being located mainly in neurons, also occur in significant amounts in gut endocrine cells; similar patterns were proposed for Met- and Leu-enkephalin (Dockray and Gregory 1980). VIP (Schultzberg et al. 1978, 1980, Dimaline and Dockray 1979, Dockray and Gregory 1980) and bombesin-like peptides (Dockray et al. 1979, McDonald et al. 1979, Walsh et al. 1979) are confined to nerve elements; at least in mammals, they have minimal representation in gut endocrine cells (Dockray and Gregory 1980).

5.3. THE FUNCTION OF NEUROPEPTIDES AS NEUROREGULATORS

The neuropeptides belong to the group of endogenous substances that serve as signal-carrying agents in interneuronal or neuro-effectorial communication; they may function as neurohormones, neurotransmitters or neuromodulators (Palkovits 1984, 1986, Krieger 1985). To fulfill such a role, the endogenous substances must be released from the communicating neuronal element in a manner that enables the target to perceive it as an information. To do this, the targets must have receptors (specific binding sites coupled to one or more effector mechanisms) for the substance. The neurohormonal aspects are discussed elsewhere; in this section the neuropeptides will be considered simply as information-carrying substances and no attempts will be made to make a further distinction between neurotransmitter or modulator role (Barchas et al. 1978, Cooper and Meyer 1984). The patterned release and the existence of receptors are taken as major criteria supplemented occasionally with some minor ones such as the demonstration of manufacturing or degrading enzymes, axonal transport, etc.

(1) Adrenocorticotrop hormone (ACTH). Immunocytochemical studies revealed ACTH in axons and nerve terminals throughout the brain (Watson et al. 1978, Pelletier and Leclere 1979). ACTH seemed to be sequestered in 60-80 nm vesicles (Pelletier and Leclere 1979). ACTH-processing enzymes could be demonstrated in certain CNS areas; axonal transport of ACTH was also detected (Larsson 1980). Very preliminary studies indicated saturable recognition sites of high affinity for ACTH in the brain with differential distribution across various regions (Akil and Watson 1980, Akil et al. 1984). Given intracerebroventricularly to rats, ACTH(1-24) selectively decreased the acetylcholine content of the hippocampus (Botticelli and Wurtman 1980).

(2) Angiotensin II. Specific high- and low-affinity angiotensin II binding sites have been demonstrated in the central nervous system of the rat (Bennett and Snyder 1976,

Sirett et al. 1977, Simonnet et al. 1984, Saavedra and Plunkett 1986). Electrophysiological effects on cultured mouse spinal cord neurons suggested that angiotensin II receptors may modulate the permeability of the Cl-channel in a way opposite to that of GABA-receptors (Simonnet et al. 1984). Angiotensin II is capable of inducing β-endorphin-like immunoreactivity release in rat pituitary cells (Sobel 1984). An interaction, at least in part at presynaptic level, between angiotensin II and central catecholaminergic neurons has been demonstrated (Simonnet and Giorguieff-Chesselet 1979, Fuxe et al. 1980a).

(3) Bradykinin. Neural bradykinin receptors were shown to exist (Manning and Snyder 1983).

(4) Bombesin. Specific binding sites for bombesin exist in rat brain membranes. The binding is the highest in the synaptosomal fraction and the binding sites exhibit a distinct regional distribution in the brain (Moody et al. 1978, Pert et al. 1980).

(5) Calcitonin gene-related peptides (CGRPs) have a highly uneven distribution in rat brain (Amara et al. 1985). A Ca^{2+}-dependent, K^{+}-stimulated release of α-CGRP from cultured rat trigeminal ganglion cells was also demonstrated (Mason et al. 1984). Calcitonin receptors were detected in rat mesencephalon by autoradiographic and functional means (Fabbri et al. 1985).

(6) Carnosine has been suggested to function as a neurotransmitter at olfactory nerve synapses (Margolis 1978). Both the carnosine-synthetizing and -destroying enzymes are present in olfactory tissue; the neuronal transport of this dipeptide could also be demonstrated. Labeled carnosine binds to glomerular layer membranes of olfactory bulb with high affinity, in a stereospecific and saturable manner (Nadi et al. 1980). However, the subcellular distribution of carnosine does not support its neurotransmitter function.

(7) Cholecystokinin (CCK). Large molecular-weight CCK precursors were shown to be present in rat brain (Deschens et al. 1984, Beinfeld 1985). The presence of at least two processing enzymes partially purified from porcine cerebral cortex, was also demonstrated (Malesci et al. 1980). The metabolism of CCK-8 in rat brain is rather slow (Meek et al. 1983).

In the CNS areas rich in CCK-like immunoreactive material, CCK-8 appears to be contained by the purified synaptosomal and synaptic vesicle fractions (Emson et al. 1980c). A Ca^{2+}-dependent, K^{+}-induced release of this octapeptide from cortical slices (Emson et al. 1980c) or cortical synaptosomal preparations (Pinget et al. 1979) was also demonstrated. Specific CCK receptors are also present in brain membrane preparations (Innis and Snyder 1980, Saito et al. 1981, Hays et al. 1981). Electrophysiological findings (Emson et al. 1980b, Skirboll et al. 1981) make it likely that CCK-8 is a good candidate to serve as an excitatory neurotransmitter in certain forebrain areas and perhaps in the spinal cord (Jeftinija et al. 1981).

The coexistence of CCK with dopamine is extensively documented both in the nigrostriatal and mesolimbic system (Hokfelt et al. 1980b, 1986). It was shown that CCK may affect the function of the dopaminergic neuron at several levels both at pre- and postsynaptic sites (for review see Hokfelt et al. 1986, Nair et al. 1986). The effect of CCK on dopamine release is a rather controversial issue (see Hokfelt et al. 1986); it appears, however, that if the experimental conditions are properly controlled, a powerful inhibitory action can be demonstrated (Markstein and Hokfelt 1984, Voigt and Wang 1984).

The occurrence of CCK in GABA-ergic forebrain neurons has also been described (Somogyi et al. 1984). CCK facilitates GABA release from cerebral cortical slices (Sheehan and de Belleroche 1983). The codistribution of CCK- (Zarbin et al. 1983) and GABA-binding sites (Chan-Palay 1978) in the hippocampus is indicative of possible interactions of CCK and GABA at postsynaptic level (for discussions see Nunzi et al. 1986).

(8) Luteinizing hormone-releasing hormone (LH-RH). Ca^{2+}-dependent K^{+}-evoked release of this peptide was demonstrated from isolated rat hypothalamic preparation (Goddard et al. 1981). The possible transmitter role of LH-RH (or a very similar peptide) in peripheral autonomic ganglia was suggested by Jan et al. (1979). The LH-RH-like substance (as determined by radioimmunoassay) is present in nerve terminals. It can be released from the terminals in a Ca^{2+}-dependent manner either by the stimulation of preganglionic fibers or by exposure to high potassium media (Jan et al. 1979). Concurrent electrophysiological evidences have rendered it probable that the slow excitatory postsynaptic potential in frog sympathetic ganglia is mediated by an LH-RH-like peptide (Jan et al.1979, Brown 1982).

(9) α-melanocyte-stimulating hormone (α-MSH). The axonal transport and the storage of the peptide in nerve terminals were demonstrated by Larsson (1980). A calcium-dependent release of α-MSH was described from hypothalamic synaptosome preparations (Warberg et al. 1979).

(10) Neuropeptide Y (NPY) appears to be a major neuropeptide in mammalian sympathetic neurons (Heym and Lang 1986); it may coexist with norepinephrine (Lundberg et al. 1982b). Postsynaptically it acts in a complementary fashion with norepinephrine in blood vessels whereas presynaptically it appears to inhibit the release of norepinephrine, as demonstrated in rat vas deferens (Lundberg et al. 1982c).

(11) Neurotensin, a tridecapeptide, has been postulated to serve as a possible neurotransmitter in the CNS (Bisette et al. 1978). Unevenly distributed, specific neurotensin receptors were shown to be present in the cat striatum (Goedert et al. 1984). Neurotensin was also shown to enhance the release of dopamine from striatal slices (Starr 1982).

(12) Opioid peptides. This group is perhaps most abundantly discussed among the neuropeptides. The localization of various endogenous opioids in neurons, their occurrence in nerve terminals, is well documented. The precursors of opioid peptide families, the way of processing (see section 5.1.7) as well as the β-endorphin (Marks 1978, Graf et al. 1978), enkephalin (Craves et al. 1978, Malfroy et al. 1978, 1979), and Met-enkephalin-Arg^{6}-Phe^{7}-degrading enzymes (Yang et al. 1981) were also characterized. The stimulation-induced, calcium-dependent release of enkephalins (Bayon et al. 1978, Henderson et al. 1978, Iversen et al. 1978b, Osborne et al. 1978) and of β-endorphin (Herz et al. 1978, Osborne et al. 1980) from various CNS-preparations was detected in vitro.

There is an overwhelming body of evidence that there are specific opiate receptors connected with neuronal membranes (Simon and Hiller 1978, Simon 1981). With very few exceptions, the opioid agonists act as inhibitors of neuronal activity. This inhibitory action can be brought about both by pre- and postsynaptic points of attack (Zieglgansberger and Fry 1978, Herz et al. 1980, Zieglgansberger 1980). Both natural and synthetic opioid agonists can affect the release of a number of neuroregulators (for review see Ronai and Szekely 1982, Chesselet 1984).

(13) Somatostatin (SRIF). The putative precursor (Schally et al. 1980), the axonal transport (Finley et al. 1981) and the localization of this tetradecapeptide in nerve terminals (Petrusz et al. 1977, Wakabayashi et al. 1977, Bennett 1981, Kewley et al. 1981) were demonstrated. The enzymes responsible for its biodegradation were also studied (Benuck and Marks 1978). A calcium-dependent, potassium- or veratrine-evoked release of somatostatin could be demonstrated from slices and synaptosomal preparations of hypothalamus and extrahypothalamic regions (e.g., cerebral cortex) in vitro (Wakabayashi et al. 1977, Berkowitz et al. 1978, Iversen et al. 1980, Bennett 1981, Kewley et al. 1981). Biogenic amines are also potent releasers of somatostatin in vitro (McCann et al. 1980, Bennett 1981). Upon microiontophoretic application, somatostatin ex-

erted a depressant effect on central neurons as detected by electrophysiological means (Renaud et al. 1975). Somatostatin was shown to enhance the release of dopamine (Starr 1982, Chesselet and Reisine 1983), norepinephrine (Tsujimoto and Tanaka 1981) and serotonin (Tanaka and Tsujimoto 1981) from various CNS preparations in vitro.

(14) Tachykinins. Substance P (SP) has long been proposed to serve as an excitatory neurotransmitter (Otsuka and Konishi 1977).

SP was shown to be concentrated in the synaptosomal fraction of neural tissue (Schenker et al. 1976, Lembeck et al. 1977). The production in neuronal somata and its axonal transport (Sperk and Singer 1982) as well as the enzymic degradation (Marks 1978, Blumberg et al. 1980) of the undecapeptide could be demonstrated. Metallo-endopeptidases with SP as preferred substrate have been shown to be present both in the rat central nervous system and parotid gland (Wormser et al. 1984). The stimulus-induced release of SP was shown to occur in vitro from nerve terminals of rat substantia nigra slices (Jessell 1978), of rat trigeminal nucleus (Jessell and Iversen 1977), spinal cord (Otsuka and Konishi 1976) and brain synaptosomic preparations (Schenker et al. 1976, Lembeck et al. 1977). Specific ^{3}H-SP binding sites in rat brain crude synaptic membrane preparations have recently been disclosed (Hanley et al. 1980). The indications that SP receptors may also be heterogenous now should be regarded as certainty.

SP, when injected in the vicinity of putative target cells, elicits an increase in neuronal firing rate, mostly by a postsynaptic site of action (Krnjevic 1977, Otsuka and Konishi 1977, Otsuka and Takahashi 1977, Nicoll et al. 1980b, Hosli et al. 1982, Lamour et al. 1983). The excitatory action was seen in the spinal cord (Krnjevic 1977, Otsuka and Konishi 1977, Otsuka and Takahashi 1977, Nicoll et al. 1980a,b, Hosli 1982), cuneate nucleus (Krnjevic 1977), locus coeruleus (Engberg et al. 1981), substantia nigra (Walker et al. 1976), hypothalamic (Ogata and Abe 1981) and cortical neurons (Phillis and Limacher 1974, Lamour et al. 1983). The compound depolarizes the

postsynaptic membrane, however, SP has also presynaptic effects (Randic et al. 1982). As to the interaction of substance P with other transmitters/modulators, it was shown to decrease the release of dopamine in the nigrostriatal system (Siebergeld and Walters 1979, Starr 1982) although, under certain conditions, an increase could also be detected (Starr 1982). It enhances the release of serotonin (Reubi et al. 1978, Reisine et al. 1982). In the neuronal system originating in the medulla oblongata, where substance P/serotonin, TRH/serotonin and TRH/substance P/serotonin coexistence could be detected (Hokfelt et al. 1978, Johansson et al. 1981, Chan-Palay 1982) a counteraction of serotonin autoreceptor-mediated inhibition by substance P, has been suggested (Mitchell and Fleetwood-Walker 1981). Substance P may also influence at a postsynaptic site serotonin- (Fasmer and Post 1983) and TRH- (Sharif and Burt 1983) receptor-mediated events in the spinal cord.

(15) Thyroid-stimulating hormone (TSH). Neural cell cultures consisting of dispersed cells from various rat brain areas (amygdala, hypothalamus, thalamus and cerebral cortex) release TSH-like immunoreactive material into the incubation medium (Hojvat et al. 1982).

(16) Thyrotropin-releasing hormone (TRH). The synaptosomal location as well as the release of TRH upon depolarisation (Bennett et al. 1975, Bennett 1981) were reported; biogenic amines were also shown to liberate TRH from hypothalamic slices and synaptosome preparations (Charli et al. 1978). The existence of neural TRH receptors is well-established (Sharif and Burt 1983, Manaker et al. 1985, Mantyh and Hunt 1985).

TRH increases membrane conductance and depolarizes frog motoneurones (Nicoll et al. 1980a). In the CNS, depressant neuronal actions of TRH were also recorded (Renaud and Martin 1975, Renaud et al. 1975).

(17) Vasoactive intestinal polypeptide (VIP). While monitoring the incorporation of ^{32}S-methionine into VIP, a large-molecular weight precursor of the active peptide was de-

tected in the CNS (Emson et al. 1980b). The occurrence of axonal transport was also demonstrated (Emson et al. 1980a, Said et al. 1980). VIP is concentrated in synaptosome-rich preparations (Giachetti et al. 1977, Said et al. 1980, Sims et al. 1980) in vesicles (Emson et al. 1978).

VIP can be released by depolarizing agents in a calcium-dependent manner from rat hypothalamus (Emson et al. 1978), cerebral cortical or amygdala (Besson et al. 1982) slices as well as from cortical synaptosomes (Giachetti et al. 1977).

High affinity, specific binding of labelled VIP to receptors in brain membranes was also disclosed (Robberecht et al. 1978, Taylor and Pert 1979).

When applied microiontophoretically to cortical neurones, VIP exerted excitatory action (Phillis et al. 1978, Lamour et al. 1983). An interaction at postsynaptic level with acetylcholine has been disclosed in the cholinergic nerves of cat salivary gland, where the neuropeptide coexists with acetylcholine (Lundberg et al. 1982a).

5.4. THE DISTRIBUTION OF NEUROPEPTIDES AND THEIR RECEPTORS IN THE NERVOUS SYSTEM

Whenever a biologically active endogenous substance is detected, one of the very first steps in the course of its research is to map the distribution of the compound and its receptor in the organism. The distribution pattern may cast light on the possible physiological functions of the substance or at least it may serve as a starting point for further investigations.

The close neuropeptide content/receptor density matches are the exception rather than the rule (Herkenham 1987). Besides the technical artifacts, the mismatch appears to stem mainly from the fact that at present, contents and not release sites and patterns are correlated with functional receptor densities. Apart from neurohormonal type of action one should expect a reasonable spatial relatedness of release sites and target receptors both in the case of synaptic and parasynaptic

communications. It is of interest that whereas the neuroregulator substances appear to be distributed conservatively across species, receptor distributions show striking species differences (Herkenham 1987).

As it was mentioned above the coexistence and co-release of more than one information-carrying substance from the very same neuron is a rule rather than an exception. A collection of hitherto revealed coexistences is given in Table 5.2.

As Palkovits (1986) pointed out, although the neuropeptides occur in most brain areas, they show a highly uneven distribution. There are some cell groups/areas, which are particularly rich in neuropeptides either in the sense that some neuropeptides occur in the given spot in prominently high concentrations or many neuropeptides exist in the area in question in varying quantities (Palkovits 1984, 1986). In none of the brain sites would occur a "pure" neuropeptidergic innervation; the neurons liberating the neuropeptides are constituents of an assembly working with "classical" neurotransmitters as well as with peptide neuroregulators. Neuropeptides often coexist with classical transmitters within the same neuron; when thinking of neurotransmission the functional consequences of this coexistence have to be taken into consideration (Costa 1982, Hokfelt et al. 1986).

The areas/systems rich in neuropeptides are as follows (Palkovits 1984, 1986):

All the known neuropeptides occur in the hypothalamus in nerve terminals as well as in cell bodies. Within the hypothalamus, the neuropeptide concentrations are prominent (in fibers and nerve terminals) in the median eminence.

The limbic system is also rich in neuropeptides: the most prominent members are the bed nucleus of the stria terminalis, central maygdaloid nucleus and lateral septal nucleus. The limbic cortex contains many neuropeptides (mostly in fibers and nerve terminals although the concentrations of individual peptides, with the exception of VIP, CCK, NPY and dynorphins/neoendorphins are moderate (Roberts et al. 1984, Jones and Hendry 1986).

Table 5.2. The coexistence of neuropeptides with classical transmitters and with each other in the brain and peripheral neural elements[1]

Classical transmitter or neuropeptide	Coexisting neuropeptide(s)	Occurrence in the nervous system (species)	Reference
1.Dopamine	1. Cholecystokinin	ventral mesencephalon (rat, man)	Hokfelt et al. (1980a,b)
	2. Neurotensin	ventral mesencephalon (rat)	Hokfelt et al. (1984)
	3. Enkephalin	carotid body (cat)	ref. in Lundberg and Hokfelt (1983)
2.Norepinephrine	1. Enkephalin	locus ceruleus (cat) sympathetic ganglia (rat)	Charnay et al. (1982), Di Giulio et al. (1978), Schultzberg et al. (1979), Ariano and Tress (1983); for restrictions see Schultzberg et al. (1979), Pelto-Huikko et al. (1980), ref. in. Hervonen et al. (1981)
		SIF cells (guinea-pig, cat, man)	Pelto-Huikko et al. (1980), ref in Lundberg and Hokfelt (1983)
	2. Dynorphin-releated peptides	sympathetic ganglia (guinea-pig)	Lang et al (1984, Heym and Lang (1986), but see Dalsgaard et al. (1983)
	3. Opioid peptide(s)[2]	Splenic nerve (bovine)	Klein et al. (1982)
	4. Neuropeptide Y[3]	medulla oblongata (rat, man)	Hokfelt et al. (1983b), Everitt et al. (1984)
		locus ceruleus (rat)	Everitt et al. (1984)
		sympathetic ganglia (rat, cat, man, guinea-pig)	Lundberg et al. (1982b,c, 1983), ref. in Lundberg and Hokfelt (1983)
	5. Neurotensin	adrenal medulla (cat)	ref. in Lundberg and Hokfelt (1983)
	6. Vasopressin-like peptide	sympathetic ganglia (rat, monkey)	Hanley et al. (1984)
	7. Somatostatin	sympathetic ganglia (rat, guinea-pig)	Hokfelt et al. (1977b), Lundberg et al. (1982b)
	8. Substance P	sympathetic ganglia (rat)	Robinson et al. (1980), Kessler et al. (1981, 1984), Kessler and Black (1982), Adler and Black (1984), Bohn et al. (1984)
	9. Galanin	brainstem (rat)	Meleander et al. (1986)
3.Epinephrine	1. Neuropeptide Y[3]	medulla oblongata (rat)	Everitt et al. (1984)
	2. Neurotensin	medulla oblongata (rat)	Hokfelt et al. (1984)
	3. Enkephalin-related peptides	adrenal medulla (several species)	e.g. Stern et al. (1979), Kilpatrick et al. (1981)

4.Serotonin	1. Substance P	medulla oblongata (rat)	Chan-Palay et al. (1978), Hokfelt et al. (1978b), Chan-Palay (1982)
	2. TRH	medulla oblongata (rat)	Johansson et al. (1981)
	3. Substance P+THR	medulla oblongata (rat)	Johansson et al. (1981)
	4. Enkephalin	medulla oblongata, pons (cat) SIF cells, superior cervical ganglion (guinea-pig)	Glazer et al. (1981) Hunt and Lovick (1982) Kanagawa et al. (1986)
5.Acetyl-choline	1. Vasoactive intestinal polypeptide	cortex (rat)	Eckenstein and Baughman (1984)
		autonomic ganglia (cat)	Lundberg et al. (1979b, 1982a), Lundberg and Hokfelt (1983), for rewiew see Heym and Lang (1986)
	2. Substance P	pons (rat)	Vincent et al. (1983)
	3. Galanin	septum, diagonal band (rat)	Meleander et al. (1986)
	4. Enkephalin	cochlear nerves (guinea-pig) preganglionic nerves (cat)	Altschuler et al. (1983) ref. in Lundberg and Hokfelt (1983), Heym and Lang (1986)
	5. Neurotensin	preganglionic nerves (cat)	ref. in Lundberg and Hokfelt (1983)
	6. LHRH	sympathetic ganglia (bullfrog)	Jan and Jan (1983)
	7. Somatostatin	heart (toad)	ref. in Lundberg and Hokfelt (1983)
	8. Substance P + Leu-enkephalin	ciliary ganglion (avian)	Erichsen et al. (1982)
6.GABA	1. Somatostatin	thalmus (cat) cerebral cortex, hippo-campus (cat, rat, monkey)	Oertel et al. (1983a) Hendry et al. (1984a) Schmechel et al. (1984), Somogyi et al. (1984), Jones and Hendry (1986), Nunzi et al. (1986)
	2. Motilin	cerebellum (rat)	Chan-Palay et al. (1981), Chan-Palay (1982)
	3. Cholecystokinin	cerebral cortex, hippo-campus (cat, monkey)	Hendry et al. (1984a), Somogyi et al. (1984), Jones and Hendry (1986), Nunzi et al. (1986)
	4. Neuropeptide Y[3]	cerebral cortex (cat, monkey)	Hendry et al. (1984a), Jones and Hendry (1986),
	5. Substance P	cerebral cortex (cat, monkey)	Jones and Hendry (1986)
	6. Enkephalin	striatum-subst. nigra (rat)	Aronin et al. (1984)
	7. Opioid peptide(s)[4]	striatum, amygdala (rat)	Oertel et al. (1983b)
7.Somato-statin	Neuropeptide Y[3]	cerebral cortex (rat, man, cat, monkey)	Vincent et al. (1982b), Hendry et al. (1984b), Jones and Hendry (1986)

Table 5.2. cont.

8.Substance P	1. TRH	see above under "serotonin"	
	2. Neuropeptide Y^3	cerebral cortex (cat, monkey)	Jones and Hendry (1986)
	3. Leu-enkephalin	n. intercollicularis, stratum cellulare internum of hypothalmus, periaqueductal grey (avian), see also above under "acetylcholine"	Erichsen et al. (1982)
	4. CGRP	cardiac and perivascular nerves (rat, guinea-pig)	Wharton et al. (1986)
9.Vasoactive intestinal polypeptide	PHI-27	autonomic ganglia (several species incl. man)	Bloom et al. (1983), Fahrenkrug et al. (1983)
10.Vasopressin	1. Enkephalins	neurohypophysis (rat)	Martin and Voigt (1981)
	2. Dynorphin	neurohypophysis (rat)	Watson et al. (1982a), Martin et al. (1983)
11.Oxytocin	1. Enkephalins	neurohypophysis (rat)	Martin and Voigt (1981) Martin et al. (1983)
	2. Corticotropin releasing factor	neurohypophysis (rat)	Sawchenko and Swanson (1985)
12.Corticotropin releasing factor	1. Oxytocin	(see above)	
	2. Enkephalin	paraventricular nucleus,	Hokfelt et al. (1983a)
	3. PHI-27	parvocellular neurons (rat)	Sawchenko and Swanson (1985)
	4. Neurotensin	(in separate subsets)	

Footnotes to Table 2

1: For recent reviews see Cuello (ed., 1982), Chan-Palay and Palay (eds., 1984), Lundberg and Hokfelt (1983), Heym and Lang (1986), Hokfelt et al. (1986), Jones and Hendry (1986), Nunzi et al. (1986).

2: The opioid peptide content was determined by radioreceptor assay, thus, the chemical identity of opioid substance(s) cannot be specified.

3: Neuropeptide Y stands for the NPY-like immunoreactivity detected as such and also for the peptides referred to previously as "Avian pancreatic polypeptide"-like or "Bovine pancreatic polypeptide"-like substances. For discussion see Tatemoto (1982); further explantations can be found in footnotes to Table 1 and in the text.

4: Because of the non-selectivity of antiserum used, the chemical identity of opioid peptide(s) cannot be specified.

In the extrapyramidal system high concentrations of enkephalin-related peptides (globus pallidus), tachykinins, dynorphins/neoendorphins (substantia nigra) and CCK (claustrum) are present. Of the brain stem autonomic centers/areas, the nucleus of the solitary tract should be mentioned first. Next to the hypothalamus, this is the area enriched most with neuropeptides. Many neuropeptides occur in this nucleus (partly in fiber nerve terminals, partly in cell bodies), some of them in high concentrations. The parabrachial nuclei and the locus ceruleus are also rich in neuropeptides.

5.5. PARTICIPATION OF NEUROPEPTIDES IN THE INTEGRATION OF COMPLEX CNS FUNCTIONS

The involvement of neuropeptides has been suggested in a great many CNS functions; of all the possible implications only those are surveyed here in which the role of the neuropeptides ranks among the decisive factors. Thus, their role in pain control, thermoregulation, food and water intake, sleep mechanisms, extrapyramidal function, simple behavioral patterns and higher brain functions (learning, memory, social behavior) are discussed. The action of neuropeptides is described with the understanding that in none of the functions do they serve as sole determinants; rather they act as constituents of a complex circuitry. Their effect, in most cases, is neuromodulator-like, i.e., the neuropeptides dampen or enhance the release or the effect of other neuroregulators involved in the control of a certain CNS function.

5.5.1. NEUROPEPTIDES IN PAIN CONTROL

When discussing pain one has to differentiate clearly between pain perception, pain tolerance and pain reaction. Pain perception with the subserving nerve mechanisms provides the ability of perceiving a stimulus, generated in the environment or within the organism, as potentially harmful and

avoidable. It serves to localize the stimulus; furthermore it is the prerequisite of initiating an adequate sequalae of reactions in order to escape the potential danger. Pain tolerance involves the attitude of the individual towards the stimulus, the subserving neural mechanisms determine the motivational value of perceived stimulus. Pain reaction is the "output" side of pain phenomenon; the term covers motor and vegetative reactions as well as complex behavioral patterns accompanying pain.

In their classical model often referred to as the "gate control theory", Melzack and Wall (1965) outlined the neuronal circuitry subserving pain perception and pain control. The afferents from "nociceptors" have their first synapse in the dorsal horn of spinal cord; the ascending pathways (see the competent textbooks) convey information towards the higher relay centers. Among these the periaqueductal grey (PAG) matter of the mesencephalon has a distinguished significance. More rostral centers mediate motivation and initiate vegetative reactions and complex behavioral patterns accompanying pain. The PAG is in connection with the "more rostral" centers as well as with some medullary nuclei which are the sites of origin of fibers descending to the dorsal horn via the dorsolateral funiculus. These descending fibers, either directly or indirectly, exert an inhibitory control on conveying the nociceptive information at spinal level.

By incorporating the recent neuroanatomical, neurochemical, electrophysiological and pharmacological findings (e.g., Yeung et al. 1977, Beitz 1982a,b, for review see Basbaum and Fields 1978, 1984, Watkins and Mayer 1982, Zieglgansberger 1980), the theory was extended and enriched in fascinating new details but no major amendement seemed to be necessary. Endogenous opioids have a key role in this pain-controlling circuitry, and the interaction (at more than one level) between substance P and opioid peptide-containing neurons constitutes also central feature of the extended model (see below). Although many other neuropeptides may have a role in pain control (see also below), neither their physiological/pathological significance nor their relation to the

abovementioned circuitry, has been established as yet. The only exception is neurotensin (Beitz 1982a) for which a tentative role within the framework of this theory, has been proposed (Basbaum and Fields 1984).

Exogenous opioids and endogenous opioid systems may control nociception at spinal as well as supraspinal level. Primary afferents carrying "noxious" information terminate mainly in the outer layer of substantia gelatinosa (lamina IIo). Neurons located in IIo may relay nociceptive input from primary afferents to marginal zone (lamina I) (Price et al. 1978); neurons in IIo might be one of the targets of descending inhibitory pathways.

Substance P was suggested to function as the transmitter at the first synapse of "pain" afferentation (Lembeck 1953, Otsuka and Takahashi 1977); even if its transmitter role can be debated (Krivoy et al. 1977, North 1979, cit. by Marx 1979) it is clear that the peptide has an important role in the signal transmission at this junction. Opiate receptors could be localized to the nerve terminals of primary afferents (LaMotte et al. 1976); opiates and opioid peptides are capable of inhibiting the stimulation-induced release of substance P from these terminals (Otsuka and Konishi 1976, Jessel and Iversen 1977). The dorsal horn of spinal cord contains opioid peptides both of proenkephalin A and pro neoendorphin/dynorphin family (see section 5.1). However, while opioid peptide-containing terminals have been shown to form synaptic contacts with dentrites and cell bodies in the dorsal horn of spinal cord, no synapses on axon terminals have been found (for discussion see Basbaum and Fields 1984). The lack of synapses on axon terminals suggest that opioid peptide-containing neurons probably exert a nonsynaptic type of control at dorsal horn terminals liberating substance P. It should be mentioned that in substantia gelatinosa "islet" cells, besides the opioids, several other neuropeptides such as substance P, neurotensin, "avian pancreatic polypeptide" (i.e., probably neuropeptide Y) and cholecystokinin are also present (Gibson et al. 1981, Hunt et al. 1981); further neuropeptides may occur in fibers and nerve terminals. The descending inhibitory pathway originates in the

rostral medulla and carries serotonin and substance P; the coexistence of these neuroregulators within this pathway has been described (Hokfelt et al. 1978, Chan-Palay 1982). In one of the medullary nuclei of origin (nucleus reticularis gigantocellularis lateralis) enkephalin-serotonin coexistence has also been detected in the rat (Glazer et al. 1981). These descending fibers bring about an inhibitory action in dorsal horn nociceptive neurons including interneurons and a population of spinothalamic and spinoreticular neurons (Willis et al. 1977, for discussion see Basbaum and Fields 1984). The inhibition may be direct (the one exerted by serotonin) or indirect (serotonin, substance P) brought about by affecting a population of spinal interneurons, among them the opioid ones; to lead to an inhibitory final result (Basbaum and Fields 1984). The involvement of a spinal opioid link in the inhibitory control by the descending pathway on spinal nociceptive neurons is demonstrated best by the experiment showing that analgesia produced by the microstimulation of the ventromedial medulla in the rat could be blocked by lumbar intrathecal administration of opioid antagonist naloxone (Zorman et al. 1982). In a highly tentative form, the recruitment of spinal opioid interneurons by collaterals of substance P-containing nociceptive primary afferents (Basbaum and Fields 1984) or by myelinated A afferent fibers (Zieglgansberger 1980) has been proposed. In the dorsal horn μ, δ and κ type opioid receptors have been shown to be present; exogenously administered κ-receptor stimulants exert their analgesic action mostly if not exclusively at spinal level (for discussion see Basbaum and Fields 1984), whereas μ - and possibly δ receptor agonists may act both at spinal and supraspinal sites. Of the latter two receptor types, in general, μ receptors appear to have a dominant role in the mediation of analgesia while the contribution of δ receptors is doubtful (Ronai et al. 1981, for discussion see Akil et al. 1984).

The mesencephalic periaqueductal grey receives nociceptive input from the spinal cord either directly or indirectly, relayed by pontine reticular formation and nucleus cuneiformis (Mehler 1969, Gebhart 1982). It receives input also from fron-

tal and insular cortex, amygdala and hypothalamus (Beitz 1982b, Mantyh 1982). Projections from PAG run to the medullary nuclei which give rise to the descending antinociceptive fibers (Beitz 1982a, Mantyh 1983) and, to a lesser extent, directly to the spinal cord dorsal horn (Mantyh and Peschanski 1982). The PAG ranks among the most sensitive brain sites to the local application of opioids (Yaksh et al. 1976, Yeung et al. 1977, for review see Szekely 1982a) to elicit analgesia; furthermore, focal stimulation of this area produces analgesia, which, at least in part, can be suppressed by opiate antagonists (Mayer and Price 1976, Watkins and Mayer 1982). It should be regarded as the most relevant site of analgesic action of systemically given opioid agonists (at least so in experimental animals) since microinjection of the opiate antagonist naloxone into the PAG abolishes the analgesic action of systemically injected morphine (Yeung and Rudy 1980 and references therein, for discussion see Basbaum and Fields 1984). PAG is enriched with opioid peptides as well as with opioid receptors especially in higher mammals. In the midbrain circuitry subserving pain control, as proposed by Basbaum and Fields (1984), an enkephalinergic local neuron, activated either directly by ascending nociceptive fibers or indirectly by the intervention of a substance P-ergic interneuron, stimulates, by disinhibitory mechanisms, an excitatory output neuron connecting PAG with medullary nuclei involved in the control of nociceptive mechanisms (see below). To the disinhibitory mechanism the participation of a third, inhibitory interneuron, impinging upon the output neuron, must be assumed. The transmitter of the third interneuron is unknown; this neuron would serve also as a target for β-endorphinergic terminals. For the transmitter of the excitatory output neuron neurotensin is a good candidate: the connection is verified morphologically (Beitz 1982a); furthermore, intracisternally administered neurotensin has been shown to exert a strong analgesic effect (Kalivas et al. 1982). The medullary nuclei, receiving the excitatory input from PAG (Beitz 1982a, Mantyh 1983b) and projecting to the dorsal horn via the dorsolateral funiculus, are the nucleus raphe magnus, nucleus reticularis gigantocel-

lularis (pars α) and nucleus reticularis paragigantocellularis. Here, again, local opioid mechanisms are also involved; the nucleus reticularis paragigantocellularis is the most sensitive site in the brain to elicit analgesia by local application of opioid agonists (Akaike et al. 1978). The local opioid activation of descending inhibitory neurons takes place probably by disinhibitory mechanism (Basbaum and Fields 1984); opioid peptide-containing neural elements are present in this area (Beitz 1982c). The extended version of gating model helps understanding the action of exogenously administered opiates as well as the mechanisms by which endogenous opioid systems may control nociception. The model may offer an explanation also for the complex role of substance P in nociception. It is known for a while, that substance P, in addition to its assumed role as a transmitter/modulator of primary nociceptive afferents, is capable of producing analgesia which could be antagonized by opioid antagonists (Stewart et al. 1976, Frederickson et al. 1977). It should be mentioned that several groups were unable to confirm this finding (Goldstein and Malick 1979, Growcott and Shaw 1979). Substance P has no direct effect on opiate receptors (Terenius and Wahlstrom 1975); this fact might indicate that substance P, in certain circumstances, is capable of activating endogenous opioid systems (Stewart et al. 1976). This activation, according to the model put forward by Basbaum and Fields (1984) may take place at several sites of gating circuitry (see above).

The neuronal circuitry described above is wired to affect mainly the perception aspect of pain phenomenon. In the majority of experimental procedures the measures of pain perception are detected although there are numerous methods by which the pain tolerance component could also be assessed; no systematic survey to evaluate comparatively the results of these different kinds of pain-analysing studies is available as yet. The limbic areas are the candidates for mediating the "pain tolerance" component; this aspect is particulary relevant to the therapeutic action of opioids. It should be realized, however, that limbic areas are capable of affecting pain perception, too; when this happens it is likely to take place by

an effect on the "classical" gating circuitry. In this context, it is to be recalled that PAG is considered as a constituent of limbic midbrain circuitry (LaMotte et al. 1978 and references therein).

Under physiological conditions the effectiveness of suppression of pain perception by endogenous opioids is low (Frederickson 1978). In stressful situations (reviewed by Watkins and Mayer 1982, Akil et al. 1984) and also by analgesic manipulations like acupuncture, electroacupuncture (Sjokund and Eriksson 1976, Mayer et al. 1977, Stewart et al. 1977) and focal brain stimulation (Reynolds 1969, Mayer and Price 1976, Watkins and Mayer 1982) powerful pain suppressing endogenous mechanisms are activated, which at least in part, involve endogenous opioids (Terenius 1978, Watkins and Mayer 1982, Akil et al. 1984). Placebo analgesia is also brought about by recruiting endogenous opioid mechanisms (Levine et al. 1978). The possible role of endogenous opioids in pain control during labor remains to be established (for discussion see Akil et al. 1984).

There are several other neuropeptides which are analgesic when given centrally (i.e., into the cerebral ventricles or directly into the brain) but whose relation to the gating circuitry has not been established as yet. Of these, bombesin has been reported to exert a naloxone-resistant analgesic action in rats when injected into the periaqueductal grey (Pert et al. 1980) but not in mice given intracisternally (Nemeroff et al. 1979). PAG injections of vasoactive intestinal polypeptide and calcitonin analogs (Fabbri et al. 1985) also produce naloxone resistant analgesia in the rat. Somatostatin, when given intracerebroventricularly had analgesic effect in rats but not in mice upon intracisternal application (Nemeroff et al. 1979). A probably nonopioid-mediated analgesia has also been reported for CCK-8 and the related peptide caerulein in mice upon peripheral administration.

5.5.2. NEUROPEPTIDES AND THERMOREGULATION

Several neuropeptides are capable of affecting body temperature upon central (intracerebroventricular, intracisternal, etc.) administration; some of them may have a role in the physiological thermoregulatory mechanisms.

TRH has been shown to exert a hypothermic effect when given centrally to rabbits, rats and mice whereas in cats, depending on the site of injection in the brain,it may produce hypothermia, hyperthermia or may have no effect (Myers et al. 1977). In a responsive species it reverses the hypothermia produced by many pharmacological agents (for review see Nemeroff et al. 1984). One of the possible neuroanatomical substrates mediating TRH hyperthermia is the preoptic-anterior hypothalamic area, which is known to play a role in thermoregulation. Immunoreactive TRH is present in this area (Hokfelt et al. 1975); microinjections of the peptide into the preoptic-anterior hypothalamus produces hyperthermia in the rat (Cohn et al. 1980, Bosch and Rips 1981) and inhibits the firing frequency of local, warm-sensitive neurons (Salzman and Beckman 1981). Although the high doses necessary to produce thermoregulatory response even upon local microinjections raise question about the physiological significance of these TRH effects (Carino et al. 1976, for discussion see Nemeroff et al. 1984) the finding that intracerebroventricular microinjection of TRH antisera results in marked hypothermia in rats suggests that TRH may function as an endogenous thermoregulatory agent (Prasad et al. 1980).

Neurotensin has a powerful hypothermic effect upon intracisternal-injection in rats and mice both in a cold room (4 °C) and at ambient temperature of 22-23 °C (Bissette et al. 1976, Nemeroff et al. 1977, Brown et al. 1978, Jollicoeur et al. 1981); at ambient temperatures higher than 30 °C the peptide produces no significant effect on body temperature (Mason et al. 1980). Neurotensin did not alter the colonic temperature of several poikilotherms such as fish, frogs and lizards. Of the endotherms, it was ineffective in the pigeon and rabbit and also in two representatives of obligate hibernators, wood-

chuck and ground squirrel (Prange et al. 1979). Neurotensin-responsive brain sites are found in the medial preoptic area, anterior hypothalamus, ventral tegmental area of Tsai, periaqueductal grey, floor of the fourth ventricle, and the spinal tract of the trigeminal nerve of the rat (Martin et al. 1981, Kalivas et al. 1982); all these sites contain endogenous neurotensin (Jenners et al. 1982). All these findings point to the possibility that this peptide plays a physiological role in thermoregulation.

Bombesin is also a potent hypothermogenic agent when given centrally to rats or mice both at low (4°C) temperature and at 25°C (Brown et al. 1978, Mason et al. 1980). In the rabbit, bombesin produced increases and decreases in rectal temperature (Lipton and Glyn 1980). As with many putative endogenous thermoregulatory agents the preoptic area-anterior hypothalamus is the region where bombesin may act. Bombesin essentially disrupts thermoregulation and renders animals poikilothermic (Mason et al. 1980, Tache et al. 1980). Thus, in a responsive species, at 4°C it induces a fall in rectal temperature, at 25°C decreases rectal temperature while increasing skin temperature and at higher (36°C) ambient temperatures bombesin induces a hyperthermic response.

Centrally administerd β-endorphin, similarly to centrally or peripherally injected opiates, increases body temperature at very low doses and decreases it at high doses (Blasig et al. 1979). Met-enkephalin elicited only hyperthermic responses. Both effects, according to the majority of reports, could be antagonized by the opiate antagonist naloxone (for review see Szekely 1982b). β-endorphin when injected directly into the preoptic area or into the spinal subarachnoideal space had hyperthermic effect while administering it into the lateral ventricles the body temperature decreased (Martin and Bacino 1979).

It is still an open question whether endogenous opioids are involved directly in central thermoregulation (Holaday et al. 1978a,b, versus Blasig et al. 1979). The increase of body temperature under stressfull conditions is due, at least in part, to the activation of endogenous opioid mechanisms (Blasig et al. 1978). Endogenous opioids play an important role in spon-

taneous hibernation (Kromer 1980). Neonatal β-endorphin or naloxone administration rendered the animals chronically insensitive to thermal stimuli (Sandman et al. 1979); no data are available, however, on the basal body temperature of treated animals.

5.5.3. NEUROPEPTIDERGIC REGULATION OF FOOD AND WATER INTAKE

5.5.3.1. FOOD INTAKE

Feeding behavior is governed by mechanisms operated by food intake - inducing agents and satiety signals (Anand 1961, Morley et al. 1984); the integrating centers for these impulses are located in the hypothalamus. The vast species differences in the feeding habits and digestive machinery (think of hibernators, monogastric or ruminant animals, poultry etc.) are a warning that no uniform regulatory pattern should be expected.

According to the model put forward by Morley and his coworkers (1984), food intake is initiated by a dopaminergic-opioid mechanism in the lateral hypothalamus controlled by a series of primary inhibitory as well as disinhibitory agents. Primary inhibitory agents of neuropeptide type are corticotropin releasing factor, calcitonin, neurotensin and bombesin. The release and action of disinhibitory substances (norepinephrine, GABA) are kept in check by secondary central (e.g., TRH-related peptides, Reichelt et al. 1978, Morley et al. 1984) and peripheral satiety factors. A potent endogenous satiety substance extracted from human plasma and urine, with a not quite closely defined structure designated as satietin, was described by Knoll (1979). Superimposed on this system are the modulatory effects of substances derived from nutrients and of hormones produced by endocrine cells.

The first candidate of peripherally generated satiety signals was the gut peptide cholecystokinin (Gibbs et al. 1973, 1976, Smith and Gibbs 1984). Other gastrointestinal peptides possibly contributing to postprandial satiety are bombesin

somatostatin and pancreatic glucagon. Claims that CCK and bombesin affect feeding through aversion rather than satiety have not been confirmed. The satiety effect of CCK, somatostatin and glucagon is mediated by the vagus nerve, while bombesin is not. Whilst in monogastric animals the CCK-induced events leading to satiety take place mostly at the periphery, CNS targets for centrally generated peptide appear to be prevalent in ruminant sheep (Baile and Della-Fera 1984).

The evidences suggesting a distinguished role for opioid peptides in apetite control are numerous (for review see Yim and Lowy 1984). Acute (Tepperman et al. 1981), but not chronic, opiate and opioid peptide (Morley and Levine 1981) administration induces food intake in a number of species. Acute (Margules et al. 1978, Lowy and Yim 1982, Shimomura et al. 1982, Yim and Lowy 1984), but not chronic (Shimomura et al. 1982) injections with opiate antagonists tend to reduce feeding. In genetically obese mice and rats, elevated β-endorphin levels were found in the pituitaries of both species and in the plasma of obese rats (Margules et al. 1978). The physiological relevance of tissue levels has been debated (Bray and York 1979), whereas the elevated plasma levels may have physiological significance. The latter notion is strongly supported by the finding that several - but not all - hyperphagia-inducing manipulations cause elevated plasma β-endorphin levels. It is of interest that in hamsters the mechanisms regulating food intake on a short-term basis seem to lack the opiate sensitive component (Lowy and Yim 1982). Not only β-endorphin, but even more potently, dynorphin was also found to be an appetite stimulant The opioid receptors primarily involved in the initiation of feeding appear to be of κ-type (Yim and Lowy 1984). Some endogenous opioid peptides may be mobilized in anticipation of impeding famine (Margules 1979); as Margules (1979) put it, β-endorphin-induced overeating may be regarded as a preparatory measure for the starvation that never comes. Under certain conditions, μ-opioid receptors may mediate suppression of feeding (Shimomura et al. 1982, Yim and Lowy 1984). This might be in keeping with the proposed drive-reducing reward factor role of endogenous opioids (Belluzzi and Stein 1977, Stein and Belluzzi 1980).

5.5.3.2. WATER INTAKE

Several neuropeptides have been implicated in thirst and sodium appetite in different species; the best established of these is the octapeptide angiotensin II which induces normal drinking behavior in all vertebrates tested (Fitzsimmons 1979, 1980). In addition, it also causes an increase in sodium appetite (Fitzsimmons 1980). Intracranial renin stimulates sodium appetite and thirst, mediated ultimately by local generation of angiotensin II (Fitzsimmons 1984). Other endopeptides which might elicit an enhancement of sodium intake through direct or indirect mechanism are vasopressin (Garrigues and Montastruo 1969), ACTH (Weisinger et al. 1977), prolactin, and oxytocin (Denton et al. 1977). Antidiuretic hormone is known to reduce water consumption mainly through an indirect action i.e., due to its water retaining effect. However, ADH was reported to stimulate drinking in the dog (Bellows 1939, Barker et al. 1953, Fitzsimmons 1980). MIF was reported to suppress deprivation-induced fluid intake in rats (Olson et al. 1980). The substance P-related eledoisin and substance P itself affect drinking behavior in pigeons (Evered et al. 1977) but both peptides act as antidipsogenic agents in cats (Fitzsimmons and Evered 1978). In hamsters, in contrast to feeding, drinking behavior appears to be an opiate sensitive mechanism (Lowy and Yim 1982).

5.5.4. NEUROPEPTIDES IN THE EXTRAPYRAMIDAL MACHINERY

The "chemical neuroanatomy" of basal ganglia, in terms of neurotransmitters, receptors and morphological cell types of neuronal pathways as well as the locations and interconnections of these pathways, is now extensively documented (for recent reviews see Groves 1983, Martin 1984, Stahl 1986). Four neuropeptides and their receptors, namely opioid (proenkephalin A- and dynorphin-related) peptides, CCK-8, substance P (tachykinins) and somatostatin occur in prominent quantities in the extrapyramidal system. In brief, striatal neurons sup-

ply with opioid peptide-containing fiber-terminals the pallidum and substantia nigra. The majority of striato-nigral opioid neurons is probably dynorphin/neoendorphin-ergic. The opioid receptors are densest in the striatum; μ receptors occur in dense patches while the δ receptors are diffusely distributed. A considerable proportion of striatal opioid receptors is located on dopaminergic nerve terminals.

The CCK-8 supply of the striatum originates mostly from cell bodies located in the piriform cortex and claustrum (Meyer et al. 1982); a minority is contained by nigro-striatal neurons, where in a highly species-dependent manner, CCK-dopamine coexistence has been disclosed (for discussion see Hokfelt et al. 1986).

Two tachykinins, substance P and substance K are present in striato-nigral descending projections. High densities of substance P receptors are present in the striatum whereas low densities are found in the substantia nigra. In the latter nucleus there is a high density of substance K and neuromedin K binding sites; it is possible that in the substantia nigra substance K is the physiologically relevant tachykinin.

Somatostatin is present in striatal interneurons of aspiny 1 type, possibly in coexistence with GABA (for discussion see Stahl 1986).

At present, movement disorders are generally interpreted in terms of imbalance of classical transmitter systems in the basal ganglia (for recent discussions see Newman and Calne 1986, Stahl 1986); no real attempts to incorporate systematically the implications from the studies on the occurrence and possible functions of neuropeptides in the extrapyramidal machinery are apparent in the literature.

Information is available on several interactions of neuropeptides with classical neurotransmitters in the striato-pallido-nigro-striatal circuitry. As to the classical transmitters, acetylcholine is present mostly in striatal interneurons; GABA occurs in striatal interneurons as well as in descending nigral projections (see e.g., Stahl 1986).

Dopamine resides within nigrostriatal ascending neurons. Corticostriatal projections contain glutamate, serotoninergic

input originates from upper brain stem sources while the transmitter(s) of thalamocorticostriatal excitatory projection is (are) unknown.

Opioid (peptide and nonpeptide) agonists have been reported to inhibit agonists with a strong effect on μ-receptors, (Loh et al. 1976, Subramanian et al. 1977, Vizi et al. 1977) to stimulate δ-receptor agonists, (Chesselet et al. 1981, Lubetzki et al. 1982) or not to affect (Lubetzki et al. 1982) striatal dopamine release. Furthermore opioid agonists have been found either to stimulate striatal acetylcholine release (probably indirectly, Vizi et al. 1977) or to have no effect on acetylcholine liberation (Jhamandas and Elliott 1980, Jones and Marchbanks 1982). They can inhibit GABA release both in the striatum (Brennan et al. 1980) and in the substantia nigra (Iwatsubo and Kondo 1978). It must be put on record hereby that catalepsy-like state seen upon the peripheral or central administration of opioid peptide agonists (Bloom et al. 1976, Jacquet and Marks 1976) is not analogous with catalepsy produced by neuroleptics (Dill and Costa 1977, Havlicek et al. 1978, Browne et al. 1979).

CCK-8 may affect striatal dopaminergic neurotransmission both at pre- and postsynaptical level (for review see Nair et al. 1986).

A number of investigators reported an inhibitory effect on dopamine turnover and release (Fuxe et al. 1980b, Mashal et al. 1983, Markstein and Hokfelt 1984, Voigt and Wang 1984) as well as on dopamine binding (decrease in binding sites; Mashal et al. 1983) whereas other studies point to an enhancement of dopaminergic function (Kovacs et al. 1981, Hamilton et al. 1984). Although electrophysiological studies show an excitatory effect on dopaminergic neurons (Skirboll et al. 1981, Bunney et al. 1982), the "release" experiments reporting on an inhibitory interaction, appear to be the better controlled ones (Markstein and Hokfelt 1984). Dopamine, in a certain concentration range, has been shown to enhance the release of CCK immunoreactive substance from striatal slices (Meyer and Krauss 1983).

Substance P has been suggested to exert a tonic excitatory influence on the nigro-striatal dopaminergic pathway (Nicoll et al. 1980b); this effect is not mediated directly at nigral dopaminergic cell bodies (Collingridge and Davies 1982). Exogenously administered substance P has been reported to enhance the release of serotonin in the substantia nigra both in vivo (Reisine et al. 1982) and in vitro (Reubi et al. 1978); dopamine release was found either stimulated or inhibited by substance P in in vitro striatal preparations (Starr 1982).

Somatostatin has been reported to stimulate dopamine release in the striatum, both in rats (Starr 1982) and cats (Chesselet and Reisine 1983).

In the brain of patients with Parkinson's disease there was a loss of striatal μ opiate receptors (Reisine et al. 1979, Rinne et al. 1983, 1984) whereas the number of enkephalin binding sites was enhanced in the striatum as well as in certain limbic areas (Rinne et al. 1983, 1984). Reduced enkephalin levels (Taquet et al. 1981) particularly in the substantia nigra (Rinne et al. 1984) have also been found in parkinsonian brains. High (Agnoli et al. 1980) but not low (Price et al. 1979) doses of naloxone have been reported to exert beneficial effect against tremor, bradykinesia and rigidity in patients with Parkinson's disease; this points to the possible involvement of opiate receptors other than μ.

CCK-8 levels have been found decreased in parkinsonian patients in the zona compacta and reticulata of the substantia nigra but not in striatal, mesolimbic or mesocortical dopaminergic projection areas (Studler et al. 1982). Substance P levels were also reduced in the substantia nigra, and, in patients who had not received levodopa treatment, also in the putamen (Rinne et al. 1984). The levels of substance P in the substantia nigra appeared to correlate with the severity of hypokinesia. Decreased levels of somatostatin, especially in demented cases, have been found in the neocortex and cerebrospinal fluid of parkinsonian patients (Dupont et al. 1982, Epelbaum et al. 1983, Rinne et al. 1984) and in Huntington's dementia as well as in family members of patients with Huntington's dementia (Schroter 1981).

Cholecystokinin, Met-enkephalin and substance P concentrations have been found to be reduced in the striatum and in the substantia nigra in Huntington's disease (Emson et al. 1980a,d); as it is suggested by the results of most recent studies the changes in neuropeptide levels (not infrequently, in positive direction) may be even more numerous (for review see Beal and Martin 1986).

5.5.5. NEUROPEPTIDES AND SLEEP

A nonapeptide that elicits a number of characteristics of orthodox sleep following application in rabbits, was isolated from blood dialysates of rabbit brain after the stimulation of hypnogenic areas of the thalamus (Monnier et al. 1975, Schonenberger and Monnier 1977) and was designated as δ-sleep inducing peptide because of the characteristic occurrence of sleep spindles and waves. Delta-sleep inducing peptide is present also in human milk (Graf et al. 1984); its involvement in the regulation of the sleep-wake cycle of neonates is unclear. In rat pups fed orally with the peptide, it appears in the plasma. Sleep inducing peptides with lower molecular weight were isolated from the brain and cerebrospinal fluid of the rat and goat, respectively (Fencl et al. 1971, Minone and Uckizono 1974). A fourth sleep peptide with structural resemblance to oxytocin and vasopressin was also described (Pavel et al. 1977). A slow-wave sleep promoting factor, derived from human urine, containing muramic acid, alanine, glutamic acid and diaminopimelic acid, resembles the sleep factors found in the CSF and brain extracts of sleep-deprived animals (Krueger et al. 1982).

Peptide hormones and possible neuroregulators known as sleep inducers are arginine vasotocin (Pavel et al. 1977) and growth hormone (Mendelson et al. 1980, Drucker-Colin 1981). It is noteworthy that extremely small amounts (on the picogram scale) of intracerebroventricularly administered vasotocin are capable of eliciting sleep in cats (Pavel et al. 1977); the effect of growth hormone is, more than likely, an indirect one

(Drucker-Colin 1981). Other neuropeptides like substance P (Hecht et al. 1980) and ACTH (and its analogs) (Urban and DeWied 1978) were also reported to act as possible sleep regulators. Endogenous opioid mechanisms might also influence sleep patterns (Sitaran and Gillin 1982). A detailed study of the interaction of various endogenous peptides (and synthetic analogs thereof) with a barbiturate effect (Bisette et al. 1978) has shown that the list of neuropeptides as potential sleep modifiers might be even more numerous.

5.5.6. NEUROPEPTIDES AND BEHAVIOR: THEIR EFFECTS ON SIMPLE BEHAVIORAL PATTERNS AND HIGHER BRAIN FUNCTIONS

The assessment of the behavioral actions of a neuropeptide may proceed in several steps. At an early stage, the behavioral effects of centrally or peripherally administered neuropeptide are registered and the possible implications of the results thus obtained, are speculated. Most neuroactive peptides when given centrally evoke autonomic changes and may alter the incidence of simple behavioral components which constitute the normal behavioral repertoire of an animal (yawning, grooming, motility, etc); however, especially, at high doses, the neuropeptide may provoke bizarre stereotypies (freezing, staring, explosive motor behavior, etc) (for review see De Wied and Jolles 1982, Nemeroff et al. 1984, Dantzer et al. 1986, Panksepp 1986). When registering effects upon peripheral administration of neuropeptides, one is confronted with several interpretational problems. The main issue is the penetration of peripherally given peptide into the brain; although some exceptions have been suggested to occur, it is generally agreed that the great majority of peptides crosses the blood-brain barrier very poorly (for recent discussions see Banks and Kastin 1985a,b, 1986). Special exceptions are the cases where the CNS targets lie outside the blood barrier (circumventricular organs). If peripheral targets initiate behavioral effects one must find the links which mediate the results. The third issue which is relevant also to the actions

of centrally given peptides but more so at the periphery, is the metabolic stability of natural peptides and peptide fragments. Although there is a wide range in the metabolic stability of natural peptides/peptide fragments it occurs quite often that the reported duration of action (or the entire time-course of action) of a peptide is far beyond the expectations based on its biological half-life.

A more advanced approach in studying the behavioral effects of neuropeptides is to correlate inborn, pathological, drug-induced or state-dependent differences (changes in the levels of neuropeptide in tissue samples) biological fluids with specific behavioral patterns. The testing of exogenously administered neuropeptides or drugs known to affect selectively the function of endogenous neuropeptide system in question in properly constructed experimental behavioral models, is also informative.

If the involvement of a neuropeptide in a certain function or pathophysiological process appears to be well established in experimental animals, or if there is experimentally well-founded reason to assume that the peptide may be involved in functions/disorders special for humans, then the neuropeptide, its synthetic analogues and antagonists or drugs affecting the function of endogenous neuropeptide otherwise might be recommended for clinical trials (with the obsrevation of safety factors, of course).

The distribution of neuropeptides in the CNS suggests that they may affect a number of behaviors by influencing arousal, mood and motivational processes. The effects of neuropeptides on sexual behavior, social behavior, learning and memory processes and their possible involvement in psychiatric disorders, will be discussed below.

5.5.6.1. NEUROPEPTIDES AND SEXUAL BEHAVIOR

Luteinizing hormone releasing hormone (LHRH) has been shown to stimulate sexual behavior both in female (Moss and McCann 1973, Pfaff 1973, Sakuma and Pfaff 1980) and male (Moss et al.

1975, Moss and Foreman 1976, for review see Moss and Dudley 1984) independently of its hormonal action (Kastin et al. 1980). The brain sites where LHRH microinjections/infusions induce lordosis behavior in female rats are the medial preoptic area, arcuate-ventromedial hypothalamic region and the periaqueductal grey in the midbrain (Riskind and Moss 1980, Sakuma and Pfaff 1980). The role of endogenous brain LHRH in mating behavior is demonstrated by the findings that antiserum against LHRH injected into the third ventricle or the periaqueductal grey (Kozlowski and Hofstetter 1978, Sakuma and Pfaff 1980) or a potent LHRH antagonist analog when infused into the third ventricle or arcuate-ventromedial hypothalamus (Moss et al. 1979) reduces lordosis behavior in overectomized estrogen-progesterone-eprimed female rats. The effect of LHRH on sexual behavior is not limited to rats, it also pertains to ring doves (Cheng 1977).

Endogenous opioids have also been implicated in sexual activity (for review see Henry 1982). They may be involved in either of the three ways: in modulating gonadotrop hormone release, in the euphoria or orgasm and, independently, in the suppression of sexual activity.

An endogenous opioid peptide similar or identical to Leu-enkephalin has been proposed to serve as a natural euphorigen or reward transmitter (Belluzzi and Stein 1977). Therefore, Henry (1982) has raised the possibility that there is an endogenous opioid-ergic component to sexual orgasm. A number of factors which might provoke the release of endogenous opioids are known as potential enhancers of human sexual response (for review see Komisaruk 1978, Henry 1982). Ejaculation has been shown to increase circulating β-endorphin levels in the male hamster (Murphy et al. 1979). After orgasm is achieved, β-endorphin which has been released may act to turn off the sexual drive (Henry 1982). Intraventricular injection of β-endorphin (Meyerson and Terenius 1977) or an enzimatically stable enkephalin analogue (Gessa et al. 1979) reduced mounting behavior in male rats. On the other hand a facilitation of copulatory activity was observed after the peripheral administration of the same enkephalin analogue (Bohus 1979).

The well-documented impairment of sexual function among narcotic addicts is mediated mostly by effect on hormone regulation.

Opioid antagonists increase the occurrence of ejaculation, decrease ejaculation latency and decrease the number of intromissions before ejaculation in rats (Hetta 1977, Myers and Baum 1979). In a group of male Sprague-Dawley rats selected because of their relative lack of sexual activity, high dose of opioid antagonist naloxone increased significantly mounting, intromission and ejaculation (Gessa et al. 1979).

In a pilot study naloxone has been found to be ineffective in three women with low sexual arousability whereas in males it reduced the time from erection to ejaculation (Goldstein and Hansteen 1977); another opioid antagonist, naltrexone, has been reported to produce spontaneous erection (Mendelson et al. 1979).

Peptides related to ACTH have been reported to influence sexual behavior, but their effects are rather controversial (for review see De Wied and Jolles 1982).

5.5.6.2. NEUROPEPTIDES AND SOCIAL BEHAVIOR

Based on the recognition that the symptomatology and dynamics of narcotic addiction and social attachments have many common features, Panksepp and his coworkers (Hermann and Panksepp 1978, Panksepp et al. 1979, 1980, 1985, Panksepp 1980) started investigating systematically the possible involvement of endogenous opioid systems in social behavior. The assumption central to their theory was that, since endogenous opioids, among many other functions, may be the mediators of the so called drive-reduction reward (Belluzzi and Stein 1977, for discussion see Panksepp 1986) social contacts may be rewarding due to the activation of relevant endogenous opioid systems. Their experimental approach to this issue was to analyze pharmacologically social attachement-seeking behavior and behaviors seen after social separation. Reactions to social isolation in young animals (distress syndrome with vocal-

ization as leading symptom), which are common in several species including humans, are reduced very effectively by exogenously administered opioid agonists in pups, young guinea pigs and chicks while exacerbated by the opiate antagonist naloxone (Hermann and Panksepp 1978, Panksepp et al. 1980). Naloxone counteracts contact-comfort in chicks (Panksepp et al. 1980). Low doses of morphine reduce social proximity-maintenance time in socially housed rats and guinea-pigs (Hermann and Panksepp 1978, Panksepp et al. 1979). The effects of naloxone on social contact-seeking were inconsistent, which, if the theory is correct, is to be expected: naloxone-treated animals may desire social contact but may obtain less satisfaction from social interactions (for discussion see Panksepp et al. 1980). The number of specific opiate binding sites in the brain have been reported to be enhanced in Swiss albino mouse strain and reduced in C57Bl/67 mice after long-term social isolation (Bonnet et al. 1976 and references therein). If receptor up-regulation is taken as an indication of an adaptive response to a functional deficiency of endogenous opioid substances and down regulation as a result of an opposite process then the pain- and morphine sensitivities in the two strains corresponded to the direction of experimental changes. Brief isolation in young rats enhances pain sensitivity and reduces the analgesic efficacy of morphine (Panksepp 1980, Panksepp et al. 1980) whereas sustained isolation enhances the responsivity to morphine (De Feudis 1978).

In dogs, in BALB but not in Swiss-Webster mice and in about 50% of Long Evans hooded rats, naloxone treatment disrupted certain elements of maternal behavior (Panksepp et al. 1980). The mother may be source of opioid-mediated reward in two ways: by inducing endogenous opioids through contact and by producing "exogenous" opioids (casmorphins) present in the milk (the access of ingested casmorphins to relevant targets is not a settled question) (Zioudrou and Klee 1978, Henschen et al. 1980, for discussion of latter issue see Terenius and Nyberg 1986, Wunderink et al. 1986). Exogenously administered opioid agonists are known to enhance prolactin and growth hor-

mone release; although the role of endogenous opioids in the physiological regulation of the release of these hormones in humans is doubtful (for discussion see Grossman and Rees 1983) it would be attractive to believe that in psychosocial dwarfism, which results from emotionally-induced deficiencies in growth hormone secretion (for discussion see Panksepp et al. 1980), the inadequate endogenous opioid release due to the lack of environmental social stimuli, is a likely factor in the pathomechanism.

Juvenile social play in rats, which is a major behavioral pattern from which adult social competence arises, is enhanced by morphine and diminished by naloxone treatment (Panksepp et al. 1985). Dominance-submission relations are also affected by opioid agonists and antagonists.

In psychopathy (DSM-III code 301.70), in addition to genetically determined predisposing factors, among the environmental factors the insufficient recriutment of endogenous opioids by social, parental stimuli in the early postnatal period may have an important contribution to the pathomechanism (Kulcsar, personal communication). These afflicted children would engage "abnormal" mechanisms to activate the endogenous reward circuit; these mechanisms, when fixed, may serve as a basis for developing antisocial behavior.

5.5.6.3. NEUROPEPTIDES, LEARNING AND MEMORY

The neuropeptides hitherto implicated in learning and memory processes are ACTH and related peptides, opioids, vasopressin, oxytocin and somatostatin (for review see De Wied and Jolles 1982, Koob and Bloom 1982, 1983, Kovacs and Telegdy 1982, Banks and Kastin 1986, Davis 1986). When discussing the effects of drugs or endogenous substances/mechanisms on learning and memory processes one must differentiate between effects on neural substrates/processes directly involved in memory formation and influences brought about by affecting variables such as arousal, motivation etc., which are not "intrinsic" i.e., not directly related to memory formation.

For such a distinction targets in anatomical as well as functional terms, should be defined. Technically, a substance affecting/involved in processes intrinsic to the memory is expected to influence primarily the consolidation and retrieval of conditioned responses both in aversively and appetitively motivated tasks; it should have long-term effect and should be effective/involved in some amnestic states or disorders with a primary impairment of cognitive functions.

ACTH and related peptides [the most extensively studied one is ACTH(4-10)], a fragment, which is devoid of adrenocortical effects] facilitated primarily the acquisition of aversively motivated conditioned behaviors but their delaying effect on the extinction, could also be demonstrated (for review see De Wied and Jolles 1982). They affect learning and, to a lesser extent, extinction in certain appetitively motivated tasks. They have a short-term effect; this effect, in all probability is due to an increased arousal state in certain limbic structures and consequently, enhanced attentive ability to and increased motivational value of external cues (Donovan 1978, Urban and DeWied 1978). An opioid link in these actions of ACTH-related peptides has also been suggested (De Vito and Brush 1984).

Exogenously administered nonpeptide opioid agonists injected post-training or pre-test, disrupt learning and performance in aversively motivated tasks; naloxone has an opposite effect under similar experimental conditions (for review see Koob and Bloom 1983).

Opioid peptides produced a confusing diversity of effects (see Koob and Bloom 1983). Peripherally injected β-endorphin, α-endorphin and Met-enkephalin (in rather low doses!) have been reported to delay the extinction in pole-jumping avoidance task whereas γ-endorphin and des-tyrosine γ-endorphin have been found to facilitate the extinction in the same test in rats (De Wied 1978, De Wied et al. 1978a,b). Opposite effects for α- and γ-endorphins on extinction have been reported in food-rewarded but not water-rewarded experimental learning situations (Koob et al. 1981). However, others found that peripherally administered enkephalins, synthetic enkephalin

analogues or β-endorphin impaired rather than facilitated acquisition/retention in aversively motivated tasks (for references see Koob and Bloom 1983). In the majority of cases, the effects cannot be characterized as opioid either because they cannot be antagonized by naloxone/naltrexone or the peptide fragment producing the effect is devoid of opioid activity (des-tyrosine γ-endorphin).

Until recently, vasopressin appeared to be a promising candidate as an agent affecting processes "intrinsic" to memory formation. A clearcut prolongation of extinction can be demonstrated in aversively motivated learning situations (De Wied et al. 1976, Van Wimersma Greidanus and De Wied 1976, for review see De Wied and Jolles 1982, Koob and Bloom 1982, 1983) and although this issue is less extensively studied, also in appetitively motivated learning (Bohus 1979, Hostetter et al. 1977, Koob and Bloom 1983). Vasopressin has a relatively long-lasting effect; furthermore, it is effective in counteracting a number of amnestic states and cognitive dysfunctions (Lande et al. 1972, Rigter et al. 1974, Bookin and Pfeiffer 1977, Oliveros et al. 1978, Asin 1980, Weingartner et al. 1981) although some uncertainties as to its clinical effectiveness have also been reported (Swaab et al. 1986). Vasopressin levels change with the age in some but not all areas (Swaab et al. 1986). In Alzheimer's disease arginin-vasopressin (AVP) levels have been found to be lower in the globus pallidus but not in other brain areas as compared to age-matched controls (Rossor et al. 1980c). In a less precisely quantified study, it has been indicated that fewer cerebrocortical areas contained detectable amount of AVP in demented patients than in controls (Nakamura et al. 1984). Decreased AVP levels in the CSF have been found in patients with primary dementia and parkinsonism but not in patients with cerebrovascular disease, multiple sclerosis, or dementia due to normal pressure hydrocephalus (Sorensen et al. 1983, Sundquist et al. 1983).

Recently, critical comments have been made as regards the importance of vasopressin in memory processes (Gash and Thomas 1983); the opposing views and arguments were confronted

directly in the same periodical where the original, criticizing article was published (De Wied 1984 versus Gash and Thomas 1984).

The effect of oxytocin on learning-memory processes is the opposite of that of vasopressin as demonstrated in aversively motivated learning situations (Van Ree et al. 1978a, for review see Kovacs and Telegdy 1982) i.e., it diminishes memory consolidation and retrieval.

In Alzheimer's disease and senile dementia of the Alzheimer type there is a significantly reduced amount of somatostatin in the cerebral cortex as compared to the brains of age-matched controls dying without a history of neurological or psychiatric illness or of intellectual impairment (Davis et al. 1980, Rossor et al. 1980b, Davis 1986 for review see Beal and Martin 1986). Other forebrain areas, although the somatostatin levels tended to be moderately lower (Rossor et al. 1980b), were not affected significantly. Reduced CSF concentrations of somatostatin have also been reported in patients with Alzheimer's disease (Francis and Bowen 1985). In the light of the high percentage of somatostatin-NPY colocalization in cortical neurons (Vincent et al. 1982, Jones and Hendry 1986) the cortical NPY loss, reported by Beal and his coworkers (Beal et al. 1986, Beal and Martin 1986) is not surprising; however, earlier findings (Allen et al. 1984) are at variance with these data. Cortical corticotropin releasing factor levels were also found reduced in Alzheimer patients (Bissette et al. 1985, De Souza et al. 1986). AVP was the only other neuropeptide for which altered brain level could be demonstrated (see above), but the alteration did not occur in the cortex. Other neuropeptides in the brain such as VIP (Rossor et al. 1980a) TRH, LHRH, substance P (Yates et al. 1983 but see Crystal and Davies 1982) CCK (Rossor et al. 1981) Met-enkephalin (Rossor et al. 1982) and neurotensin (Yates et al. 1985) were spared in Alzheimer-type dementia (for review see Beal and Martin 1986).

5.5.6.4. NEUROPEPTIDES IN PSYCHIATRIC DISORDERS

Although the localization of neuropeptides to brain areas regulating mood and affect is conspicuous, and the anxiolytic and antidepressant properties of morphine-like drugs have long been known (see Verebey et al. 1978), considerably more attention was paid to the role of neuropeptides in schizophrenia-related psychoses than affective disorders. Opioid peptides, cholecystokinin and, in a type-dependent fashion, somatostatin and vasoacitve intestinal polypeptide have been implicated in schizophrenia whereas somatostatin, TRH and opioid peptides in affective disorders.

In 1976 two groups showed that intracerebroventricular injection of β-endorphin elicited rigid immobility in rats (Bloom et al. 1976, Jacquet and Marks 1976). Bloom et al. (1976) relating this effect to schizophrenic catatonia proposed that an excess in central opioids might have a role in the pathophysiology of schizophrenia, while Jacquet and Marks (1976) by paralelling the very same effect with the cataleptic side-effect of certain neuroleptics, suggested that β-endorphin may have therapeutic properties as a neuroleptic.

Although closer analyses proved unequivocally that the rigid immobility produced by β-endorphin is not analogous either with neuroleptic-induced catalepsy or catatonia, the finding and its interpretations started the investigations in two opposite directions.

Following the strategy of measuring opioid activity rather than the levels of chemically defined substances in the CSF, Terenius and his coworkers (1976, Lindstrom et al. 1978) reported on elevated opioid activities in schizophrenics in a fraction, separated by gel-chromatography, designated as fraction I. Furthermore, activity levels in this fraction were reduced upon neuroleptic treatment. According to subsequent chemical analyses (for discussion see Terenius and Nyberg 1986) this fraction contains β-endorphin, dynorphin A, dynorphin A(1-8) and dynorphin B; however, their levels are 10 times less than it is predicted from the opioid activity measured in this fraction. Dupont et al. (1978) found no signifi-

cant alterations in liquor opioid activities of schizophrenic patients. Domschke et al. (1979) reported markedly elevated liquor β-endorphin levels in acute schizophrenics, while the concentrations tended to be lower in chronically ill patients. Others (Emrich et al. 1979) could detect no clear-cut changes in CSF β-endorphin levels in schizophrenia. Plasma β-endorphin concentrations showed no conclusive alterations in schizophrenics (Emrich et al. 1979, Brambilla et al. 1986). Furthermore, no differences were found in the β-endorphin levels between control and schizophrenic brains in any of the areas studied (Lightman et al. 1979). The so called "Leu^5-β-endorphin" theory of schizophrenia (Palmour and Ervin 1979, James and Hearn 1980) appears to be of dubious value (Hollt et al. 1979, Lewis et al. 1979). The opiate antagonist naloxone reduced hallucinations (Gunne et al. 1977) at least so in a subpopulation (Watson et al. 1978) of schizophrenic patients (for review see Berger et al. 1980).

The marked improvement of schizophrenic patients maintained on a milk- and cereal-free diet (Dohan and Grasberger 1973, Singh and Kay 1976), the relatively high occurrence of gluten-dependent enteropathy and schizophrenia, the significant similarity in serum amino-acid pattern in both celiacs and schizophrenics (Manowitz 1978) taken together with the finding that opioid substances are present both in wheat gluten and milk (Zioudrou and Klee 1978, Henschen et al. 1980, Terenius and Nyberg 1986) raises the possibility of contribution of nutritional opioid factors to schizophrenia in certain cases (for discussion see Wunderink et al. 1986), although again, the access of ingested opioids to relevant targets in the organism, remains to be established.

As to the "neuroleptic" line of research, a fragment of β-endorphin devoid of opioid activity, destyrosine γ-endorphin [β-endorphin(2-17)] has been reported to possess neuroleptic-like properties in some behavioral tests (De Wied 1978, De Wied et al. 1978b). Furthermore, from the finding that α-endorphin [β-endorphin(1-16)] and γ-endorphin itself [β-endorphin(1-17)] have opposite effects in certain behavioral tests (amphetamine-like and neuroleptic like respec-

tively) a number of psychotic symptoms were interpreted in terms of an "inborn error of the generation of β-endorphin fragments" (De Wied 1978). In the initial clinical trials des-tyrosine-γ-endorphin has been shown to produce a marked alleviation of psychotic symptoms in a high proportion of schizophrenics (Van Ree et al. 1978b, Verhoeven et al. 1979). Subsequent therapeutic attempts were met with mixed success; now that the number of patients treated by the same group is close to 100, some positive response tendencies still seem to prevail (Van Ree et al. 1986). Hebephrenics and paranoid schizophrenics responded best; the therapeutic effect was less in catatonics while residual type of schizophrenics and schizoaffective psychotics did not respond at all.

β-endorphin itself, although some positive therapeutic effects have been reported in a very early "open" study (Kline et al. 1977), produced no obvious improvement in schizophrenics (Berger et al. 1980). There were some positive findings with a synthetic enkephalin analogue, FK-33-824 (Jorgensen et al. 1979, Nedopil and Ruther 1979), but these therapeutic results were not quite conclusive (Jorgensen et al. 1979).

One of the most conclusive findings in the brain of schizophrenic patients was the reduction of the cholecystokinin levels in limbic areas (Roberts et al. 1983, for discussion see Nair et al. 1986, Tofferdell and Smith 1986). The "dopaminergic hyperactivity" theory of schizophrenia is still the most widely accepted one among the various proposals on the pathomechanisms of schizophrenia; as it was discussed previously, endogenous cholecystokinin may interact with the dopaminergic mechanisms at several levels. This interaction may be particularly relevant in the mesolimbic dopaminergic pathways where CCK-dopamine coexistence has been found in a considerable proportion of neurons (Hokfelt et al. 1980b). The direct CCK-dopaminergic interactions have been reviewed recently by Nair et al. (1986); the same review treated also the effects of CCK and related peptides in pharmacological tests used for neuroleptic screening. In general, CCK and related peptides have an inhibitory effect on dopaminergic mechanisms (for some details and exceptions see the "Extrapyramidal " paragraph of

this section) and give positive results in the majority of neuroleptic screening tests. Interestingly, the most fascinating proposal on the possible role of endogenous CCK-deficiency in the pathomechanism of schizophrenia is based not on a direct interaction of CCK- and dopamine-containing neuronal systems, although the possibilities of direct interactions were also taken into consideration (Tofferdell and Smith 1986). Areas where reduced CCK levels were found in schizophrenics (temporal cortex, amygdala, hippocampus) are known to project to the nucleus accumbens/vantral striatum which receive dopaminergic input from the ventral tegmental area. In the latter areas, dopaminergic input produces inhibitory, the inputs originating from the former sites produce excitatory effects as detected by electrophysiological methods. CCK-containing terminals exert an excitatory influence on the abovementioned excitatory projection neurons, which may use excitatory amino acid transmitters. Thus, according to the hypothesis put forward by Tofferdell and Smith (1986) CCK-deficiency (which may be the primary event) would reduce the excitatory input to an area where dopaminergic mechanisms are inhibitory, thereby the balance would be shifted towards dopamine-mediated events.

At the clinical trials with CCK and related peptides, although improvement has been observed in 8 out of 11 studies (Itoh et al. 1982, Moroji et al. 1982a,b, Van Ree et al. 1984, for review see Nair et al. 1986) the magnitude of improvement was very small. In all but one of the investigations the patients were maintained on neuroleptics.

Vasoactive intestinal polypeptide levels were found to be enhanced in the amygdala of type I (Crow et al. 1980) schizophrenics (Roberts et al. 1983) which might have a bearing on the positive symptoms. On the other hand, the loss of somatostatin in the hippocampus in type II schizophrenia is likely to relate to the negative symptoms (Roberts et al. 1983).

It should be mentioned that substance P levels were reported to be higher in the hippocampus in both type I and type II schizophrenics (Roberts et al. 1983); the possible implications of this finding are uncertain as yet.

Somatostatin levels have been found to be lower in the CSF of depressed patients than in normal controls (Gerner and Yamada 1982, Post et al. 1982, Rubinow et al. 1983); in patients studied longitudinally values were significantly lower in depressive compared with manic or euthymic phases of the illness (ref. in Post et al. 1982). No general correlation has been detected between the severity of depression and CSF somatostatin levels (Rubinow et al. 1983). However, CSF somatostatin concentrations were significantly and inversely related to CSF 5-hydroxy-indolacetic acid and norepinephrine concentrations, and suprisingly, to sleep duration (Rubinow et al. 1983). As it was apparent in the previous paragraphs, CSF/brain somatostatin levels are characteristically lower in diseases accompanied by disturbed cognitive function; disturbed cognitive function is a recognized manifestation of depression (ref. in Rubinow et al. 1983).

Terenius et al. (1976), Lindstrom et al. (1978), Terenius and Nyberg (1986) reported increased opioid activities in fraction I of CSF (see above) in manic phase of the illness as well as in endogenous depression. Others (Post et al. 1982) found no significant changes in the opioid activities or β-endorphin levels in the CSF of patients with affective illness as compared to controls. However, anxiety scores were reported to correlate positively with CSF opioid activities by both groups.

Examining plasma opioid activity in patients with bipolar affective disorders, Pickar et al. (1980) found mania associated with significantly higher levels than depression. Others, though some minute variations were also noted, could detect no conclusive changes in plasma β-endorphin levels of patients with primary or secondary affective disorder (Brambilla et al. 1986).

As to the clinical studies, naloxone has been reported to produce beneficial effects in mania in 50% of patients (Judd et al. 1978) but others did not see similar effects (Pickar et al. 1982). Positive result with systematically given β-endorphin was found in one-double-blind study (Gerner et al. 1980) which could not be corroborated by others (Pickar et al.

1981). A distinct improvement in endogenous depression was reported upon the administration of a synthetic enkephalin analogue, FK-33-824 (Krebs and Roubicek 1979).

TRH was reported to possess antidepressant effect in a number of experimental models used for antidepressant testing (Plotnikoff et al. 1972, Prange et al. 1978). In spite of initial promising data (Prange et al. 1972) on the beneficial effect of TRH in clinical trials, the general opinion now is less optimistic (Prange et al. 1978, Nemeroff et al. 1979).

5.5.7. MISCELLANEOUS EFFECTS OF NEUROPEPTIDES

Neuropeptides, notably opioids and cholecystokinin have been implicated in certain components of epileptic and postictal events (Frenk et al. 1977, Urca et al. 1977, Frenk 1983, Cain and Corcoran 1984, Iadarola et al. 1986).

A variety of neuropeptides have been shown to affect the development of the brain (for review see Handelmann 1985). The physiological relevance of these experimentally demonstrated effects remains to be established. In this context, it should be mentioned that several polypeptide growth factors as well as their receptors are present in the brain; their physiological function is obscure at present (for review see Herschman 1986, and also Chapter 8).

It is known (Stein et al. 1976) that the immune system is subject to some modulations by the brain. At the periphery, lymphocytes can be pointed out as potential targets for neurohormonally released peptides. Specific, non-opiate receptors for β-endorphin have been shown to be present on lymphocytes moreover, lymphocytes are capable of producing β-endorphin (Smith and Blalock 1981). Endogenous opioids and non-opioid relatives thereof stimulate a number of lymphocyte reactions (proliferative responses, natural killer activity) known to be crucial in natural resistance toward malignant cells and in the immediate host defense of viral and other infections (Gilman et al. 1982, Kay et al. 1984).

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CHAPTER 6

ENDOGENOUS PEPTIDES IN THE INTEGRATION OF GASTROENTEROPANCREATIC FUNCTIONS

J. MENYHART

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INTRODUCTION

Opinions as to the nature of the control of gastroenteropancreatic (GEP) functions have changed several times in the past, and especially markedly in the last two decades. Pavlov, in his ingenious neural control theory attributed an almost exclusive role to the nervous reflexes in coordinating gut functions. However, Pavlov's theory was practically swept away, when Bayliss and Starling discovered secretin in 1902, which also signaled the birth of a new branch of biological sciences: by generalizing their observations on secretin, and introducing the term hormone they became the founders of endocrinology. Due to the rapid acceptance of the idea of humoral regulation by the scientific community, the neural control theory was replaced by the humoral control theory. However, progress in the molecular field of gastrointestinal endocrinology remained disappointingly slow for long in comparison to that in other field of endocrinology. This was primarily due to the peculiar character of the endocrine system operating in the gut.

In the classical glandular endocrine system (GES), homologous populations of cells are grouped into discrete organs, the endocrine glands, whose function could relatively easily be studied by organ extirpation, the classical method of endocrinology. In the gut, however, endocrine cells of one type are singularly scattered amongst endocrine cells of other type, and amongst nonendocrine cells throughout an extended area, often along the entire length of the alimentary canal. Such an arrangement of cells is characteristic of the diffuse endocrine system (DES) first described by Feyrter (1938). For

obvious reasons, organ extirpation proved to be inapplicable for studying DES cells' functions, and more sophisticated approaches had to be applied to achieve this goal. Thus, it was not until 1961, that the first gut hormone, secretin was finally obtained in a chemically pure form. Since then, however, we have been witnessing an unparalleled progress in peptide chemistry, separation techniques and in microdetermination of peptides, which allowed the isolation, quantitation as well as tissular localization in individual cells and tissue extracts of peptides present in an infinitesimally low concentration. Due to this progress in methodology, the number of identified gastrointestinal peptides has substantially increased in recent years, and the product of most gut endocrine cells has also been identified (for details see Solcia et al. 1981). Owing to the progress outlined above, our view on gastrointestinal control mechanisms has taken a new shape which, in its present form, represents a compromise between the neural and humoral control theories. This view finds its origin in the recognition that both DES cells and nerves have their own share in integrating GEP functions, and that both endocrine and neuronal cells accomplish their function through producing and liberating humoral agents. The latter may act as true hormones, or as paracrine/neurocrine secretory products liberated from endocrine or neural cells that act on neighbouring structures by diffusion. The finely tuned control of gastrointestinal functions is ascertained by a precise interplay among these humoral agents of dual origin. In addition, in recent years, gut peptide hormones have been detected in a steadily increasing number in the CNS, especially in the brain. These brain-gut peptides seem to function as true or local hormones in the gut, indicating that the old and rigid distinction between neurotransmitters and hormones has become obsolete. It has also been recognized that, in the brain, some peptides of neural origin may behave as hormones by acting via the circulation, while some peptides originating from gut endocrine cells may act only locally, as the classical neurotransmitters do. Thus, the borderline between the neural and humoral (endocrine) controls also starts to become blurred, and the idea of

the unity of the body's control systems is gradually gaining acceptance.

At present, the number of peptides detected in the GEP system exceeds twenty. The majority of these peptides have been fully identified. The structure of a minority, however, is only partially known, or still entirely unknown. The latter include GEP peptides detected only immunochemically as well as those few whose existence was only postulated by their observed effects. In this chapter, mainly the gut peptides with an established structure and/or function are discussed, but mention is also made of those that presently are less well characterized. They are discussed in a succession determined by the site of their origin. However, the production of most GEP peptides is not restricted to a single region or organ within the GEP system. Consequently, a particular peptide is classified as gastric, intestinal or pancreatic if the density of its producing cells, or its immunochemical concentration is highest in the stomach, intestine, or in the pancreas, respectively. As to the nomenclature of peptide-producing cells in the GEP system, the propositions accepted at the 1981 Los Angeles convention are applied in this chapter. (For recent reviews on various aspects of gut peptides see Sundler et al. 1983, Go et al. 1984, Miller 1984a,b, Said 1984a, Tache 1984, Dockray 1987, Johnson 1987, Walsh 1987, Yamada 1987).

6.1. GASTRIC PEPTIDES

6.1.1. GASTRIN

Gastrin sequences determined by direct amino acid analysis and those derived from cDNAs have equally been reported and the structure of porcine preprogastrin (Yoo et al. 1982) as well as the organization of the human gastrin gene have also been determined (Wiborg et al. 1984). The 34 amino acid gastrin, also termed big gastrin (G-34), is the longest form hitherto recognized in various species whose primary structure displays significant interspecies sequence homologies

(Fig.6.1). Most of the other known forms of gastrin are C-terminal fragments of various length of G-34 which include G-17 (little gastrin), G-14 and G-13 (mini gastrin), G-6 (hexagastrin) and G-4 (tetrin). Two additional forms detected by radioimmunoassaying chromatographic plasma fractions have not been chemically identified. Of these "component 1", a somewhat larger molecule than G-34 probably is a precursor for G-34 (Rehfeld et al. 1974), whereas "big-big gastrin" (Yalow and Wu 1973) having an apparent molecular weight near the size order of serum proteins may be an artifact. Finally, G-5 or pentagastrin with a structure Boc-β-Ala-Trp-Met-Asp-PheNH$_2$ is one of the several synthetic gastrin products (Schon et al. 1978a,b) that is widely used for experimental and diagnostic purposes. Evidence has been presented indicating that at least some of the smaller forms may be generated from the larger ones (Dockray and Walsh 1975, Dockray et al. 1978b, Pauwels and Dockray 1982). It should be noted that G-17, the first form that was isolated from porcine antral tissue corresponds to the C-terminal 17 residues of G-34, however, the glutamyl residue in G-34 corresponding to the N-terminus of G-17 is transformed to pyroglutamate through cyclization (Gregory and Tracy 1964, Gregory et al. 1964, 1979b). Although the C-terminal end of G-34 seems to play a major role in its biological activity, the N-terminal tryptic heptadecapeptide fragment of G-34, termed NTG-34, was found to be the predominant immunoreactive form in human antral mucosa. NTG-34 is also present in the plasma and may have some biological function (Pauwels and Dockray 1982). All forms of gastrin that are

```
                                    10                                  20
h: pGlu-Leu-Gly-Pro-Gln-Gly-Pro-Pro-His-Leu-Val-Ala-Asp-Pro-Ser-Lys-Lys-Gln-Gly-Pro-
p:   -   -   - Leu -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
d:   -   -   -   -   -   -   -   -   -   -   -   -   - Leu-Ala -   -   -   -   -
r:   -   -   -   -   -   -   -   - Gln -   -   -   -   -   -   -   -   -   -   -
                                  Gln - Phe-Ile -   -   -   -   -           Arg

                                30                  34
h: Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Try-Gly-Trp-Met-Asp-PheNH2
p:   - Met -   -   -   -   -   -   -   -   -   -   -   -
d:   - Met -   -   -   - Ala -   -   -   -   -   -   -
r: Pro Met -   -   -   -   -   -   -   -   -   -   -   -
```

Fig. 6.1. Amino acid sequences of human (h), pig (p), dog (d) and rat (r) gastrin-34s. Dashes indicate residues identical to those in the human peptide

larger than G-6 may exist both in sulfated and nonsulfated forms, the sulfate group being covalently attached to the only tyrosyl residue, the sixth from the C-terminus (Dockray et al. 1979a). The nonsulfated and sulfated forms are collectively named gastrin-I (G-I) or gastrin-ns (G-ns) and gastrin-II (G-II) or gastrin-s (G-s) family of gastrins, respectively. The biological activities of the various molecular forms, sulfated or nonsulfated, are essentially identical.

Both bioassays (Loveridge et al. 1974, Bugat et al. 1976, Vigna and Grobman 1977, Gregory 1979) and radioimmunoassays (RIAs; Rehfeld et al. 1972, Dockray and Taylor 1977) have been elaborated for quantitating gastrin in various biological sources. However, difficulties may arise from crossreactivity with unwanted sequences of the antisera with inadequate region-specificity, (reviewed by Dockray 1978). Normal basal plasma concentration of gastrin was reported to range between 6 and 20 pmol/l, depending on the region-specificity of the antisera employed (Bloom and Polak 1980, Rosenquist and Walsh 1980).

Gastrin is principally localized in G cells that are most abundantly present in the antrum, but are also found in the upper intestine (Greider et al. 1972, Bloom and Polak 1979). Immunoreactive gastrin-containing cells were also detected in fetal pancreas (Larsson et al. 1977, Track et al. 1979). Concentrations of the various molecular forms of gastrin vary at different areas of the gut and according to the species investigated (Berson and Yalow 1971, Nillson et al. 1975, Vaillant et al. 1979a). G-34 is the main form in the plasma, but the latter also contains G-17, G-14, component-1 and NTG-34. Tissues in general contain much more sulfated forms than the plasma.

Stimulation of gastric acid secretion, or more correctly, the postprandial control of gastric acid secretion is the main and the best-established action of gastrin, and perhaps the only one that is of physiological importance (McGuigan et al. 1971, Walsh and Grossman 1975, Berkowitz et al. 1976). In this respect, the larger forms are more effective than the smaller

ones, probably due to a faster biodegradation of the latters, especially tetrin. The gastrin-induced acid response appears to require an interaction among several humoral agents (Soll and Walsh 1979) and also the contribution of multiple neural mechanisms mediating both stimulatory and inhibitory cholinergic impulses, mainly via the vagus nerve (Debas et al. 1975, 1976, Walsh and Grossman 1975, Lam and Lai 1976, Impiccatore et al. 1977, Hirschowitz and Gibson 1978a,b, 1979, Schafmayer et al. 1978, Taylor et al. 1979), but the participation of β-adrenergic (Stadil and Rehfeld 1973, Kronberg et al. 1974, Jarhult and Uvnas-Wallensten 1979) and dopaminergic mechanisms (Uvnas-Wallensten et al. 1978) has also been suggested. In man, the cholinergic mechanisms are predominantly inhibitory in character (Grossman 1979). In most mammals, a majority of gastrin released postprandially originates from the gastric antrum, but in some species the duodenum is an additional gastrin source. The acid response is inhibited by several gut hormones and certain prostaglandins but is potentiated by histamine. The natural products of protein digestion are the main stimuli for gastrin release (Elwin 1974, Richardson et al. 1976), although stomach distension inducing a receptor mediated reflex mechanism is also an effective stimulus (Debas et al. 1975, Schiller et al. 1980). Gastrin release can be enhanced by hydrocortisone, calcium and gut peptides, certain prostaglandins, and by high intragastric H^+ concentrations.

Mainly in animal experiments and in pharmacological doses, gastrin was also shown to elicit biological responses different from gastric acid stimulation. These include a trophic action directed on the epithelial cells both in the gut and the pancreas, effects on gut smooth muscles, and a stimulation of various gut secretory products.

Both high and low affinity binding sites for gastrin have been reported to exist (Baur and Bacon 1976, Lewin et al. 1976, Soumarmon et al. 1977, Takeuchi et al. 1979a,b).

G-34 is cleared from the circulation more slowly than are the smaller forms, and all forms are cleared more slowly in humans than in dogs. A multiorgan system appears to participate in the elimination of various gastrin forms but the enzy-

matic mechanisms involved in gastrin degradation remain to be elucidated.

Hypergastrinemia is associated with a number of pathological conditions, such as gastrinomas (gastrin-producing tumors), primary hyperfunction of the antral G cells, achlorhydria (gastric acid inhibits gastrin release), and the Zollinger-Ellison syndrome (Zollinger and Ellison 1955). The latter is characterized by a tumorous growth of non-beta pancreatic islet cells secreting G-17 and G-34 in excessive amounts (Gregory et al. 1969). Stomach is the only organ affected by hypergastrinemia often responding to this condition with developing severe forms of peptic ulcer disease. Chronic renal failure, the rarely occurring primary hyperplasia of the G cells and multiple endocrine adenomatosis (MEA) are also associated with hypergstrinemia. On the other hand, primary G cell deficiency is practically non existent (for reviews on various aspects of gastrin see Wunsch 1984, Walsh 1987).

6.2. PANCREATIC PEPTIDES

6.2.1. PANCREATIC POLYPEPTIDE

Pancreatic polypeptide (PP), a member of a family of related peptides that also includes peptide YY (PYY) and neuropeptide Y (NPY), was first discovered in chicken pancreas (Kimmel et al. 1968) and subsequently in pancreatic extracts of several mammalian species (Lin and Chance 1972, Floyd et al. 1977). The primary structures of PPs derived from various species are shown in Fig.6.2 (for details and references see Chance et al. 1979, Meyers and Coy 1980a, Walsh 1981, Mutt 1983). A detailed analysis of the organization of the human PP gene as well as the isolation of a cloned cDNA-encoding human PP have also been accomplished in recent years (Leiter et al. 1985, Takeuchi and Yamada 1985).

The 95 amino acid human PP precursor (M_r 10,432; Leiter et al. 1984), and also the precursors of several other mammalian PPs have been shown to give rise to stable icosapeptide pro-

```
                                                  10                  20
h:   Ala-Pro-Leu-Glu-Pro-Val-Tyr-Pro-Gly-Asp-Gln-Ala-Thr-Pro-Glu-Gln-Met-Ala-Gln-Tyr-
p/c:  -  Ser  -  -  -  -  -  -  -  -  -  Asp  -  -  -  -  -  -  -  -  -  -
b:    -  -  -  -  -  -  Glu  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
o:    -  Ser  -  -  -  Glu  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
r:    -  -  -  -  -  -  Met  -  -  -  -  Tyr  -  -  His  -  -  Arg  -  -  -
a:   Gly  -  Ser-Gln  -  Thr  -  -  -  -  Asp  -  Pro-Val  -  Asp-Leu-Ile-Arg-Pro-
                                                  30                  36
h:   Ala-Ala-Glu-Leu-Arg-Arg-Tyr-Ile-Asn-Met-Leu-Thr-Arg-Pro-Arg-TyrNH2
p/c:  -  -  Asp  -  -  -  -  -  -  -  -  -  -  -  -  -  -
b:    -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
o:    -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
r:   Glu-Thr-Gln  -  -  -  -  -  -  Thr  -  -  -  -  -  -  -
a:   Tyr-Asp-Asn  -  Gln-Gln  -  Leu  -  Val-Val  -  -  His  -  -
```

Fig. 6.2. Amino acid sequences of human (h), porcine (p), canine (c), bovine (b), ovine (o), rat (r) and chicken = avian (a) pancreatic polypeptides. Hyphens indicate residues identical to those in the human peptide.

ducts (Fig.6.3) corresponding to the C-terminal part of the precursor molecule, that could be exclusively localized by immunochemical means to PP-storing cells in the pancreas (Schwartz and Hansen 1984, Schwartz et al. 1984). The PP portion (M_r 4,200) of the common precursor proved to be well conserved among various mammals (see Fig.6.2), as compared to the icosapeptide portion (see Fig.6.3) though the C-terminal halves of the latter are also remarkably conserved. On the other hand, the avian PPs (aPP) differ rather significantly from their mammalian counterparts. The C-terminal hexapeptide sequence shared by most mammalian PPs (mPP) appears to be important for biological activity (Lin and Chance 1978).

```
                                                  10                  20
h: His-Lys-Glu-Asp-Thr-Leu-Ala-Phe-Ser-Glu-Trp-Gly-Ser-Pro-His-Ala-Ala-Val-Pro-Arg
c: Asp-Arg-Gly-Glu-Met-Arg-Asp-Ile-Leu  -  -  -  -  -  -  -  -  -  -  -  -  -  -
o: Asp  -  -  Gly  -  -  Asp  -  Leu  -  Cys  -  -  -  -  Ser  -  -  -  -
```

Fig. 6.3. Amino acid sequences of human (h), canine (c) and ovine (o) pancreatic icosapeptides. Dashes indicate residues identical to those in the human peptide

In general, mPPs have similar biological activities which, however, differ significantly from those of their avian counterparts. While mPP from a given species also elicits biological responses in other mammals, mPPs are practically ineffective in avian species, and vice versa. mPPs characteristically inhibit secretory processes mainly in the pancreas and also in the stomach (Greenberg et al. 1978a,b, 1979, Lin and Chance 1978, Taylor et al. 1978a,b, Parks et al. 1979), but they also affect smooth muscle activity in the gut with a consecutive decrease in biliary output into the duodenum that is accompanied by an increased gastric emtying and intestinal transit. Contractility of the lower esophageal sphincter (Rattan and Goyal 1979), DNA synthesis in the pancreas (Greenberg et al. 1977) and food intake are also affected by mPPs (Malaisse-Lagae et al. 1977). On the other hand, aPPs predominantly elicit metabolic effects with lipid metabolism as the main target (Floyd et al. 1977, Kimmel et al. 1968), although their trophic effects have also been postulated (Laurentz and Hazelwood 1979).

The mPP-containing cells have been identified as F or D1 cells depending on the species (Heitz et al. 1976, Gersell et al. 1979). In man, the pancreas appears to be the main site of PP production (Adrian et al. 1976), but the stomach and the upper small intestine also contribute to PP production in other mammals. PP-containing cells have been identified both in the endocrine and exocrine pancreas (Larsson et al. 1976b) with a distribution pattern just the opposite to that reported for glucagon (Gersell et al. 1979). In avian species aPP-containing cells are predominantly present in the exocrine pancreas (Larsson et al. 1974).

Postprandially, there is a huge increase in plasma levels of PP. Food components (mainly proteins), neural (mainly cholinergic/vagal) and hormonal factors (VIP, GIP, CCK, G34, cerulein), are all able to stimulate PP release. Available evidences indicate that the main physiological role of PP and of its postprandially elevated serum concentrations are to modulate pancreatic secretory response to feeding.

Although satisfactorily sensitive and specific bioassays are unavailable for PP, this peptide can be measured without serious difficulties by RIA. In mammals most measurements were performed with antibodies raised against bovine PP that react with mPPs but not with aPPs (Chance et al. 1979). By using this type of antisera, fasting values of PP in human and dog plasma have been found to range between 10 and 30 pmol/l, but significantly higher values have also been reported in humans (Schwartz et al. 1976, Adrian et al. 1977, Floyd et al. 1977).

Extremely high PP plasma levels have been detected in a number of pathological conditions such as certain mixed cell endocrine tumors of the pancreas (Polak et al. 1976, Bloom et al. 1978c, Schwartz et al. 1979),tumors consisting purely of PP cells (Bordi et al. 1977, Lundquist et al. 1978), PP cell hyperplasia (Larsson et al. 1976a, Larsson 1977), and diabetes mellitus (Floyd et al. 1977). On the other hand, PP release is markedly diminished or is entirely absent following total pancreatectomy or in conditions that are accompanied by severe pancreatic insufficiency (Adrian et al. 1979, Valenzuela et al. 1979). However, no specific adverse consequences of PP surplus or PP deficiency have been recognized so far.

6.2.2. PANCREATIC SPASMOLYTIC PEPTIDE

Pancreatic spasmolytic peptide (PSP) is a 106 amino acid peptide of M_r 11,698 having the following sequence:

pGlu-Lys-Pro-Ala-Ala-Cys-Arg-Cys-Ser-Arg-Gln-Asp-Pro-Lys-Asn-
Arg-Val-Asn-Cys-Gly-Phe-Pro-Gly-Ile-Thr-Ser-Asp-Gln-Cys-Phe-
Thr-Ser-Gly-Cys-Cys-Phe-Asp-Ser-Gln-Val-Pro-Gly-Val-Pro-Trp-
Cys-Phe-Ser-Pro-Leu-Pro-Ala-Gln-Glu-Ser-Glu-Glu-Cys-Val-Met-
Gln-Val-Lys-Ala-Arg-Lys-Asn-Ser-Gly-Tyr-Pro-Gly-Ile-Cys-Pro-
Glu-Asp-Cys-Ala-Ala-Arg-Asn-Cys-Cys-Phe-Ser-Asp-Thr-Ile-Pro-
Glu-Val-Pro-Trp-Cys-Phe-Phe-Pro-Met-Ser-Val-Glu-Asp-Cys-His-
Tyr. PSP was thus far isolated only from the porcine pancreas (Thim et al. 1985). The amino acids at positions 26 through 55 and 76 through 105 show sequence similarities to

several kringle regions of plasminogen and prothrombin, whereas the amino acids at positions 14 through 49 and 63 through 98 show pairwise identical residues indicating that these two domains may be derived from a common ancestral gene.

PSP inhibits gastrointestinal motility,thereby eliciting a spasmolytic action. This peptide also inhibits stimulated gastric acid secretion. The physiological role of PSP is unclear at present (for more detail and references see Thim et al. 1985).

6.2.3. SOMATOSTATIN

Structurally identical 14 amino acid somatostatins (SS14; Fig.6.4) of M_r 1540 have initially been identified in, and isolated from, the hypothalami of several mammalian species (Krulich et al. 1968, Krulich and McCann 1969, Brazeau et al. 1973, Burgus et al. 1973, Schally et al. 1975, 1976) as potent inhibitors of growth hormone release and of a multitude of other functions (hence the name paninhibin). From porcine hypothalamus an SS22 form (see Fig.6.4) has also been isolated (Bohlen et al. 1980). Subsequently, however, SS14s structurally identical with the hypothalamic peptides were also found in the pancreas of a number of species, including teleostean fish, angler-fish, pigeon (Speiss et al. 1979), guinea pig and also in the stomach of the latter species (Conlon 1984), indicating a high degree of evolutionary conservation of this peptide. SS14, also called SS-I may exist in a linear and a cyclic from, respectively. The linear form, also termed dihydrosomatostatin, represents a reduced form, whereas the cyclic form in which the cysteine residues at positions 3 and 14 are disulfide-bridged, represents an oxidized form of the peptide. Analysis of cloned cDNAs from various species have strongly indicated that a whole family of SS genes may exist (for details and references see Rutter and Shen 1984, Tavianini et al. 1984), though only a single SS14 form has been isolated from mammals. On the other hand, it was convincingly demonstrated that SS28, also termed SS-II is the predominant form

in mammalian intestinal mucosa (Baskin and Ensinck 1984, Baldissera et al. 1985). SS28 is a 14 amino acid extended form, and may be a pro-form, of SS14. It is also interesting to note that the amino acid sequences of SS14 and SS28 predicted from a cloned cDNA prepared from a pancreatic somatostatinoma (SSoma) and a medullary carcinoma, respectively, proved to be identical to amino acid sequences of the corresponding peptides isolated from porcine and ovine hypothalami and from porcine intestine (Rutter and Shen 1984). Apart from SS14, SS22 and SS28 forms have also been isolated from catfish pancreatic islets and porcine intestinal extracts, respectively (Oyama et al. 1980, Pradayrol et al. 1980). In 1984, the first glycolsylated SS was reported by Andrews et al. (1984). This was an SS22 glycopeptide isolated from the pancreatic extract of channel catfish whose amino-acid sequence differed in residues at positions 5 (Thr instead of Arg) and 19 (Arg instead of Ser) respectively, from that of the peptide reported by Oyama et al. (1980; see Fig.6.4).

The Brockmann organ of the teleostean fish *Lophius piscatorius* is an endocrine pancreas containing excessive amounts of SS. A cloned cDNA isolated

```
                                                  10                                  20
SS28 I : Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-
                                                  10                                  20
SS28 II: - Val-Asp - Thr-Asn-Asn-Leu-Pro - - - - - - - - - - - -
                                1                       10
SS22  :                   Asp-Asn-Thr-Val - Ser-Lys-Pro-Leu-Asn - Met - Tyr-
                                                        1
SS14 I :                                                - - - - - -
                                                        1
SS14 II:                                                - - - - - Tyr-

                                                  28
SS28 I : Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys
                                                  28
SS28 II: Tyr - - Gly - - - -
                                      20          22
SS22  : - - - Ser-Ser - Ala -
                                10                14
SS14 I : - - - - - - - - -
                                10                14
SS14 II: - - - Gly - - - -
```

Fig. 6.4. Amino acid sequences of mammalian brain and gut somatostatins (SS) (SS28 I and SS14 I), catfish pancreatic SS (SS22) and angler-fish SSs from the Brockmann organ (SS28 II and SS14 II). Hyphens indicate residues identical to those in SS28 I

from this organ was shown to encode a proSS containing at its C-terminal end a new SS14, termed SS14-II to distinguish it from SS14-I isolated from mammalian hypothalami and pancreatic tissues. SS14-II has been identified as a Tyr(7), Gly(10) derivative of SS14-I (for references see Morel et al. 1984). Attempts to detect SS14-II in mammalian neural or pancreatic tissues have failed thus far. On the other hand, in angler-fish Brockmann organ, both SS14-I and SS28-II have been detected. This new SS28 form differs from SS28-I in its N-terminal end, and its C-terminal 14 amino acid fragment is identical with SS14-II (for details and references see Morel et al. 1984). The primary structures of the hitherto discussed SS species are demonstrated in Fig.6.4 (for additional sequence data and species-dependent variations see Walsh 1987).

Amino acid substitution experiments revealed the residues whose presence at the proper position is required for biological activity (Holladay et al. 1977, Vale et al. 1978) and synthetic SS analogs possessing more advantageous therapeutic capability than the parent molecules have also been prepared (for details see Barnes et al. 1978, Meyers and Coy 1980b).

Processing enzymes capable of converting SS28 and other precursors into SS14 have been found both in hypothalamus (Lauber et al. 1979) and the islet tissues (Noe et al. 1978, 1979), but the biodegradation routes remain largely unknown. SS is cleared from the circulation with extreme rapidity and the kidneys appear to play a major role in SS removal.

In vivo and in vitro bioassays as well as RIA methods are equally available for measuring SS in biological fluids. However, due to several reasons (multiple molecular forms of SS, unstability of the molecules, insufficient recovery, non-specific plasma interferences, the likely existence of an SS carrier protein, etc.), measurement of SS especially in plasma still encounters serious difficulties. As a result, values reported for fasting human plasma concentrations scatter significantly (range: 17-274 pg/ml; Kronheim et al. 1978; for an improved RIA method see Arimura et al. 1978).

SS cells were identified as D cells, a particular type of endocrine cells within the GEP system. These cells are most abundantly present in the pancreas but also occur in the antral and duodenal mucosa of mammals (Polak et al. 1975). SS-like immunoreactivities (SS-LI) have been detected in gastric and intestinal mucosa throughout the entire small intestine, in the pancreas (McIntosh et al. 1978, Zyznar et al. 1979) and also in gut nerve fibers (Hokfelt et al. 1975). The relative abundance of SS-LI and the distribution of larger SS-LI forms in various gut regions vary from one species to another (for details see Chayavialle 1983).

Deserving the name paninhibin, SS is one of the most powerful inhibitors of the functions of the glandular and diffuse endocrine systems. Apart from inhibiting the release of the majority of GEP peptides, SS inhibits the effects of several GEP peptides on their target tissues. Moreover, SS also inhibits the interdigestive migrating myoelectric complexes, acetylcholine release and intestinal motility. It also affects the blood flow in the GEP system and inhibits fluid absorption, and the absorption of various sugars and amino acids from the intestine. In contrast to these, SS increases ileal sodium and chloride absorption.

Instead of being a circulating hormone, SS is more likely a local hormone acting in a paracrine fashion. Available evidence suggests that a tonic inhibition of the release of various hormones is the most likely physiological effect of SS.

Specific high-affinity binding sites for SS have been reported to exist, predominantly intracellularly, in gastric mucosal and pancreatic islet cells (Reyl and Levin 1982, Reubi et al. 1982). A certain proportion of the intracellular receptors becomes translocated to the plasma membrane upon glucose-stimulated insulin release (Draznin et al. 1982, Reyl and Lewin 1982) pointing to a peculiar kinetic behavior of SS receptors.

Local administration of fat and amino acids and local acidification of the stomach and duodenum stimulate SS-LI release from the pancreas and stomach. Glucagon, secretin, VIP, bombesin and pentagastrin stimulate, whereas Met-enkephalin, sub-

stance P and metacholine inhibit SS release (for recent reviews on gut SSs see McIntosh 1985, Walsh 1987).

Although D cells are frequently present in mixed endocrine cell tumors, SSomas consisting purely of D cells are relatively rare. Such tumors are characterized by high SS levels in the plasma and are often associated with low gastric acid secretion, diabetes mellitus, steatorrhoea, weight loss, gall stones, decreased insulin tolerance, and delayed gastric emptying. A few other endocrine and neural tumors also produce SS-LI, though ordinarily in small amounts only. Pathological conditions causally related to SS deficiency are presently unknown, though D-cell population may be reduced in nesidioblastosis, a condition associated with excessive insulin release.

6.2.4. PANCREASTATIN

Pancreastatin, a 45 amino acid peptide with the following primary structure:

Gly-Trp-Pro-Gln-Ala-Pro-Ala-Met-Asp-Gly-Ala-Gly-Lys-Thr-Gly-Ala-Glu-Glu-Ala-Gln-Pro-Pro-Glu-Gly-Lys-Gly-Ala-Arg-Glu-His-Ser-Arg-Gln-Glu-Glu-Glu-Glu-Glu-Thr-Ala-Gly-Ala-Pro-Gln-Gly.

It has been isolated and sequenced from porcine pancreas as a potent inhibitor of glucose-induced insulin release from isolated perfused pancreas (Tatemoto et al. 1986). The C-terminal portion of the molecule is important for biological activity.

Besides inhibiting insulin release, this peptide stimulates the release of somatostatin upon glucose stimulation from the perfused pancreas. It may be important in the regulation of insulin secretion, in the pathogenesis of diabetes mellitus and also in fighting the latter disease (for more details see Tatemoto et al. 1986, Efendic et al. 1987).

6.2.5. CHOLECYSTOKININ-RELEASING PEPTIDE

Cholecystokinin-releasing peptide (CCK-RP) is a 61 amino acid peptide having the following primary structure:
Gly-Asn-Pro-Pro-Ala-Glu-Val-Asn-Gly-Lys-Thr-Pro-Asn-Cys-Pro-Lys-Gln-Ile-Met-Gly-Cys-Pro-Arg-Ile-Tyr-Asp-Pro-Tyr-Cys-Gly-Thr-Asn-Gly-Ile-Thr-Tyr-Pro-Ser-Glx=Cys-Ser-Leu-Cys-Phe-Glu-Asn-Arg-Lys-Phe-Gly-Thr-Ser-Ile-His-Ile-Gln-Arg-Arg-Gly-Gly-Cys, that was recently isolated from rat pancreatic juice (Iwai et al. 1987). The sequence corresponding to residues 24 through 33 closely resembles those present in highly conserved regions of the pancreatic secretory trypsin inhibitors (PSTI) or Kazal type inhibitors:
Ile-Tyr-Asx-Pro-Val-Cys-Gly-Thr-Asx-Gly.

CCK-RP is a potent CCK-releaser, and consequently a potent stimulant of the pancreatic enzyme secretion. Physiologically, this peptide may act as a mediator of pancreatic enzyme secretion in response to dietary protein intake. CCK-RP is trypsin sensitive. When food proteins which serve as substrates for trypsin enter the intestine, they prevent inactivation of CCK-RP by trypsin. Speculating that CCK-RP may monitor the amount of food protein intake through a competition between endogenous (CCK-RP) and exogenous (food proteins) trypsin substrates in the small intestine, CCK-RP was also designated "monitor peptide". Rat CCK-RP may be one of the rat PSTIs or a related peptide. Available data suggest that Kazal type inhibitors may play a previously unrecognized multiple physiological role.

6.2.6. THYROTROPIN-RELEASING HORMONE

Thyrotropin-releasing hormone (TRH)-like immunoreactivities have been found in pancreatic, especially in islets extracts and also in gut extracts. TRH stimulates glucagon release, inhibits PP release and affects intestinal motility. Centrally administered TRH stimulates gastric acid secretion (Tache et al. 1980c). However, presently available data preclude to ex-

actly define the physiological role what TRH might play in the GEP system.

6.2.7. INSULIN

Although insulin primarily is a metabolic hormone and a growth factor (see Chapters 3 and 8), insulin, or more likely insulin-induced hypoglycemia affects a number of secretory functions in the GEP system, the effects being predominantly stimulatory in nature. It should also be noted that both parasympathetic/cholinergic and sympathetic neural mechanisms are involved in mediating the majority of the insulin-induced secretory effects.

Similarly, the primary effect of insulin or insulin-induced hypoglycemia on gastric tone and motility appears to be of stimulatory character which is mediated by cholinergic/vagal mechanisms. As to the effect of insulin on the motility of esophagus, duodenum and lower small intestine, both stimulatory and inhibitory effects have been reported, the stimulatory effects being mediated through a vagal mechanism. Colonic motility may be stimulated or inhibited by insulin depending on the species investigated. The fact, however, that insulin induces hypoglycemia, stimulates both parasympathetic and sympathetic mechanisms and the release of a whole array of GEP peptides, that all have their own effects on the gastrointestinal functions, presents substantial difficulties in assessing insulin effects on various GEP functions.

6.2.8. GLUCAGON

Similarly to insulin, glucagon is a hormone primarily involved in the carbohydrate metabolism (see Chapter 3) which, however, elicits characteristic effects also in the GEP system. Glucagon has an overall inhibitory effect on gastric secretion, which apparently is unrelated to its hyperglycemic effect and presumably is the result of its direct metabolic

action on gastric mucosal cells. In a majority of reported experiments, glucagon was shown to inhibit pancreatic secretion in various species. The peptide decreases gastric and increases intestinal mucosal blood flow. Most investigators also attribute a choleretic effect to glucagon which may, or may not be related to its hepatic blood flow-increasing effect. Glucagon inhibits gastric, duodenal and jejunal motility, and delays the propulsive movement. Its inhibitory activity on ileal movement appears to be less pronounced. Colonic motility is reduced, but the tenia coli are contracted by glucagon. It has a relaxing effect nearly on all sphincters in the gut. The mechanism of action of glucagon on gut smooth muscles is unclear at present, but neural mechanisms do not seem to play a major role.

6.3. INTESTINAL PEPTIDES

6.3.1. CHOLECYSTOKININ

Cholecystokinin (CCK) and pancreozymin (PZ), formerly thought to be separate entities, have been identified as a single peptide (Mutt and Jorpes 1968) and named CCK-PZ, or CCK for short referring to the gallbladder-contracting potential as its most characteristic effect. CCK is a member of the gastrin-cholecystokinin family of peptides to which the cerulein-like amphibian skin peptides also belong (see Chapter 13).

CCK exists in multimolecular forms that differ in the length of the peptide chain: molecules with 4 (CCK-4), 5 (CCK-5), 8 (CCK-8), 22 (CCK-22), 33 (CCK-33), 39 (CCK-39) and 58 (CCK-58) residues have been hitherto identified (Fig.6.5) either in the intestine or in the brain of various mammalian species indicating that CCK is also a gut-brain peptide (Mutt and Jorpes 1968, Mutt 1976, Dockray et al. 1978a, Eysselein et al. 1982, Morley 1982, Tatemoto 1983, Tatemoto et al. 1984b, Walsh 1987). The human CCK gene encodes a 115 amino acid prepro-form containing several common residues with the gas-

trin precursor (Takahashi et al. 1985, 1986). The CCK gene is present as a single copy gene within the haploid human genome suggesting that the same gene is expressed both in the gut and the brain. The assumed existence of CCK species larger than CCK-39 has become a reality when CCK-58 was isolated and sequenced both from intestinal and brain sources in a number of mammalian species (Eysselein et al. 1982, Eng et al. 1983; for a review see Walsh 1987). The porcine brain CCK-58 differs in the Val(2)-Gln(3)-Lys(4)-Val(5)-Asp(6) sequence as well as in the Ser(9), Val(32) and Met(34) residues from the canine intestinal peptide shown in Fig.6.5 (Tatemoto et al. 1984b).

The molecular forms smaller than CCK-58 correspond to the C-terminal fragments of various length of CCK-58. Gastrin peptides as well as members of the cerulein-like amphibian skin peptides have the same C-terminal pentapeptide sequences as CCK peptides have. Similar substances have been detected both in the gut and the brain of birds and some primitive vertebrates indicating a striking evolutionary conservation of these peptides (Dockray 1979a,b,c, Holmquist et al. 1979).

```
                                    10                                  20
h: Val-Ser-Gln-Arg-Thr-Asp-Gly-Glu-Ser-Arg-Ala-His-Leu-Gly-Ala-Leu-Leu-Ala-Arg-Tyr-
p: Ala-Val  -  Lys-Val  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
r:  -  -  Leu-Arg-Pro  -  Ser  -  -  -  -  Arg  -  -  -  -  -  -  -  -  -  -  -  -
d:  -  -  -  -  -  -  -  -  Pro  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
                                    30                                  40
h: Ile-Gln-Gln-Ala-Arg-Lys-Ala-Pro-Ser-Gly-Arg-Met-Ser-Ile-Val-Lys-Asn-Leu-Gln-Asn-
p:  -  -  -  -  -  -  -  -  -  -  -  -  -  Val  -  Met-Ile  -  -  -  -  Ser-
r:  -  -  -  Val  -  -  -  -  -  -  -  -  -  -  Leu  -  -  -  -  Gly-
d:  -  -  -  -  -  -  -  -  -  -  -  -  -  -  Val  -  -  -  -  -  -
                                    50      +                   58
h: Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-PheNH2
p:  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
r:  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
d:  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
```

Fig. 6.5. Amino acid sequences of human (h), porcine (p), rat (r) and dog (d) cholecystokinin-58s (CCK-58). The cross-marked tyrosine residue at position 52 is sulfated in all sequences. Other known forms of CCK represent the C-terminal fragments of various length of CCK-58: N-termini of CCK-39, CCK-33, CCK-8 and CCK-5 are Tyr(20), Lys(26), Asp(51) and Gly(54), respectively. Dashes indicate residues identical to those in the human peptide

Structural conditions essential for biological activity and for full potency of CCK peptides have been determined by Yajima et al. (1977), Bodanszky et al. (1978b), Gillessen et al. (1979) and by Willanueva et al. (1982). The smaller forms are more potent in vivo than are the larger ones but the formers are more rapidly destroyed in, and cleared from, the circulation in vivo. The CCK-8-like substance present in the brain, where larger forms including CCK-58 are also present, likely is identical to that in the intestine (Robberecht et al. 1979). CCK-58 appears to be the major circulating form in canine blood (Eysselein et al. 1987). Biosynthesis of various CCK forms is likely accomplished by enzymatic cleavage of the precursor and by fragmenting larger forms (Straus et al. 1978).

CCK-III detected in porcine intestinal extracts (Tatemoto 1980) is a structurally unidentified peptide with CCK-like biological activities whose biochemical properties, however, are different from those of other known CCK-species. Unique CCK-peptides not found in other species have been isolated from intestinal extracts of guinea pigs, an animal species renown for producing irregular peptides if compared to other species.

Under experimental conditions, CCK affects practically all elements of the GEP system. In the pancreas, CCK is to be regarded as a major hormonal regulator of pancreatic acinar cell functions probably through mobilizing one of the intracellular calcium compartments (Korc et al. 1979). CCK stimulates pancreatic enzyme and electrolyte secretion (Wormsley 1969) and also exerts an insulinotropic effect presumably by senzitizing pancreatic B cells (Szecovka et al. 1982). It stimulates somatostatin release (Ipp et al. 1977a,b, Fujita et al. 1979, Yamada et al. 1980) and exerts a trophic action on the pancreas (Folsch et al. 1978, Peterson et al. 1978).

Stimulation of gallbladder contractions and relaxation of the Oddi's sphincter are the most characteristic effects of the CCK-peptides (Takashi et al. 1982). CCK enhances bile flow in the liver (Shaw and Jones 1978).

In the gastrointestinal canal, CCK inhibits gastric acid secretion (it is a candidate for enterogastrone) and enhances the release of enteropeptidases (Konturek et al. 1972a,b, Gotze et al. 1978). By causing vasodilatation, CCK enhances blood flow in various gut areas (Guth and Smith 1976, Chou et al. 1977, Richardson and Withrington 1977). CCK also plays a role in controlling food intake through a central mechanism (Gibbs and Smith 1977, Della-Fera and Baile 1979). The effect of CCK on the smooth muscles in various gut areas may be direct (Yamagishi and Debas 1978, Fara et al. 1979) or neurally mediated (Behar and Biancani 1977, Stewart and Burks 1977). The involvement of receptor mechanisms has been demonstrated in a number of CCK actions (Jensen et al. 1980, Sankaran et al. 1982).

Various nutritional components (Go et al. 1970, Meyer and Jones 1974, Meyer et al. 1976), intraluminal calcium and magnesium (Holtermuller et al. 1976) and neural, mainly cholinerg mechanisms (Debas and Yamagishi 1978, Singer et al. 1980) all stimulate CCK release.

Due to several reasons, none of the presently available methods, RIAs and bioassays, has the sensitivity and sepcificity that would be required for exact quantitation of CCK-peptides. By using an improved RIA method (Lonovich et al. 1980, Byrnes et al. 1981, Walsh et al. 1982) fasting plasma values of CCK were estimated to be less than 0.2 pmol/l upon physiological stimulation, which is too low for a satisfactory stimulation of pancreatic enzyme secretion. Consequently it is probable that circulating CCK peptides should interact with other factors, perhaps neural reflexes to produce a normal postprandial stimulation of pancreatic enzyme secretion (Walsh et al. 1982).

I cells, the CCK-containing endocrine cells, have been identified throughout the entire duodenal and jejunal mucosa (Buffa et al. 1976, Buchan et al. 1978b), while another cell type distinct both from the G and the I cells, containing a peptide whose C-terminal tetrapeptide sequence is common both to gastrin and CCK was detected in the gastric antral, jejunal and the ileal mucosa.

Although CCK has been implicated in biliary (Hepner 1975) and celiac diseases (Low-Beer et al. 1975) as well as in pancreatitis (Harvey et al. 1973), in the absence of reliable RIA methods these assumptions require further confirmation.

6.3.2. SECRETIN

Secretin is a member of the glucagon-secretin family of peptides also including vasoactive intestinal polypeptide, gastric inhibitory and gastrin-releasing peptides, peptide HI as well as helospectin and helodermin, the latter two being a 38 and 35 amino acid peptide, respectively, isolated from G l i a m o n s t e r venom (see the review by Dockray 1987). The 27 amino acid mammalian peptides of about M_r 3,050 (Fig.6.6) present in the small intestine and probably also in the brain (Chang et al. 1985) share a high degree of sequence homology (Jorpes and Mutt 1961, Jorpes et al. 1962, Mutt et al. 1970, Carlquist et al. 1981, 1985, Shinomura et al. 1987) but differ significantly from the chicken peptide (Nilsson et al. 1980). A secretin-like molecule that differs in its N-terminal portion from authentic secretin has also been isolated from intestinal mucosal extracts (Tatemoto and Mutt 1978).

Chemically synthesized secretin (Bodanszky et al. 1966, Wunch 1972, Coy et al. 1982) has full potency and the same biological activities as the natural peptide (Begliner et al. 1982). The whole molecule is required for biological activities (Makhlouf et al. 1978). Secretin is unstable in aqueous solution (Jaeger et al. 1978).

```
                                  10                                  20
h: His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Glu-Gly-Ala-Arg-Leu-Gln-
c: - - - - - Leu - - - - - Tyr - Lys-Met - Gln-Asn - Gln-Val -
                                  27
h: Arg-Leu-Leu-Gln-Gly-Leu-ValNH2
c: Lys-Phe-Ile - Asn - MetNH2
```

Fig. 6.6. Amino acid sequences of human (h) and chicken (c) secretins. The pig/beef peptides are identical but differ from their human counterpart in Asp(15), while the dog peptide in Ser(16)

Secretin is a potent stimulator of pancreatic electrolyte and volume secretion and a weak stimulant of pancreatic enzyme secretion (Ward and Bloom 1974, Schaffalitzky de Muckadell and Fahrenkrug 1978, Schaffalitzky de Muckadell et al. 1978, 1979a,b). This peptide also stimulates the biliary output of water and gastric pepsin secretion (Gardiner and Small 1976, Jansson et al. 1978) but inhibits gastric acid (and gastrin) secretion (Henriksen et al. 1974, Vatn et al. 1974, Dalton et al. 1976, Chiba et al. 1980, Ruoff and Becker 1982). It may or may not have an insulinotropic effect (Lerner 1977, Fahrenkrug et al. 1978c, Begliner et al. 1982). The ability of secretin to evoke a hypertrophic response in the gut is uncertain (Johnson and Guthrie 1978) but it definitively affects the function of a number of intestinal smooth muscles (Hubel 1972, Valenzuela 1976, Wingate et al. 1978).

Neural mechanisms do not seem to participate in secretin-mediated effects (Lee et al. 1978a) while the involvement of specific membrane receptors and cAMP as a second messenger is highly probable (Domschke et al. 1976, Gardner et al. 1976, Milutinovic et al. 1976, Robberecht et al. 1977). Acidification of the upper small intestine especially the duodenum (Meyer et al. 1970) bile and bile salts (Osnes et al. 1978, Hanssen et al. 1980), but not food components (Boden et al. 1974, 1978, Rayford et al. 1978), are effective stimuli of secretin release.

The kidneys appear to play a major role in a metabolic, but not a urinary removal of secretin from the circulation (Lehnert et al. 1974, Curtis et al. 1976a,b, Fahrenkrug et al. 1978b).

Secretin is present in S cells that are distributed in a varying concentration throughout the entire length of the small intestine (Bussolati et al. 1971, Polak et al. 1971), while immunoreactive secretin-containing cells could be detected also in cultured fetal pancreas (Miller et al. 1978).

Less sensitive bioassays (Scratcherd et al. 1975) and more sensitive RIAs (Schaffalitzky de Muckadell and Fahrenkrug 1977, Tai and Chey 1978) are equally available for measuring

secretin. Since, however, the sensitivity of available RIAs ordinarily does not surpass the limit of 5 pmol/l (Boden et al. 1978), the low basal plasma concentration of secretin (0,1-1,4 pmol/l) does not allow us to follow its subtle but physiologically important changes by RIA.

Gastrinomas (Straus and Yalow 1977) as well as multiple endocrine tumors of the pancreas (Schmitt et al. 1975) have been demonstrated to be accompanied by high plasma levels of secretin. Attempts to demonstrate a reduced secretin output in duodenal ulcer patients have failed so far (Bloom and Ward 1975, Isenberg et al. 1977). On the other hand, an impeded secretin release upon duodenal acid stimulus may be causally related to the decreased bicarbonate output seen in patients with celiac sprue (O'Connor et al. 1977, Besterman et al. 1978a, Rhodes et al. 1978).

6.3.3. GASTRIC INHIBITORY PEPTIDE

Gastric inhibitory peptide (GIP) is also a member of the glucagon-secretin family of peptides that was originally isolated from partially purified porcine CCK (Brown and Dryburg 1971) as a 43 amino acid peptide having a close resemblance to its M_r 5,105 human counterpart, the only difference being in residues Arg(18) and Asp(36) (Moody et al. 1984). Primary structure of the human peptide is as follows:
Tyr-Ala-Glu-Gly-Thr-Phe-Ile-Ser-Asp-Tyr-Ser-Ile-Ala-Met-Asp-Lys-Ile-His-Gln-Gln-Asp-Phe-Val-Asn-Trp-Leu-Leu-Ala-Gln-Gln-Lys-Gly-Lys-Lys-Ser-Asn-Trp-Lys-His-Asn-Ile-Thr-Gln.

GIP is instabile in the plasma due to its extreme susceptibility to proteolytic degradation. The majority of plasma GIP corresponds to the above shown form but a larger species (M_r 8,000) is also present as a minor component. Immunoreactive GIP (IR-GIP) detected in tissue extracts appears to be a different molecular species (Bacarase-Hamilton et al. 1984) with a postulated physiological significance (Brown et al. 1975, Sarson et al. 1980).

The entire molecule seems to be required for full biological activity, although the N- and C-terminal portions of GIP may be responsible for different biological activities (Moroder et al. 1971, Taminato et al. 1977, Yajima et al. 1977).

Neither reliable bioassays nor truly sensitive RIAs have been elaborated in the past. Fortunately, however, the plasma levels of GIP are relatively high that enabled many investigators to measure GIP, in spite of the low sensitivity of available RIAs (Cataland et al. 1974, Morgan et al. 1978), but non-specific plasma interferences may affect the reliability of measured values (Kuzio et al. 1974, Bloom 1975, Falko et al. 1975, Creutzfeldt et al. 1976).

GIP was localized in K cells, a particular type of endocrine cells that are most abundantly found in the villus epithelium and the upper crypt cells in the small intestine (Buchan et al. 1978a). On the other hand, immunoreactive GIP could be demonstrated in varying concentrations in duodenal, jejunal, ileal and gastric antral extracts (Polak et al. 1973, O'Dorisio et al. 1976) as well as in pancreatic A cells and in glucagon-containing gut cells (Smith et al. 1977, Alumets et al. 1978, Leduque et al. 1982).

Inhibition of gastric acid and pepsin secretion as well as stimulation of intestinal secretion are the best-established actions of exogenous GIP (Pederson and Bloom 1972, Brown et al. 1975). Among other effects, GIP stimulates the release of glucagon (Fujimoto et al. 1979), pancreatic polypeptide (Adrian et al. 1978) and of pancreatic somatostatin (Ipp et al. 1977b) and inhibits intestinal water and electrolyte absorption (Helman and Barbezat 1977), the transmembrane traffic of sodium in the salivary gland (Dennis and Young 1978) and motor activities of the stomach and the lower esophageal sphincter (Brown et al. 1975, Sinar et al. 1978). While the candidacy of GIP for "enterogastrone", a postulated duodenal substance released by fat that is capable of inhibiting gastric acid output has been questioned (Maxwell et al. 1980, Yamagishi and Debas 1980), this peptide remains a promosing candidate for "incretin", a postulated intestinal substance enhancing glucose-stimulated insulin release (Brown and Otte 1978,

Creutzfeldt 1979, Ebert et al. 1979a,b, Mueller et al. 1982, Takemura et al. 1982). Since the insulinotropic effect, clearly the most important physiological action of GIP is highly dependent on glucose concentration, the terms glucose-dependent insulinotrôpic peptide and glucose-dependent insulin-releasing peptide (still abbreviated as GIP) were suggested to designate this peptide.

The efficacy of GIP-releasing and release-modulating stimuli, such as various food components (Cataland et al. 1974, Falko et al. 1975, Thomas et al. 1976, 1978), duodenal acidification, glucagon, somatostatin, insulin, gastrin and CCK (Brown et al. 1975, Ebert et al. 1977, 1979a,b, Sirinek et al. 1977) is species-dependent. The majority of GIP released by various stimuli originates from the upper small intestine (Thomas et al. 1977). The kidneys seem to play a major role in removing GIP from the circulation (O'Dorisio et al. 1977).

The pathological significance of exaggerated GIP responses to glucose observed in diabetes (Creutzfeldt and Ebert 1979), obesity (Creutzfeldt et al. 1978), chronic pancreatitis (Botha et al. 1978) and in duodenal ulcer disease (Arnold et al. 1978) is unclear at the present time. Apart from a few pathological conditions in which reduced GIP release occurs as a secondary phenomenon (celiac disease, the condition prevailing following bypass operations, etc.), no disease states that could be ascribed with certainty to deficiencies in GIP release have been recognised so far.

6.3.4. MOTILIN

Motilin (Mo) has hitherto been isolated only from porcine and canine small intestines as a 22 amino acid peptide of M 2,700 (Brown et al. 1973, Schubert and Brown 1974) but immunoreactive Mo has been demonstrated also in the cerebral tissue (O'Donohue et al. 1981, Chan-Palay et al. 1982). Amino acid sequences of the pig and dog peptides are as follows (residues in parentheses indicate differing amino acids in the dog sequence):

Phe-Val-Pro-Ile-Phe-Thr-Tyr(His)-Gly(Ser)-Glu-Leu-Gln-Arg(Lys)-Met(Ile)-Gln(Arg)-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln.

The entire molecule appears to be necessary for full biological activity (Itoh et al. 1978). The immunoreactive large molecular weight Mo detected in the plasma may be a promotilin, a precursor molecule (Bloom et al. 1979c).

No bioassays but relatively reliable RIAs are available for quantitating Mo in various biological fluids (Dryburg and Brown 1975, Bloom et al. 1976, Itoh et al. 1978, Lee et al. 1978b).

Following a long dispute, Mo cells, a particular type of endocrine cells have been approved at the 1980 Los Angeles conventions as a Mo-containing cell population that are most abundantly present in the duodenal and jejunal mucosa but also occur in the lower small intestine in some species. The ongoing dispute over the quantitative distribution of Mo cells in different gut areas (Bloom et al. 1976, Polak 1978) probably is due to the use of antisera of different quality recognizing different molecular forms of Mo existing in distinct cell types.

Since the earliest observations establishing Mo as an agent capable of inducing nonneurogenic gastric contractions upon duodenal alkalization (Brown et al. 1966), the role of Mo in influencing a wide spectrum of gut motility functions, directly (Strunz et al. 1975) or through neural mediation (Meissner et al. 1976, Itoh et al. 1978) has been repeatedly confirmed, though the actual effects display significant species-dependency (Ruppin et al. 1975, Strunz et al. 1976, Debas et al. 1977, Christofides et al. 1979b). The role of Mo in initiating migrating myoelectric complexes (MMC) preferably in fasting animals (Wingate et al. 1975, Itoh et al. 1976, Borody et al. 1982) remains, however, controversial (Bloom et al. 1982).Mo also stimulates pepsin secretion and increases pancreatic volume, electrolyte and protein output (Konturek et al. 1976a) as well as mucosal blood flow (Koch et al. 1976).

In fasting periods, Mo is phasically released and its plasma concentration changes accordingly (Itoh et al. 1978, Vantrappen et al. 1978, Poitras et al. 1980, Bloom et al. 1982).

The stimuli influencing Mo release act species-dependently. In man, duodenal acidification, ingested fat and water drinking (as an osmotic stimulus) rise, whereas duodenal alkalization, intravenous glucose, amino acids as well as secretin decrease the plasma levels of Mo (Mitznegg et al. 1976, 1977, Bloom et al. 1978d, Christofides et al. 1979a). It is interesting to note that, in human infants, there is a 10-fold rise in Mo plasma concentration immediately after birth, but parental feeding completely counteracts this rise (Bloom et al. 1979a). No organ is known today that would preferentially remove Mo from the circulation (Itoh et al. 1978, Shima et al. 1979).

Despite the numerous actions elicitable by Mo under experimental conditions, a clear-cut definition of its physiological function(s) needs further investigations.

Although several pathological conditions are known to be associated with elevated Mo plasma levels (see e.g., Besterman et al. 1978b, Bloom et al. 1978d, Lucas et al. 1979) the etiological role of the elevated Mo levels could not be established with certainty. Similarly, no conditions having its root in a hypoproduction of Mo have been established so far (for a review on motilin see Walsh 1987).

6.3.5. NEUROTENSIN

Neurotensin (NT) originally isolated from bovine hypothalamus (Carraway and Leeman 1973) but subsequently also from the intestinal extracts of several mammalian species and of chicken, proved to be a 14 amino acid peptide in all species hitherto studied. The mammalian peptides have identical amino acid sequences (M_r 1,673) while the avian peptide differs in three amino acid substitutions that are placed in parentheses in the following sequence:
pGlu-Leu-Tyr(His)-Glu(Val)-Asn-Lys-Pro(Ala)-Arg-Arg-Arg-Pro-Tyr-Ile-Leu (Carraway and Leeman 1975a, Carraway et al. 1978). NT bears no resemblance to other gut peptides but is strikingly similar to the Xenopsin group of amphibian skin peptides also detected in several vertebrate species (Carraway et al.

1982; see also Chapter 13). Multiple molecular forms including a large form, probably a proNT, have been suggested to exist both in the plasma and various tissue extracts. A peptide corresponding to the N-terminal 12 amino acids of NT and a smaller C-terminal NT fragment have been demonstrated in bovine intestinal and rat gastric mucosal extracts, respectively (Carraway and Leeman 1976, Carraway et al. 1978). A hexapeptide (Lys-Asn-Pro-Tyr-Ile-Leu), similar to the C-terminal end of NT, called LANT-6 (Carraway and Ferris 1983) and its Ile(2) homolog, termed neuromedin-N (Minamino et al. 1984b), have been isolated from chicken gut and the porcine spinal cord, respectively. Both high and low molecular weight forms of NT-like immunoreactivities (NT-LI) are present in the plasma. The high molecular weight form, probably corresponding to proNT, represents a large proportion of fasting plasma NT but its concentration remains unaltered upon various stimuli. In contrast to this, the smaller form corresponding in size to the natural sequenced peptide is present in a hardly detectable quantity in plasma but its concentrations rise significantly upon ileal stimulation.

The N-terminal portion of the 14 amino acid peptide is responsible for biological activity (Carraway and Leeman 1975b, Folkers et al. 1976, Rivier et al. 1977).

Reliable bioassays are unavailable for NT and the elaboration of adequate RIA methods has also encountered susbtantial difficulties. By using a somewhat improved RIA, fasting plasma levels of NT were estimated to be 10pmol/l in humans (Flaten and Hanssen 1982).

Approximately 80% of the total NT is present in the intestine and only the rest in the head region. Intestinal N cells were identified as NT-containing endocrine cells with highest density in the ileal mucosa (Frigerio et al. 1977, Polak et al. 1977). In humans, the highest concentration of NT-LI has been detected in ileal and colonic mucosal extracts and in extracts from the muscle layer.

NT was originally discovered as a potent vasodilatation-, hypotension-, and cyanosis-producing agent that also increases vascular permeability (Carraway and Leeman 1973). Its other

reported effects include the stimulation of the release of several adenohypophyseal hormones, glucagon and gastrin (Brown and Vale 1976, Carraway et al. 1976, Folkers et al. 1976, Ishida 1977, Ukai et al. 1977). NT differently affects motility in various regions of the gastrointestinal tract (Andersson et al. 1977, Rokaeus et al. 1977, Rosell et al. 1980). For instance, NT inhibits gastric and intestinal motor activity in dogs (Andersson et al. 1977, Blackburn et al. 1980). Centrally administered NT induces hypothermia, decreased gastric secretion, diminished mucosal blood flow and elicits a cytoprotective effect on gastric mucosal cells (Nemeroff et al. 1982).

Neural mediation seems to play a role in some effects of NT (Andersson et al. 1977, 1980) and specific binding sites have been described on the plasma membranes of responding cells (Kitabgi et al. 1977, Lazarus et al. 1977a,b). It is presently unclear, whether NT functions as a hormonal or as a paracrine agent.

Consumption of a mixed meal elevates the plasma level of NT (Mashford et al. 1978, Blackburn and Bloom 1979, Rosell and Rokaeus 1979) with fat as the most potent stimulus of NT release (Flaten and Hanssen 1982). In man, somatostatin infusion also stimulates NT release.

Although elevated NT plasma levels have been registered in a number of pathological conditions (MacGregor et al. 1977a,b, Besterman et al. 1978c, Bloom et al. 1978b), disease states that would be causally related either to an overproduction or to a deficient production of NT have not been published so far.

6.3.6. GUT GLUCAGON-LIKE IMMUNOREACTIVITY: GLICENTIN AND OXYNTOMODULIN

The failure of total pancreatectomy to completely eliminate glucagon immunoreactivity from the circulation of dogs was interpreted to mean that glucagon or a closely related substance(s) must be generated in organs outside the pancreas. The substances detected in the gut that react with antigluca-

gon antisera but are heterogenous and distinct from pancreatic glucagon in many of their properties (Tyler and Marri 1965, Bottger et al. 1973, Valverde et al. 1973, Moody et al. 1978) are most commonly named gut glucagon-like immunoreactants (GLIs) but the terms "gut glucagon-like immunoreactivity" (still GLI) and enteroglucagon (EG) are also in use.

Attempts to characterize gut GLIs in various species have led to the recognition of two distinct forms whose M_rs and pIs differed not only between each other but also from those of pancreatic glucagon. The larger form (M_r 7,000-12,000) was called gut GLI-I or large gut GLI, whereas the smaller form (M_r 3,000-3,500) gut GLI-II or small gut GLI. Upon further characterization of gut GLI-I from porcine intestinal tissues (Sundby et al. 1976), the molecule was first claimed to have 100 amino acid residues, hence came the name glicentin ("gli" for gut glucagon-like immunoreactant and "cent" for 100). More precise investigations, however, revealed that porcine glicentin correctly is a 69 amino acid peptide with the following primary structure:

Arg-Ser-Leu-Gln-Asn-Thr-Glu-Glu-Lys-Ser-Arg-Ser-Phe-Pro-Ala-Pro-Gln-Thr-Asp-Pro-Leu-Asp-Asp-Pro-Asp-Gln-Met-Thr-Glu-Asp-Lys-Arg-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-Lys-Arg-Asn-Lys-Asn-Asn-Ile-Ala [His(33) corresponds to the N terminus of glucagon-37/oxyntomodulin; Thim and Moody 1981a,b].

As it can be inferred from the primary structure, in the glicentin molecule the glucagon sequence (residues at positions 33 through 61) is bracketed at its both ends by the dibasic amino acid sequence, Lys-Arg that link the glucagon sequence to the N- and C-terminal extension peptides. A peptide corresponding to the C-terminal 37 residues of glicentin, termed glucagon-37 has been isolated from porcine (Bataille et al. 1982) and human intestinal tissues (Thim et al. 1984). The term oxyntomodulin is also used to designate this peptide referring to its stronger activity on adenylate cyclase in the acid-secreting fundic than in the antral portion of the rat stomach (Bataille et al. 1981). Moreover, strong evidence for

the presence of oxyntomodulin in porcine small intestine was independently presented by Holst (1982) who, however, called it enteroglucagon II, glicentin then being enteroglucagon I. It should also be noted that oxyntomodulin or glicentin is most probably identical with the peptide isolated from impure glucagon preparations by Tager and Steiner (1973) who referred to it as "proglucagon fragment" (for details see Jacobsen et al. 1977, Bataille et al. 1981). Oxyntomodulin is higly conserved among mammals. Human and hamster oxyntomodulins are identical as are the pig and cow peptides that differ from the former two in Lys(33) susbstituting Arg(33) in the human/hamster peptides. On the other hand, guinea pig oxyntomodulin differs more extensively from its mammalian counterparts, the differences being restricted to the C-terminal 17 residues of the molecule and are manifested in the following substitutions: Gln(21), Leu(23), Lys(24), Leu(27), Val(29) and Arg(33) if compared to the sequence shown above (for details and references see Conlon et al. 1985). Glicentin itself as well as the peptides corresponding to the N-terminal dotriacontapeptide and the C-terminal hexapeptide sequences of glicentin have also been detected in the porcine pancreas (Moody et al. 1980, 1981, Yanaihara et al. 1985).

Based on the presence of glucagon, glicentin and various glicentin fragments in the pancreas, a glicentin-glucagon conversion scheme has been suggested to operate in this organ through postsynthetic enzymatic cleavages at the two Lys-Arg sequences at positions 31-32 and 62-63, respectively, within the glicentin molecule yielding (1) glicentin-related pancreatic peptide (GRPP) corresponding to the N-terminal 30 amino acids of glicentin; (2) glucagon, and (3) the N-terminal glicentin hexapeptide. It is intriguing to note that the glicentin molecule so structured:

(GRPP)-(Lys-Arg)-(glucagon)-(Lys-Arg)-(hexapeptide)

is conspicuously similar to the arrangement described for porcine proinsulin (Chance et al. 1968):

(B-chain)-(Arg-Arg)-(C-peptide)-(Lys-Arg)-(A-chain).

These similarities may be fortuitous or may reflect the requirements for the enzymes responsible for postsynthetic

cleavages. It is worth mentioning that the glicentin segment extending from His(33) to Met(59) shows a significant sequence homology with secretin (see Fig.6.6).

The structures and the nucleotide sequences of the glucagon genes and the predicted amino acid sequences of encoded gene products have been determined in several species. Sequence analysis of human and hamster glucagon mRNAs revealed that in the predicted sequence of the biosynthetic precursor (preproglucagon) the signal peptide is immediately followed by the sequence of glicentin, whereas the remaining C-terminal portion contains two additional glucagon-like peptides, termed glucagon-like peptide 1 and 2, respectively, that are separated by a short spacer. The latter peptides show extensive homologies to glucagon suggesting that they were produced by triplication and subsequent sequence divergence of a single peptide. Human and hamster glucagons and glucgon-like peptides 1 are identical in structure, whereas glucagon-like peptides 2 derived from the two species differ in 3 substitutions. The preproglucagon-derived major proglucagon fragment produced in the hamster pancreas is the C-terminal sequence containing glucagon-like peptides 1 and 2. Available analytical data suggest that the cleavage of the majority of the human proglucagon molecules does not lead to the production of glucagon-like peptide 1.

The only extrahepatic tissue known to produce true glucagon, but not enteroglucagon, is the dog stomach, whereas an inverse relation exists in the intestine (Larsson et al. 1975, Holst 1977). Gut GLI concentrations increase from the duodenum downward with highest concentrations in the terminal ileum and the colon. Gut GLIs represent various forms, glicentin being present in the highest concentrations (Holst 1977). Detection in the rat brain of glucagon-like peptides similar to their intestinal counterparts indicates that these peptides also belong to the gut-brain axis (for various molecular forms of human glucagon-like peptides see George et al. 1986).

No bioassays are available for gut GLIs at present, and the attempts to elaborate specific and sensitive RIA methods have also encountered substantial difficulties so far. Thus, assay-

ing enteroglucagon still depends to a significant extent on antisera raised against pancreatic glucagon. By using the so-called substractive method, in which the value obtained with pancreatic (glucagon)-specific antisera is substracted from the value obtained with antisera reacting both with glucagon and enteroglucagon (Holst 1977), the fasting value for human plasma enteroglucagon was estimated to be about 20 pmol/l, that rose to about 40 pmol/l after a mixed meal (Besterman et al. 1978a). So far, assays employing glicentin-specific antisera have only been employed for tissue extracts, but not for plasma.

Due to various reasons, information on the biological activities and the physiological role of enteroglucagon are scanty in the literature. Since pancreatic glucagon also produces intestinal effects that are mediated through receptors for enteroglucagon, some effects of enteroglucagon may be roughly deduced from the intestinal effects of pancreatic glucagon (see section 6.2.8.). However, glicentin hardly elicits any characteristic glucagon effects (Moody et al. 1978), although enteroglucagon is a more likely candidate for a trophic agent in intestinal adaptation than is gastrin (Sagor et al. 1982). Until recently, however, no biological activities for any gut GLIs, except oxyntomodulin, have been clearly established, and thus their physiological roles are only speculative at present. Oxyntomodulin and its C-terminal octapeptide fragment were shown to potently inhibit gastric acid secretion through a cAMP-independent mechanism (Jarousse et al. 1985).

GLI present in porcine intestinal extracts competes with glucagon for liver plasma membrane receptors (Bataille et al. 1973, 1974). Orally or enterally administered glucose, while is inhibitory on the release of pancreatic glucagon, simultaneously is a potent releaser of larger enteroglucagon forms (Unger et al. 1968). Intraduodenally administered fat releases both glucagon and enteroglucagon, but only enteroglucagon is released following intraileal administration (Ohneda et al. 1975). Reactive hypoglycemia also stimulates enteroglucagon release (Rehfeld and Heding 1970; for recent data on the biological activities of GLIs see Drucker et al. 1987, Holst et al. 1987).

Enteroglucagonomas may be associated with a gross intestinal mucosal hypertrophy/hyperplasia, reduced intestinal transit and with a mild degree of fat malabsorption (Gleeson et al. 1971, Bloom 1972). Hyperphagia is also associated with hyperenteroglucagonemia. Plasma levels of enteroglucagon rise markedly when newborns start oral nutrition and require a functioning gut mucosa for the first time. In babies fed parenterally, plasma levels of enteroglucagon remain unaltered (Bloom et al. 1979b). In general, enteroglucagon appears to be elevated basally in states associated with mucosal damages, and postprandially in conditions associated with a gastrointestinal hurry. Presently, no conditions are known that could be causally related to enteroglucagon deficiency.

6.3.7. UROGASTRONE

Urogastrone (UG) isolated from human urine exists in two molecular forms: β and γ (Gregory 1975). β-UG is a 53 amino acid peptide with three disulfide bonds and with the following sequence:

Asn-Ser-Asp-Ser-Glu-Cys-Pro-Leu-Ser-His-Asp-Gly-Tyr-Cys-Leu-His-Asp-Gly-Val-Cys-Met-Tyr-Ile-Glu-Ala-Leu-Asp-Lys-Tyr-Ala-Cys-Asn-Cys-Val-Val-Gly-Tyr-Ile-Gly-Glu-Arg-Cys-Gln-Tyr-Arg-Asp-Leu-Lys-Trp-Trp-Glu-Leu-Arg (γ-UG lacks the C-terminus (Gregory 1975, Gregory and Preston 1977).

β-UG is strikingly similar to the mouse epidermal growth factor (EGF), also a 53 amino acid peptide (see Chapter 8), 37 out of their 53 amino acids being in identical positions. UG-like immunoreactivities (UG-LIR) including large forms probably representing a precursor have been identified in human saliva, plasma, urine, gastrointestinal juice and in milk (Gregory et al. 1979a, Hirata and Orth 1979a,b). Larger UG-LIRs could be converted to smaller forms with enhanced biological activity upon incubation with an arginine esterase from mouse salivary gland (Hirata and Orth 1979b), while tyrptic digestion resulted in a smaller form displaying an

EGF-like mitogenic activity. While the disulfide bonds represent significant structural requirements for full biological activity, the C-terminal residue and the C-terminal dipeptide sequence do not. A fairly specific RIA method is available for measuring UG in various biological sources (Gregory et al. 1977, Hirata et al. 1980).

In humans, UG-LIR-containing cells have been found in duodenal Brunner's glands and the submandibular glands whereas extractable UG-LIR in pancreatic, duodenal, jejunal, renal, submandibular and in thyroid extracts. However, the low concentrations of UG-LIR in these extracts are in contradiction with the high concentrations detected in human urine.

Back in the thirties, two separate entities, termed anthelone and urogastrone, were supposed to have been responsible for the observed ulcer healing and gastric acid inhibitory effects of urine (Sandweiss 1943). Subsequent investigations, however, identified a single molecule, now termed UG, as an agent responsible for both mentioned effects. UG has been shown to inhibit gastric acid secretion in several mammalian species (Gregory et al. 1978) and to promote premature eye opening in newborn mice as mouse EGF does (Gregory 1975). UG stimulates DNA synthesis, proliferation, ornithine and histidine decarboxylase activities as well as amino acid uptake in fibroblast cultures equipotently with EGF and the two molecules share common receptor sites on these cells (Holenberg and Gregory 1977). Different portions of the molecule appear to be responsible for the different actions (Hollenberg and Gregory 1978).

Very little is known of the UG-releasing factors or mechanisms. Plasma levels of UG-LIR do not rise after meals nor could a circadaian rhythm be established. Despite its several effects observed in the GEP system, the physiological regulatory roles of UG remained undecided up to the present. Currently no pathological conditions are known that could be ascribed to excess or deficient quantities of UG.

6.3.8. PEPTIDE YY

The C-terminally amidated structure is a common characteristic of many biologically active pepties. Tatemoto and Mutt (1978) have elaborated a novel chemical method for the detection of peptides with such structures. Using this method a whole array of novel peptides has been identified in various biological sources without knowing anything of their biological properties at the moment of isolation. Peptide YY (PYY, where P stands for peptide while the two "Y"s refer to the one-letter symbol of tyrosine forming both termini of the peptide) was isolated by this method from porcine intestine as a 36 amino acid peptide having the following primary structure: Tyr-Pro-Ala-Lys-Pro-Glu-Ala-Pro-Gly-Glu-Asp-Ala-Ser-Pro-Glu-Glu-Leu-Ser-Arg-Tyr-Tyr-Ala-Ser-Leu-Arg-His-Tyr-Leu-Asn-Leu-Val-Thr-Arg-Gln-Arg-TyrNH_2 (Tatemoto 1982, see also Tatemoto and Mutt 1980).

PYY shows a significant homology with neuropeptide Y (see below) and pancreatic polypeptide. PYY-containing cells, a particular group of endocrine cells are especially abundant in the distal intestine in a wide spectrum of the animal kingdom, and PYY coexists with glicentin immunoreactivity in these cells (Bottcher et al. 1984). Until recently, PYY was not detected in the CNS.

Although PYY was demonstrated to inhibit gastric acid and pancreatic exocrine secretion as well as gastric, gallbladder, jejunal and colonic motility, its physiological role still remains elusive (for more details and references see Adrian et al. 1985, Walsh 1987).

Pathological conditions that could be attributed to an over- or a deficient production of PYY have not been described so far.

6.4. NEUROPEPTIDES IN THE GASTROENTEROPANCREATIC SYSTEM

Peptides discussed in the previous sections are localized, exclusively or overwhelmingly, to particular types of endocrine cells in the GEP system. A majority of them were also detected in the CNS, especially the brain forming the group of the so-called gut-brain peptides. However, there also are peptides that are localised, primarily or exclusively, in neural elements of the GEP system that are functioning as true hormones, paracrine agents and/or as neurotransmitters/neuromodulators of the extended peptidergic nervous system within the gut. Of these, the peptides displaying some well-recognized non-transmitter functions within the GEP system will be briefly surveyed in the following paragraphs.

6.4.1. GASTRIN-RELEASING PEPTIDE

Gastrin-releasing peptide (GRP), a member of the family of bombesin-like peptides (see Chapter 13) is also termed mammalian bombesin because its C-terminal portion closely resembles that of bombesin, an amphibian skin peptide. GRP has been isolated and sequenced from gastric and gut mucosal extracts of several mammalian and one avian species (McDonald et al. 1979, 1980). A cDNA-derived sequence has also been described for the human peptide (Spindel et al. 1984). In a few cases, GRP sequences were also confirmed by chemical synthesis (Marki et al. 1981, Akaji et al. 1982). GRPs hitherto sequenced from various species proved to have 27 amino acid residues (Fig.6.7) but in the human 28, 23 and 10 residue varieties may also exist (for references see Orloff et al. 1984, Spindel et al. 1984, Walsh 1987). The GRP gene maps to chromosome 18 in the human genome (Lebacq-Verheyden et al. 1987, Naylor et al. 1987).

```
                                    10                                  20
h: Val-Leu-Pro-Ala-Val-Gly-Gly-Gly-Thr-Val-Leu-Thr-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His
p: Ala-Pro-Val-Ser - - - - - - - Ala - - - - - - - -
c: Ala-Pro-Val-Pro-Gly - Gln - - - Asp - - - - - - - - -
a: Ala-Pro-Leu-Gln-Pro - - Ser-Pro-Ala - - - Ile - - - - Ser -
                                  27
h: Trp-Ala-Val-Gly-His-Leu-MetNH2
p: - - - - - - -
c: - - - - - - -
a: - - - - - - -
```

Fig. 6.7. cDNA-derived amino acid sequence of human (h) gastrin-releasing peptide and chemically determined sequences of the corresponding pig (p), dog (d) and chicken (a=avian) peptides. Dashes indicate residues identical to those in the human peptide

In contrast to the birds and amphibia, where bombesin occurs in gut endocrine cells, GRP exclusively is of neuronal origin in the gut of mammals. Following peripheral administration in vivo GRP elicits gastrin and pancreatic enzyme release gallbladder contractions and also exerts an insulinotropic effect (Deschodt-Lanckman et al. 1976, Brown et al. 1980, Tache et al. 1980a), whereas it inhibits gastric acid and pancreatic secretion following central administration through a largely unknown mechanism (Tache et al. 1980a,b, Dubrasquet et al. 1982). The secretion of GRP can be increased by electric neuronal stimulation but not by feeding. GRP/bombesin may be an excitatory transmitter of enteric interneurons participating in the vagal release of gastrin (for more details on the function of GRP see Yajima 1983).

Thus far, no pathological conditions could be ascribed to abnormalities in the plasma levels of GRP. For an exhaustive discussion of GRP/bombesin see Dockray (1987).

6.4.2. GALANIN

During attempts aimed at purifying PHI (see below) and PYY, a novel 29 amino acid peptide with a C-terminal amidated structure has been isolated from porcine intestinal extracts whose primary structure was established to be as follows:
Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-His-Ala-Ile-Asp-Asn-His-Arg-Ser-Phe-His-Asp-Lys-Tyr-Gly-Leu-AlaNH_2

(Tatemoto et al. 1983b). The term galanin was coined to designate this peptide where "g" refers to its glycine N-terminus and "alanin" to its amidated alanine C-terminus, respectively.

Although the C-terminal tetrapeptide sequence of galanin is structurally similar to those of physalaemin (see Chapter 13) and susbtance P (see below and also Chapter 5), and its N-terminal hexapeptide sequence to that of proopiomelanocortin (see Chapter 5), galanin appears to belong to a hitherto unknown peptide family. In the gut, galanin is found exclusively in neural elements. Galanin-like immunoreactivities have been detected in the gastrointestinal tract in a wide range of mammalian species (Melander et al. 1985) as well as in the brain (for references see Tatemoto 1983, Tatemoto et al. 1983b).

Contractions of the fundic, ileal, colonic and the gallbladder muscles as well as a sustained hyperglycemia are the only biological activities of galanin hitherto recognised (Tatemoto et al. 1983b). Isolated rat ileum preparations can be used for bioassaying galanin. No pathological significance has been attributed to galanin so far.

6.4.3. VASOACTIVE INTESTINAL POLYPEPTIDE

Vasoactive intestinal polypeptide (VIP) was first isolated "by accident" as a side product of secretin purification from hog intestinal extracts (Said and Mutt 1970, 1972). Subsequently it was also isolated from intestinal extracts of a large number of mammalian species, from chicken intestine as well as from mammalian brain (for refrences see Mutt 1983, Eng et al. 1986, Dockray 1987, Walsh 1987). Amino acid sequences of mammalian VIPs were found to be identical, except guinea pig VIP that, similarly to chicken VIP, differs in 4 residues from other mammalian VIPs (Fig.6.8).

Although VIP was suggested to exist in multiple molecular froms, neither the larger nor the smaller forms have been sufficiently characterized so far. VIPs derived from tumors proved to be heterogenous in size and include several large

```
                                                  10                  20
h/b/p: His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys
g.p. : -   -   -   -   Leu -   -   -   Thr -   -   -   -   -   -   -   -   Met -
c    : -   -   -   -   -   -   -   -   -   -   -   Ser -   Phe -   -   -   -   -   -

                                                  28
h/b/p: Lys-Tyr-Leu-Asn-Ser-Ile-Leu-AsnNH2
g.p. : -   -   -   -   -   Val -   -
c    : -   -   -   -   -   Val -   ThrNH2
```

Fig.6.8. Amino acid seguences of human/bovine/porcine (h/b/p), guinea pig (g.p.) and chicken (c) vasoactive intestinal polypeptides. Dashes indicate residues identical to those in the h/b/p sequence

forms (Bloom et al. 1978c). The synthesis of VIP from an M_r 17,500 precursor molecule (proVIP) has been observed in a human neuroblastoma cell line (Obata et al. 1981). More recently, the structure and several characteristics of the human and rat VIP precursors and those of the human VIP gene have been reported (Itoh et al. 1983, Nishizawa et al. 1985, Tsukada et al. 1985). It was also demonstrated that the coding sequences for VIP and peptide PHM-27 (see below) are present in two adjacent axons in the human genome and that the latter two are cosynthesized from the same precursor (Bodner et al. 1985). Structural requirements for biological activity of the molecule have been thoroughly investigated (Bodanszky et al. 1973, 1974, 1977, 1978a, Bodanszky 1977).

Porcine VIP is rapidly cleared from the circulation (Domschke et al. 1978) but the mechanism of the elimination remains controversial (Kitamura et al. 1975, Konturek et al. 1977, Ebeid et al. 1978).

Although both bioassays (Fahrenkrug 1979) and receptor assays (Laburthe et al. 1977) are available for measuring VIP, RIA was the method of choice in most reported cases (see e.g., Dimaline and Dockray 1978, Long and Bryant 1982). By using an especially sensitive RIA a skew distribution has been reported for values of fasting plasma concentrations of VIP with a mode of 1,5 pmol/l and a mean of 2,1 pmol/l, while the upper end of the normal range was found to be 21 pmol/l (Mitchell and Bloom 1978).

VIP is now thought to occur throughout the entire GEP system. However, in contrast to earlier assumptions suggesting D1 cells as VIP-containing cells, presently available evidences are more consistent with a purely neural localization of this peptide (Gaginella et al. 1978, Larsson et al. 1978, Bishop et al. 1980). VIP is present in a conspicuously high concentration in neurons of the muscle layers of the intestinal wall, especially in the plexuses of Meissner and Auerbach (Jessen et al. 1979, 1980). VIP-containing nerves are especially numerous in the gallbladder wall, the pancreas, in the regions of practically all sphincters, around the openings of the urethers, urethra and vasa deferentia (Sundler et al. 1977, Larsson et al. 1978, Alumets et al. 1979) as well as in the celiac, superior and inferior mesenteric sympathetic ganglia. VIP is also present in cultured neuroblastoma and astrocytoma cells (Said and Rosenberg 1976, Hokfelt et al. 1977). Conspricuosuly high levels of VIP were also found in the human milk (Werner et al. 1985). VIP-like immunoreactivity (VIP-LIR) has been described in the human placenta, and the cord blood (Ebeid et al. 1977), the adrenal medulla and in the respiratory tract (Said and Mutt 1977, Uddman et al. 1978). VIP is concentrated within the pulmonary and peritoneal mast cells and within the platelets (Giachetti et al. 1978).

The wide spectrum of the general cardiovascular and metabolic effects of VIP are not discussed here. VIP causes a marked vasodilation in the gut and affect practically all functions of the GEP system, including the motility (Vagne and Troitskaja 1976, Rattan et al. 1977, Ryan and Ryave 1978, Fahrenkrug 1979), endocrine and exocrine secretory (Barbezat and Grossman 1971, Makhlouf and Said 1975, Konturek et al. 1976b, Krejs et al. 1977, Schebalin et al. 1977, Ipp et al. 1978) and the absorptive functions (see e.g., Krejs et al. 1980), the actual effects being, however, species-dependent to a significant extent. This is mainly due to substantial interspecies differences existing between the innervation patterns of the abdominal organs (Larsson et al. 1978). High affinity and specific VIP receptors operating through the stimulation of intracellular cAMP formation have been described to exist

in several areas of the GEP system (Klaevman et al. 1975, Said 1975, Amiranoff et al. 1978, Carter et al. 1978).

The plasma concentration of VIP remains unaltered following the consumption of a normal meal, but intraduodenal presentation of acid, sodium or osmotic loads as well as fasting and a mechanical stimulation of the gastrointestinal mucosa or the electric stimulation of the vagus nerve, all produce VIP release (Ebeid et al. 1977, Schaffalitzky de Muckadell et al. 1977b, Bloom et al. 1978a, Burhol et al. 1978, Mitchell and Bloom 1978, Galbo et al. 1979). Stimulus-released VIP originates from neural elements of the GEP system and the release process is neurally controlled to a significant extent (Schaffalitzky de Muckadell et al. 1977a, Edwards et al. 1978, Fahrenkrug et al. 1978a).

VIP-secreting tumors (VIPomas) are the principal pathological conditions associated with VIP hypersecretion and high plasma levels of VIP. Such tumors account for a majority of the manifestations of the watery diarrhea syndrome, or WDHA syndrome (WD stands for watery diarrhea, H for hypokalemia and A for achlorhydria). VIPomas may be of endocrine and nonendocrine origin that most often develop from the pancreas, but ganglioneuronomas, ganglioneuroblastomas, astrocytomas may also secrete a large amount of VIP. However, there are tumor-associated WDHA syndromes that are not mediated by VIP, whereas other disease states (ulcerative colitis, cholera, acute intestinal infections, etc.), though are accompanied by WDHA syndrome but are associated neither with tumors nor with elevated plasma levels of VIP. There are some indications that VIP act through a local release of prostaglandins into the intestine in cases of WDHA syndromes (for details see Go et al. 1984).

Pathological conditions that could be causally related to a deficient VIP release have not been described so far (for recent reviews on VIP see Said 1984b, Dockray 1987).

6.4.4. SUBSTANCE P AND SUBSTANCE K

Substance P (SP) belongs to the tachykinin group of peptides whose members are recruited both from mammalian and amphibian organisms. (For amino acid sequence data see Chapters 5 and 13). It is confusing, however, that the two tachykinins newly isolated from porcine spinal cord were named differently by independent investigators: one that proved to be identical with substance K (SK) was named neurokinin-α (NK-α or NKA) by Kimura et al. (1983) and neuromedin L by Minamino and associates (1984a), whereas the other was termed neurokinin-β (NK-β or NKB) by Kimura et al. (1983) and neuromedin K by Kangawa and coworkers (1983). They are closely related both structurally and pharmacologically and in fact, can be regarded as naturally occurring analogs of SP. SP (M 1,348) was first detected by Euler and Gaddum (1931) in the horse brain and intestine but its primary structure was established approximately 40 years later by Chang and coworkers (1971).

SPs purified from the bovine hypothalamus and the horse intestine were found to be structurally identical (Leeman and Carraway 1977, Carraway and Leeman 1979). Multiple molecular forms have been detected in canine duodenal extracts but not in the plasma (Nilsson and Brodin 1977). Structural requirements for biological activities of SP have been reported from several laboratories (Oehme et al. 1977, Rosell et al. 1977, Yanaihara et al. 1977).

The genes encoding SP and SK have been cloned and sequenced from the bovine brain (Nawa et al. 1983) and the tissue-specific nature of the gene expression has also been established (Nawa et al. 1984; for further details and refrences see also Folkers et al. 1984).

SP present in the plasma is of intestinal origin (Gamse et al. 1978). The liver and the kidneys appear to play a major role in removing SP from the circulation (Melchiorri et al. 1977, Campbell and Ward 1979). Cellular and subcellular SP-degrading systems have been described in several organs (Ward and Johnson 1978, Heyman and Mentlein 1978, Lee et al. 1979) and in cultured endothelial cells (Johnson and Erdos 1977).

Both bioassays and RIAs are available for measuring SP in biological sources, although the preparations of appropriate samples and of highly specific antisera may encounter substantial difficulties. Normal plasma concentration of SP may be about 9 pmol/l (Leeman and Carraway 1977).

A majority of immunoreactive SP (IR-SP) was preferentially detected in neural elements in the GEP system but some was also found in a subpopulation of 5-hydroxytriptamin-containing EC cells, a particular type of endocrine-like cells in duodenal and colonic mucosa (Nilsson et al. 1975). IR-SP is distributed throughout the gut with highest concentrations in the duodenum, especially the muscle layers and with relatively small amounts in the mucosa. SP release is under a cholinergic control (Uvnas-Wallensten 1978, Jaffe et al. 1982).

As to the biological activities of SP and tachykinins, the forthcoming discussion will be restricted to the effects manifested in the GEP system. Readers interested in more general tachykinin effects, mainly neural and vascular, are referred to Chapters 5, 10 and 13, respectively.

Of the tachykinin group of peptides most investigations were carried out with physalaemin, eledoisin and SP that share many common pharmacological properties. The main effects are directed on intestinal and vascular smooth muscles, the pancreas and the salivary glands. SP is a potent stimulator of the contractions of practically all smooth muscles in the gut with ileum as the most susceptible and stomach as the least sensitive organs (Erspamer and Melchiorri 1973, Erspamer et al. 1977, Mukhopadhyay 1978). The effects of SP directed on the gastric and pyloric muscles are mediated through complex and coordinated neural mechanisms (Lidberg et al. 1982). SP has a strong atropine-resistant vasodilatory effect with a consecutive hypotension and increased blood flow and increased vascular permeability (Erspamer et al. 1977, Melchiorri et al. 1977). SP inhibits pancreatic electrolyte and insulin secretions but stimulates pancreatic volume and enzyme secretion, intestinal electrolyte transport as well as glucagon release (Thulin and Holm 1977). The stimulatory effect of SP on intes-

tinal electrolyte transport is clearly associated with its action on SP receptor-linked Ca^{2+} channels in intestinal mucosal cells. It also stimulates secretory processes in the salivary and the lacrimal glands. Physiologically, SP appears to function as a neurotransmitter/neuromodulator substance but its other mentioned pharmacological effects are unlikely to be manifested at the concentrations prevailing in the circulation.

Specific SP receptor sites operating via cGMP mediation have been described in the pancreatic acinar cells (Albano et al. 1977, Gardner and Jensen 1980). Receptor studies on other organs have indicated the existence of two subclasses of SP receptors, termed SP-P and SP-E, respectively, where P refers to a preferential physalaemin and E to a preferential eleodisin sensitivity of the receptors involved in motility functions. There is a strong indication that SP and SK are physiological ligands for the SP-P and SP-E receptors, respectively. Since both types of receptors appear to be present in the gut, both SP and SK may play a physiological role in controlling gastrointestinal motility (for more details and references see Miller 1984b).

Although some pathological conditions are associated with elevated (e.g., intestinal carcinoid tumors; Hakanson et al. 1977, Powel et al. 1978) or decreased SP levels (e.g., Hirschprung's disease, Huntington's chorea; Powel et al. 1978), the pathological significance of the altered concentrations of SP has not been established so far.

6.4.5. PEPTIDE HISTIDINE ISOLEUCINE

Peptide histidine isoleucine (PHI) was first isolated from porcine intestinal extracts (Tatemoto and Mutt 1980, 1981) but subsequently also from other mammalian sources. In the term PHI, P refers to peptide, H to the histidine N-terminus and I to the amidated isoleucine C-terminus of the peptide, respectively. All PHIs hitherto isolated from mammalian species had 27 amino acid residues (PHI-27). The human peptide has a

$MetNH_2$ C-terminus (PHM). Its primary structure is as follows: His-Ala-Asp-Gly-Val-Phe-Thr-Ser-Asp-Phe-Ser-Lys-Leu-Leu-Gly-Gln-Leu-Ser-Ala-Lys-Lys-Tyr-Leu-Glu-Ser-Leu-$MetNH_2$. The corresponding bovine and porcine peptides, termed PHI or PHI-27, differ from their human counterpart in Tyr(10) (bovine), Arg(12) (porcine) and $IleNH_2$(27) (both), respectively (Tatemoto and Mutt 1981, Carlquist et al. 1984, Tatemoto 1984).

PHI/PHM is a member of the glucagon/secretin family of peptides (other name:VIP/PHI family) and its presence was also demonstrated in porcine brain (Tatemoto et al. 1983a). PHI and VIP may act through common receptors and also have similar cell localisation (for references see Hoshino et al. 1984, Tatemoto 1984, Yanaihara et al. 1984). In the gastrointestinal tract immunoreactive PHI (IR-PHI) has been detected mainly in nerves of the lamina propria, the submucosa and the muscle layer (for references see Tatemoto 1984) where it is frequently colocalised with IR-VIP (Yanaihara et al. 1983). The analysis of cloned cDNAs has indicated that PHI and VIP are cosynthesized from a common precursor in the intestine (Christofides et al. 1983, Itoh et al. 1983), but not in the stomach where a large molecule with purely PHI-like characteristics has been detected (Yiangou et al. 1985).

The biological activities of PHI/PHM manifested in the GEP system include vasodilation, the stimulation of insulin and glucagon release, pancreatic exocrine and intestinal fluid secretion as well as an inhibition of gallbladder contractions (for further details see Tatemoto 1984, Tatemoto et al. 1984a).

Several outstanding RIA methods have been elaborated and used for establishing tissue distribution of PHI/PHM and for detecting its various immunoreactive forms (for references see Yanaihara et al. 1984). The physiological and pathological significance of PHI/PHM remains unknown for the time present (for recent data on PHI/PHM see Miller 1984a, Moroder et al. 1985).

6.4.6. NEUROPEPTIDE Y

During attempts to purify PYY from porcine brain and gut, Tatemoto and associates (1982) have found a large amount of PYY/pancreatic polypeptide (PP)-like peptide that was subsequently identified as a separate entity. The novel 36 amino acid peptide termed neuropeptide Y (NPY), where Y refers to the amidated tyrosine C terminus (Tatemoto et al. 1982), together with PP and PYY apparently are members of a new family of structurally related peptides. The brain-derived porcine peptide has the following amino acid sequence:
Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Leu-Asn-Leu-Ile-Thr-Arg-Gln-Arg-TyrNH_2.

NPY-like immunorectivity (NPY-LIR) is widely distributed both in the CNS and the peripheral nervous system. Especially dense networks of NPY-containing nerve fibers have been detected in several organs including the intestine (for references see Tatemoto 1983, Emson and De Quidt 1984). There are indications that NPY and PYY may be mutually exclusively localized in nerve cells and endocrine cells, respectively, and may represent distinct neural and intestinal forms of a fundamentally identical entity. NPY coexists with catecholamines both in the central and peripheral neurons and there are other evidence indicating that NPY and adrenergic functions may be closely related (for more details and references see Emson and De Quidt 1984 and also Chapter 5).

Apart from numerous established extragastrointestinal effects, NPY also performs circulatory and motility functions in the gastrointestinal tract and inhibits stimulated pancreatic secretion. It is, however, fair to say that the exact nature of the physiological role of NPY remains to be elucidated (for more recent data see Stanley et al. 1986).

Apart from sporadic observations implicating NPY as a hypertensive agent in patients with phaeochromocytoma (Emson et al. 1984), and the finding that in the caudate nucleus NPY content increased in parallel in patients with Huntington's chorea

(Aronin et al. 1983), we have no reliable information that would causally link NPY to any known pathological conditions.

6.4.7. BOMBESIN-LIKE PEPTIDES

Besides tachykinin- and caerulein-like peptides bombesin-like peptides (BLPs) constitute the third major group of amphibian skin peptides that also has a mammalian counterpart, namely gastrin-releasing peptide (see section 6.4.1.). Apart from bombesin, the 14 amino acid parent molecule (B14; for its structure see Chapters 3 and 5, and also Anastasi et al. 1972), the group includes 7 additional peptides (see Chapter 13).

Bombesin-like immunoreactivities (B-LIR) containing multiple molecular forms have been detected in extracts of various mammalian tissues (Erspamer and Melchiorri 1973, Wharton et al. 1978) including intestinal extracts (Walsh et al. 1979).

Although the spectrum of biological activities of all BLPs proved to be basically similar, occasionally marked species differences were found in the intensity of biological responses (Erspamer et al. 1975, Jensen et al. 1978b). Numerous structural properties of B14 required for biological activities have been determined (Brown 1978, Melchiorri 1978).

Both bioassays (Erspamer et al. 1972, 1979) and RIAs (Brown et al. 1978, Walsh et al. 1979, have been developed for measuring B14 and BLPs and radioreceptor assays have been successfully used for studying receptor sites in the brain (Moody et al. 1978b).

B-LIR, has been detected both in the gut and the brain of amphibians, birds and of mammalian species including man. In the gastrointestinal tract B-LIR may be localized either to endocrine-type cells (Lechago et al. 1978, Timson et al. 1979, Vaillant et al. 1979b) or to nerves (Dockray et al. 1979) depending on the species investigated, although available data are more consistent with a purely neural localization in human intestine (Bloom and Polak 1979).

B-LIRs detected in mammalian species could be extracted mainly from the gut and the brain. Of these large molecular forms and molecules with the size order of B14 have been equally detected (Villareal and Brown 1978, Walsh et al. 1979). B-LIRs in the stomach and intestine of mammals are differently distributed between the mucosa and the muscle layers (Dockray et al. 1979). B-LIR has also been demonstrated in rat plasma (Brown et al. 1978).

B14 itself, its C-terminal octapeptide fragment and both the large and small molecular weight B-LIRs proved to be potent stimulants of gastrin release and consecutive gastric acid secretion (Bertaccini et al. 1974, Melchiorri 1978, McDonald et al. 1979, Erspamer et al. 1979, Walsh et al. 1979). B14 stimulates pancreatic enzyme secretion, the release of PP and CCK and thereby gallbladder contractions, but inhibits motility both in the stomach and the upper small intestine (Melchiorri 1978, Taylor et al. 1979). Intravenously administered B14 inhibits liquid food intake (Gibbs et al. 1979), whereas centrally administered B14 produces hypoglycemia in rats probably through stimulating insulin release (Brown et al. 1977, 1979, Taminato et al. 1978). In contrast to somatostatin, a universal inhibitor of the release of gut hormones, B14 appears to be a universal stimulant of their release (Chatel et al. 1982).

Neural, probably vagal mechanisms are involved in some gastrointestinal effects of B14 (Hirschowitz and Gibson 1978b). Its hyperglycemic effect is associated with epinephrine and a secondary glucagon release (Brown et al. 1979). The effect of B14 on gastric acid secretion is due partly to its gastrin-releasing activity and partly to its direct effect on the acid-secreting gastric mucosal cells (Varner et al. 1980). Specific B14 binding sites demonstrated in dispersed pancreatic acini mediate cAMP-independent amylase release (Deschodt-Lanckman et al. 1976, Jensen et al. 1978).

Consumption of a meal regularly produces elevated levels of plasma B-LIR (Melchiorri 1978, Erspamer 1980), whereas neural or humoral cholinergic stimuli result in a parallel increase in gastrin-like and bombesin-like immunoreactivities.

No pathological conditions are known that could be associated with hyper- or hypoproduction of B-LIR in humans (see, however, Pert and Schumacher 1982).

6.4.8. OPIOID PEPTIDES

The use of morphine for treating gastrointestinal disorders goes more far back in the past than its use as an analgesic. The sensitivity of several isolated gastrointestinal preparations to the effects of morphine and endogenous opiates is also known for long and has been utilized for assaying opiate agonist activities. The existence of several related families of endogenous opioid peptides (OP) and at least five types of receptors on which they can potentially act are now an established knowledge that are more extensively discussed in Chapter 5 and partly in Chapter 9.

Out of five major opiate receptors ($\mu, \delta, \kappa, \sigma$ and ε ; Miller 1981) up to four receptor subtypes may coexist in some gut tissues. Morphine and many opiates with antidiarrheal activity preferentially act on the μ receptors.

The localization of opioid peptides and their receptors as well as the distribution of various opiate-cantaining nerves within the gut is rather well established (for details and references see Miller 1984b, Vincent et al. 1984 and Chapter 5). Due to their topography, the endogenous opioid-containing neurons may influence most gastrointestinal functions. Indeed, it is now clear that opiate drugs and endogenous OPs can alter intestinal motility, epithelial electrolyte transport, gut hormone secretion and neurotransmission in enteric ganglia (for references see Miller 1984b). As an overall effect, opiates cause constipation (Tokimasa et al. 1981). However, it is not really clear even now whether which of the many actions of opiates is the most important one in producing this effect.

The local effects of OPs on intestinal motility and electrolyte transport may be direct or indirect and are accomplished through events linked to different receptors, the effects on motility being mediated mostly by μ receptors whereas

those on the electrolyte transport primarily through δ receptors (for more details and references see Bitar and Makhlouf 1982, North 1982, Miller 1984b). Besides local effects, OPs can also regulate gut motility and electrolyte transport via central mechanisms (see e.g., Brown and Miller 1983, Galligan et al. 1984).

6.4.9. HEAD ACTIVATOR

It is interesting to note that a undecapeptide, termed head activator (Ha) originally isolated from the unicellular *Hydra attenuata* and the sea anemone *Anthopleura elegantissima* species (see Chapter 13) in which Ha accomplishes CNS-mediated morphogenetic functions could also be isolated from human brain and intestine, respectively (Bodenmuller and Schaller 1981). As to the function of Ha in humans, no convincing evidence has been presented so far.

6.5. CANDIDATE PEPTIDES IN THE GEP SYSTEM

The term candidate peptide refers to a number of GEP peptides with unknown or insufficiently characterized structure whose physiological significance is still debated (see e.g., Grossman 1974).

Chymodenin (CD) is assumed to have 74-76 amino acid residues (M_r 9,000) and a blocked N-terminus (Adelson et al. 1980). Partial sequence analysis revealed a pentapeptide sequence within the molecule that is homologous to the N-terminal regions of peptides belonging to the glucagon family. Since the molecule also contains the trypsin-sensitive sequence Asx-Asx-Arg-Arg, CD may be a precursor to one or more smaller peptides. In spite of the availability of adequately sensitive RIA methods the presence of interfering substances in the plasma did not allow thus far to establish CD as a circulating hormone. This peptide probably is localized to the

duodenum but its cellular localization remains undetermined for the time present (Adelson and Rothman 1975). CD causes a lysis of the zymogen granules in vitro and markedly and rapidly stimulates the secretion of pancreatic chymotrypsinogen without affecting other peptides in the pancreas or gut (Adelson and Rothman 1975). Even so, however, establishing CD as a true gastrointestinal hormone requires further investigations (for a review see Adelson et al. 1980).

V i l l i k i n i n (VK) is a postulated peptide having the capability of stimulating villous motility (pumping movement promoting lymph flow and agitating the chyme) that was detected in mucosal extracts of the upper small intestine (Kokas and Ludany 1934). Analytical data suggested a peptide with a M_r 2,000 (pig) or 2,500 (dog) and with free N- and C-termini as the active agent within the purified extracts (Kokas et al. 1980). However, VK should be more extensively characterized before it can be accepted as a single hormonal peptide of physiological importance.

I n t e s t i n a l p h a s e h o r m o n e (IPH). Evidence collected in various mammalian species strongly argued for the existence of an humoral agent of intestinal origin that would stimulate meal-induced gastric acid secretion (for references see Orloff et al. 1980). The postulated agent, termed IPH, could be detected in the portal blood, and the jejunum appeared to be its major source (Orloff et al. 1970b). Circumstantial evidence did not support its identity with any other known acid secretagog agents (Orloff et al. 1970a). Analytical data suggested a small peptide as the active agent (M_r~1,000-3,500; Orloff et al. 1980). Its relation to entero-oxynthin (see below) remains unclear for the time present.

E n t e r o - o x y n t h i n (EO). The term EO was first used by Grossman (1974a) to designate a postulated agent stimulating the intestinal phase of gastric secretion. Attempts to purify this agent from porcine intestinal extracts provided some supporting evidence for the existence of EO (Chandler et al. 1972, Orloff et al. 1977, Vagne 1980). Neither its structure nor its relation to IPH have been established so far.

G a s t r o n e s. Several body fluids and tissue extracts have been reported to inhibit gastric acid secretion and the term gastrone was coined to commonly designate the active ingredients present in the aforementioned biological sources. Since not a single gastrone has been structurally characterized to a sufficient extent gastrones are distinguished according to their tissue origin. Accordingly, sialogastrone (in the saliva), antrogastrone (in the antral juice), bulbogastrone (in the duodenal bulb), enterogastrone (in the small intestine), chylogastrone (in the lymph), urogastrone (in the urine) and vagogastrone (in the vagus nerve) have been distinguished.

The antral mucosa was indicated as the site of the production of antrogastrone (Hood et al. 1953, Code 1958). Attempts to purify antrogastrone from gastric juice have yielded two major fractions with gastrone activity (Fiasse et al. 1968, Glass and Code 1968, Lopes and Glass 1975). One, termed "gastrone A", was a large molecular weight component and resembled, or was identical with, sialogastrone and had only moderate biological activity. The other fraction, termed "gastrone B" was biologically more active and proved to consist of a mixture of glycopeptides with M_rs ranging between 3,000 and 9,000, respectively. Subsequently collected data suggested that "gastrone A" is related to sialo- and antrogastrone whereas "gastrone B" to bulbo- and chylogastrone, respectively. On the other hand, urogastrone (see section 6.3.7.) is claimed to be unrelated to any gastrones mentioned above.

Several observations have indicated the existence of a bulbar mechanism capable of inhibiting gastric acid secretion (Pavlov 1910, Andersson and Uvnas 1961, Andersson et al. 1967). Results of subsequent investigations were consistent with a humorally mediated effect, and the hypothetical agent was named bulbogastrone (Andersson et al. 1973, Nilsson 1974). In full accord with this assumption, extracts from the bulbar duodenal region elicited a gastrone action (Andersson et al. 1973). Although this bulbogastrone has not been further characterized, circumstantial evidence suggested its noniden-

tity with any other known gut peptides. It was also suggested that bulbogastrone would be a competitive inhibitor of gastrin (Nillson 1980).

Enterogastrone, another postulated acid-suppressing agent is thought to be present in the mucosa of the upper small intestine, from where it is released when digested fat establishes a contact with the mucosal surface. The identity of enterogastrone and urogastrone has not been reassuringly excluded, and the finding that the commonly used enterogastrone preparations are grossly contaminated with CCK also indicates that more studies are needed before a meaningful conclusion can be drawn on the existence of enterogastrone.

Inhibition of gastric acid secretion accompanied by a moderate reduction in the volume of gastric juice, but not its pepsin content, is the only effect attributable to gastrones so far. The mechanisms of their actions are unclear at present as is their exact physiological significance.As to their pathological significance, a defective operation of the bulbar mechanism (bulbogastrone) has been implicated as a direct (Shay et al. 1942, Johnston and Duthie 1964) or indirect causative factor in duodenal ulcer disease (Dragstedt 1942, Nilsson 1975; for more details on bulbogastrone see Nilsson 1980).

A n t r a l c h a l o n e. For the time present, antral chalone (the name originates from Jordan and Sand 1957a,b) remains a purely hypothetical agent that assumedly is formed in the gastric mucosa upon acidification of the antrum and suppresses gastric acid secretion. Despite some supporting evidence presented in favor of the existence of antral chalone, it should be stressed that the antral chalone concept has never gone beyond the sphere of a strictly physiological research and speculative argumentation, and in contrast to gastrones, the isolation and structural characterization of antral chalone has never been attempted. There is an increasing suspicion that antral chalone may be identical with the previously discussed "gastrone A", and somatostatin must also be considered as a serious contender for the role of antral chalone.

Gastrozymin (GZ), a pepsin secretion-stimulating principle of uncertain physiological importance (Blair et al. 1953, Vagne 1980) was partially purified from a starting material described by Mutt (1978).

Water and sodium absorption-inducing peptide is a postulated agent with a capability of stimulating water and sodium absorption from the duodenum of rats, that was separated from a GZ-containing fraction (Pansu et al. 1978, Pansu 1980). Its existence as a distinct entity remains to be confirmed.

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CHAPTER 7

ENDOPEPTIDES IN THE INTEGRATION OF IMMUNOLOGICAL FUNCTIONS. THE IMMUNOPEPTIDES

F. ANTONI, and J. MENYHART

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INTRODUCTION

Immunopeptides can be defined as peptides involved in immune processes either as active participants of physiological immune mechanisms, or passive targets of immune reactions. The above definition is purely functional, therefore the immunopeptides are no exceptions to the general rules of peptide chemistry, isolation, analysis and synthesis, which are described in other chapters.

It is a striking feature of the molecular logic and economy of living organims that certain proteins are synthesized as precursors of enzymes, hormones and other biologically active peptides. Subtly regulated, limited proteolysis liberates in some cases both an enzyme and a potent peptide from a single protein molecule, as it will be shown in connection with the peptides originating from the proteins of the complement system.

The number of peptides known to participate in immune processes is increasing steadily. Beside the progress of peptide chemistry, this is due also to the development of in vitro cell cultures, where the presence and effects of soluble mediators can be investigated under carefully controlled circumstances. Cell-cell interactions resulting in cell differentiation, or activation of specific cellular functions (like discharge of granules or chemotaxis, i.e., directed cell migration) are mediated by numerous peptides originating from the thymus (thymus factors) and leukocytes (lymphokines or interleukins). Isolation and characterization of these factors is in progress, and it seems that their precise definition is

a precondition of progress in this very dynamic field of immunobiology (for a current concept on the immune system see Nossal 1987).

7.1. PEPTIDES AS ANTIGENS

Molecular recognition is a fundamental, characteristic ability of the immune system. While molecular and cellular recognition of self components appears to be the basis of the organization of the structure and function of living material, recognition of non-self is the specific function of the immune apparatus. It is remarkable that the classics of immunology, Ehrlich and Landsteiner approached the immunological problems from the molecular point of view already at the beginning of this century. Thus, it was Landsteiner who demonstrated that antibodies to foreign proteins were directed actually against certain parts, i.e., peptides of the protein antigen.

While the number of peptides actively participating in immune reactions may be quite large, the number of peptide antigens is virtually infinite. As we attempt to show in this chapter, it is the task of biochemists, immunologists and chemists to identify and produce those peptide antigens which, as synthetic vaccines, may revolutionize the practice of active immunization, the most important measure of preventive medicine.

Foreign polypeptides are generally immunogenic, i.e., their parenteral administration evokes an immune response. Before discussing the relationship between the structure and antigenic properties of polypeptides in detail, the immune response is considered briefly. First, the responsiveness of any individual animal to a certain antigen is genetically determined by their (immune response) genes. Synthetic polypeptides have been very useful in the elucidation of this phenomenon by the identification of responder (producing an immune response) and non-responder strains of mice with respect to synthetic antigens (McDevitt 1968, McDevitt and Chinitz 1969, McDevitt and Tyan 1968).

Thus, the responsiveness of an experimental animal to the antigen tested is an important precondition of such studies. Another crucial point is the method and schedule of immunization. The efficiency of primary immunization is profoundly influenced by the application of Freund's adjuvant or other adjuvant material. In the case of small or medium- sized antigens the (covalent) binding to a macromolecular carrier can change (generally improve) the immunogenicity of the antigen. Finally, there are a number of test systems for the demonstration of the cellular and/or humoral immune response both in vivo and in vitro (precipitin reactions, complement fixation, delayed hypersensitivity, anaphylaxis, Arthus phenomenon, antigen-induced T cell proliferation in vitro, etc.). For these reasons both the method of immunization and the test system have to be selected carefully in order to reach valid conclusions about the antigenicity of polypeptides.

In the followings we discuss predominantly the in vivo, humoral inmmune response to polypeptides, and the interaction of polypeptide antigens with specific antibodies. Advances in peptide synthesis have allowed experimentation with polypeptide antigens tailored to the needs of specific investigations. These studies have supplied an impressive body of information, and they have shaped our present concepts of antigens and immunity. Very promising results with synthetic polypeptide vaccines are mentioned as outstanding examples of progress in this field. Another important breakthrough, the production of monoclonal antibodies is still having a great impact on the elucidation of antigen-antibody interactions.

7.1.1. AMINO ACID CONFIGURATION

While both proteins and synthetic polymers containing amino acids of the (natural) L-configuration evoke immune response, early experiments with synthetic polymers of D-amino acids suggested that these were very poor immunogens (Borek et al. 1965). However, subsequent studies have demonstrated that extremely low doses of D-amino acid polymers provoke an immune

response (Jaton and Sela 1968). It was suggested that relatively small doses of D-amino acid polymers induce immunological intolerance because of the slow elimination of the antigen.

7.1.2. PEPTIDES AS HAPTENS

Small, non-immunogenic peptides can provoke the production of anti-peptide antibodies when covalently bound to a carrier macromolecule. A minimum of 4 amino acid residues (a tetrapeptide) is necesarry to elicit a hapten specific response (Schechter et al. 1970.) From these investigations it has also been concluded that the antigen combining site of the antibody accomodates at least 4 amino acid residues.

7.1.3. SIZE REQUIREMENT OF PEPTIDE ANTIGENS

Though the complexity of polypeptides, as regards amino acid composition and structure, is of prime importance in their immunogenicity (see below), efforts have been made to determine the minimum peptide sequences necessary for priming and triggering humoral and cell-mediated immune responses. Synthetic polypeptides (e.g., poly-Tyr,Glu,Ala) with M_r of 17,000 did not provoke precipitating antibodies, but elicited delayed hypersensitivity. Precipitin reactions appeared after immunization with similar polymers larger than M_r 30,000 (Stupp et al. 1966, Borek 1968). In contrast, a model compound of M_r 4,100 containing 38% Ala, 52% Glu and 10% Tyr elicited an immune response.

However, when non-amino acid groups, such as dinitrophenyl or trinitrophenyl moieties were attached to peptides of different length, minimum sequences of seven (Yaron et al. 1974) or twelve (Singh et al. 1980) amino acids were sufficient to induce an antibody response. In the latter cases, the molecular weight of the immunogens were less than 2,000 and antibodies against the peptide moiety were also demonstrated.

In view of the above, rather conflicting data the size requirement for peptide antigens needs further, careful examination. Recently two cyclic peptides of 16 and 21 amino acids (corresponding to the hepatitis B virus surface antigen) have been shown to elicit humoral immune responses (Dreesman et al. 1982, Fig.7.1) nevertheless the general rule that immunogenicity increases with the molecular weight seems to be valid for polypeptides, too.

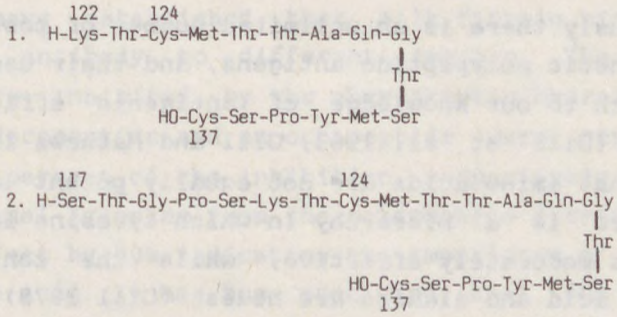


Fig. 7.1. HBsAG peptide 1 and 2 designed by Dreesman et al.(1982)

Natural polypeptides in the range of M_r 1,000-10,000 have proved to be moderately immunogenic. Polypeptide hormones of this size are poor immunogens, but antihormone antibodies can be elicited by repeated inoculations for several months using adjuvants (Senyk et al. 1971).

7.1.4. AMINO ACID COMPOSITION

The antigenic properties of complex macromolecules are generally more pronounced than those of simple structures. Thus homopolymers of either L-or D-amino acids are not immunogenic at all. It is noted, however, that they can serve as carriers for haptens (Borek and Stupp 1965). A mixture of poly-glutamic acid and poly-lysine has proved to be immunogenic, probably because the combination of these molecules by electrostatic forces yields an immunogenic structure (Gill and Doty 1962).

Synthetic copolymers of two amino acids are still poor immunogens (Gill and Doty 1961). More effective antigens are produced by the combination of three or more amino acids (Borek 1968, Gill and Dammin 1963, Stupp et al. 1966). However, one must be aware of the fact that the overall amino acid composition of the molecule does not reflect the composition of the antigenic sites (see below), and there is a significant positional effect of minor amino acid components (Gill et al. 1963).

Obviously there is an unlimited number of possible variants of synthetic polypeptide antigens, and their use have contributed much to our knowledge of antigenic efficiency. Early results (Gill et al. 1963, Gill and Mathews 1963) have suggested that amino acids are not equally potent antigenically, and there is a hierarchy in which tyrosine is very potent, lysine is moderately effective, while the contributions of glutamic acid and alanine are modest (Gill 1973). However, recent studies on complex natural proteins have not confirmed this concept directly. Beside the quality of amino acids, a series of stereochemical features of both the antigen and the antibody help in selecting antigenic sites of a protein.

7.1.5. CONFORMATION

A clear demonstration of the importance of conformation in antigenic properties has also come from studies with synthetic polypeptides. Antibodies raised against an intramolecularly cross-linked polypeptide did not react with the parent linear peptide, because they were directed against antigenic sites which depended upon the organization of the spatial structure of the cross-linked molecule (Gill et al. 1968). Denaturation of proteins was shown to abolish the binding of antibodies raised against the native protein, and disulfide bridges proved to be very important in this respect (Gerwing and Thompson 1968).

The profound significance of protein conformation will be discussed in more details in connection with the antigenic sites.

7.1.6. ANTIGENIC SITES (DETERMINANTS, EPITOPES) OF PROTEINS AND PEPTIDES

Pioneering studies of Landsteiner (1942) continued by Cebra (1961a,b,c) have established that silk fibroin binds three molecules of antibody to different regions. The binding processes were inhibited by the chymotryptic hydrolysate of fibroin. A dodecapeptide and an octapeptide were responsible for 50 and 40 percent of the inhibition, respectively. Removal of the C-terminal tyrosine from the octapeptide decreased its inhibitory effect by 50% indicating the importance of this individual amino acid. It has been concluded that the peptide fragments of silk fibroin combine with antibodies directed against certain parts of the protein where that particular sequence is exposed on the surfasce. Such a region of the protein surface has been called an antigenic site, or antigenic determinant or epitope.

An epitope contains 5-10 amino acid residues, in an area of about 2.5x2.5 nm. The side chains of the amino acids are complementary to those of the antigen-binding site of the antibody. Thus, an epitope can be defined as a restricted, specific area on the surface of a macromolecular antigen to which a specific antibody binds. As to the number of epitopes of protein antigens, experience has shown that larger proteins have more epitopes, generally one site per 5,000 dalton molecular weight. No clear-cut explanation has been put forward to explain this ratio, though it can be assumed that the number of epitopes is equal to the number of antibody molecules which can bind simultaneously to a single antigen molecule. This consideration allows, at the same time, the immunochemical interpretation of the antigenic "valence" of monomeric proteins and peptides.

Identification of the epitopes of natural protein antigens provides a chemical problem of immense complexity. One of the basic problems was recognized during the study of synthetic polypeptide antigens (Schechter et al. 1971). Namely the proximity and grouping of amino acid side chains is determined not only by the amino acid sequence of the polypeptide backbone but also by its folding, i.e., conformation. Therefore, two types of epitopes have been proposed. The first, comprising amino acids following each other in the peptide backbone, is called sequantial or continuous determinant. The second type, called conformational or discontinuous determinant consists of amino acid residues brought together by the folding of the polypeptide backbone.

Advances in protein chemistry have allowed the precise definition of epitopes of proteins during the last decade. In a long series of brilliant immunochemical investigations Atassi and coworkers determined the entire antigenic structure of whale sperm myoglobin and of egg white lysozyme (Atassi 1978, Atassi and Lee 1979) It should be noted that knowledge of the three-dimensional structure of these proteins was a precondition of the indentification of antigenic sites. Whale sperm myoglobin was shown to carry five epitopes which were all continuous (Fig.7.2).

However, each of the three epitopes of hen egg white lysozyme proved to be discontinuous (Fig.7.3). Antibody binding to the continuous epitopes of myoglobin could be inhibited by

```
                15  16                  21  22
site 1: (Ala)-Lys-Val-Glu-Ala-Asp-Val-(Ala)
                56                  62
site 2: Lys-Ala-Ser-Glu-Asp-Leu-Lys
                94                  99
site 3: Ala-Thr-Lys-His-Lys-Ile
               113                 119
site 4: His-Val-Leu-His-Ser-Arg-His
               145 146                 151
site 5: (Lys)-Tyr-Lys-Glu-Leu-Gly-Tyr
```

Fig.7.2. Amino acid sequences of the five antigenic determinants (sites) of whale sperm myoglobin. Residues in parantheses indicate an uncertainty of their involvement in that particular antigenic site. Numbers indicate position of the residues within the myoglobin molecule

synthetic peptides of the corresponding sequence. However, antibody binding to lysozyme was not inhibited by linear peptide fragments corresponding to any sequence of lysozyme. Inhibition achieved by synthetic surface-simulating peptides (Atassi 1978) designed to mimic the discontinuous epitopes (Fig.7.3). It was shown that, besides the major epitopes, antibodies are produced against many additional minor antigenic sites of protein antigens (Takagaki et al. 1980).

site 1:

	125		5		7		14	13
aar:	Arg		Arg		Glu		Arg	Lys
ssp 1:	Arg-	Gly-	Gly-	Arg-	Gly-	Glu-	Gly-	Gly-	Arg-	Lys

site 2:

	62		97	96	93	89	87
aar:	Trp		Lys	Lys	Asn	Thr	Asp
ssp 2:	Phe-	Gly-	Lys-	Lys-	Asn-	Thr-	Asp

site 3:

	116	113	114		34	33
aar:	Lys	Asn-	Arg		Phe	Lys
ssp 3:	Lys-	Asn-	Arg-	Gly-	Phe-	Lys

Fig. 7.3. Positions of the amino acid residues (aar) contributing to the three discontinuous antigenic sites of hen egg white lysozyme and the sequences of the corresponding surface simulation peptides (ssp)

Individual sera contain antibodies to different epitopes in various ratios. These epitopes most probably participate in a preferential reaction between B lymphocytes and definite surface regions of protein antigens. Much less is known about the determinants to which T lymphocytes react. The two types of determinants seem to be different (Maizels et al. 1980), and the significance of conformation is probably of minor importance with respect to T cell reactivity (Arnon 1974).

7.1.6.1. DETERMINANT SELECTION

Immunization with native antigens induces antibodies against a limited number of (major) epitopes. It has been demonstrated that not only different individuals but even different species react to the same major immunogenic determinants of lysozyme (Atassi and Lee 1979), serum albumin (Sakata and Atassi 1980) myoglobin and hemoglobin alpha chain (Kazim

and Atassi 1982) and probably of other antigens, too. Therefore, in the interaction of the immune system with the antigen, the antigenic sites have to be regarded as structurally inherent features of macromolecular antigens (Kazim and Atassi 1982).

What kind of properties distinguish an epitope from other regions of a polypeptide antigen? No clear-cut explanation is available as yet, though some basic requirements have been recognized. Evidently, an epitope has to be exposed to the environment, that is, it must be on the surface of the molecule. Considering that peptide sequences containing hydrophilic amino acid side chains are likely to be exposed on the surface of the native molecule, they have a relatively greater chance to be involved in epitopes. A search through the primary sequences of proteins of known antigenic structure has yielded encouraging results. The most hydrophilic sequences (hexapeptide) have proved to be parts of some of the antigenic sites (Hopp and Woods 1981). However, this type of search did not reveal many of the epitopes.

Better understanding of the molecular mechanism of the humoral immune response and further study of B-lymphocyte antigen receptors may provide a deeper insight into the process of determinant selection, and it may result in more powerful methods of prediction of antigenic sites of complex antigens.

7.1.6.2. ANTIGENIC DETERMINANTS: IMMUNOGENECITY VS. ANTIGENICITY

So far, we have discussed the significance of antigenic determinants in the humoral immune response against native proteins. Since antibodies are induced against the epitopes, these can be called immunogenic determinants. The necessity to distinguish between immunogenic and other types of determinants has emerged recently in connection with an important breakthrough in immunochemistry: the production of antibodies of predetermined specificity (Lerner 1982).

Immunization with various fragments or natural proteins (either proteolytic or synthetic peptides containing 15-40 amino acids) induces antibodies which react with the native, parent protein. It has been observed that polypeptide fragments unrelated to the immunogenic determinants also induce anti-protein antibodies, which bind to the corresponding regions of the protein (Green et al. 1982). Thus, a binding site of the protein can be different from the immunogenic determinants, but it is still an antigenic determinant because it is able to bind antibody induced by the corresponding fragment.

This recognition has opened new perspectives in the design of synthetic antigens. Earlier it was tacitly assumed that a synthetic peptide has to mimic the original, immunogenic epitope of the native protein to be able to induce anti-protein immunity. In view of the great difficulties of the identification of immunogenic determinants, the design of such peptides seemed to be a formidable task. It has been demonstrated that anti-peptide antibodies raised against almost any sequence (containing about 20 amino acids) of influenza virus hemagglutinin do react with the native protein (Green et al. 1982). This ability of the antipeptide antibodies seems to be independent of the location of the peptide in the native molecule. The far-reaching implications of these observations with respect to the behaviour of proteins in solution have also been recognized (Lerner 1982).

7.1.7. BIOMEDICAL APPLICATIONS OF PEPTIDE ANTIGENS: SYNTHETIC VACCINES

The conclusion of the above section is that immunization with peptides of 10-20 amino acids corresponding to partial sequences or proteins, can induce antibodies reactive with the native protein. Amino acid sequences of viral proteins can be obtained with a relative ease by decoding the nucleic acid sequences of the viral genome. Thus viral proteins predicted from the genome can be detected by the use of anti-peptide antibodies raised against synthetic sequences (Sutcliffe et al.

1980, Walter et al. 1980). Antibodies of predetermined specificity can be directed against biologically active regions of proteins, such as of the hormone calcitonin (Amara et al. 1982).

Replacement of existing vaccines by man-made, chemically defined vaccines is attracting increased attention. Antiviral vaccines containing whole viral particles are not entirely harmless. Further, the only source of hepatitis B virus is the human blood of infected persons, thus the preparation of the vaccine presents a serious problem. Therefore, the possible significance of synthetic peptide vaccines can be hardly overestimated. The first demonstration of this approach was the induction of neutralizing antibodies against tobacco mosaic virus by immunization with the C-terminal hexapeptide of the coat protein coupled to serum albumin (Anderer 1963). In recent applications, the investigators rely on protein sequences predicted from the viral genome, and exploit the latest information on the nature of antigenic determinants. Peptides corresponding to the proteins of hepatitis B virus (Lerner et al. 1981, Dreesman et al. 1982, Prince et al. 1982), foot-and-mouth disease virus (Bittle et al. 1982, Muller et al. 1982; Fig.7.4) were shown to elicit anti-virus antibodies, several of which have proved to be neutralizing antibodies. An exciting perspective is the induction of antibodies against invariable portions of influenza virus proteins, thereby combating the viruses' strategy to change.

```
141                                   150
Val-Pro-Asn-Leu-Arg-Gly-Asp-Leu-Gln-Val
                                      160
Leu-Ala-Gln-Lys-Val-Ala-Arg-Thr-Leu-Pro
```

Fig. 7.4. VP1 polypeptide of foot-and-mouth disease virus. Numbers refer to the positions of residues within the protein encoded by the viral genome

7.2. PEPTIDES ORIGINATING FROM THE COMPLEMENT SYSTEM

Activation of the complement system generates peptides of significant biological activity. Complement is a multimolecular self-assembling biological system which constitutes the primary humoral mediator between antigen-antibody complexes and cells (for a review see Muller-Eberhard 1975). It participates in a number of physiological defense mechanisms including bacteriolysis, virus inactivation, opsonization, immune adherence, chemotaxis, anaphylatoxin formation, etc. Its activation leads to cytolysis and to induction of specific cellular functions (Rapp and Borsos 1971).

Complement is present in the serum of all mammals and many lower animals, constituting as much as approximately 10% of serum globulin (Gigli and Austen 1971). The entire system consists of at least 18 different proteins. No interspecies differences are experienced in serological reactions, indicating that complement is one of the ancestral systems of the immune apparatus.

Complement can be activated by two converging routes, called the classical and the alternative pathways. The activation mechanism includes consecutive proteolytic reactions, in which active proteins are enzymatically formed from inactive precursor proteins. Therefore, the activation mechanism can be regarded as a proteolytic cascade reaction. An interesting feature of the activation mechanism is that several of the proteolytic reactions yield two biologically active molecules. After splitting, the large fragment participates in the formation of the multimolecular complex involved in the cytolytic reaction while the small fragment dissociates and induces inflammation. In the following we shall discuss the chemical and biological properties of these phlogogenic peptides, called anaphylatoxins.

Complement proteins are designated numerically (C3, C4, etc.). Fragments resulting from their cleavage (by enzymes indigenous to the complement system) are distinguished by letters, e.g., C3a and C3b. Limited proteolysis by individual specific enzymes formed during the activation affect C2, C3,

C4 and C5 in the classical pathway, and C3 and C5 in the alternative pathway. The splitting of C2 (M_r 117,000) yields two large fragments C2a (M_r 80,000) and C2b (M_r 37,000). The former participates in the subsequent activation reactions, while no biological activity of C2b has been demonstrated as yet.

C3, C4 and C5 have similar structural and functional characteristics suggesting that they derive from a common ancestral protein. The molecular weights of C3, C4 and C5 are 180,000, 206,000, and 180,000, respectively. Both C3 and C5 consist of two chains (M_r 100,000 for the α chain and 70,000 for the β chain). C4 contains three chains (M_r 93,000, 78,000 and 33,000 for the α, β and γ chain, respectively). The smaller proteolytic fragments of C3, C4 and C5 are known as anaphylatoxins.

C3a is the result of the cleavage of the α chain of C3 between Arg 77 and Ser 78 (Muller-Eberhard 1975, Gorski et al. 1979, Muller-Eberhard and Lepow 1965). The released peptide of 77 amino acids has a M_r of 8,900. The biological activities of C3a include induction of histamine release from mast cells, contraction of guinea pig ileum, skin wheal and flare reaction and chemotactic activity for polymorphonuclear leukocytes, which can be detected at concentrations as low as 10^{-8} mol/l. An inhibitor present in the serum inactivates C3a by cleaving the C-terminal arginine (Fernandez and Hugli 1976), indicating the importance of the C-terminal part of C3. In fact, the C-terminal pentapeptide Leu-Gly-Leu-Ala-Arg is as active as the intact C3a in guinea pig and man (Caporale et al. 1980). Moreover, the activity of the C-terminal octapeptide of pig C3a is three times higher than that of the pentapeptide. The activity of the octapeptide of human C3 (Ala-Ser-His-Leu-Gly-Ser-Ala-Arg) is twelve times higher than that of the common pentapeptide. As demonstrated by these investigations, structure-function relationships of C3a were successfully analyzed, yielding the conclusion that the biological activity resides in its C-terminal part (Caporale et al. 1980). Also some importance is ascribed to the side chains of the arginine and two leucine residues. Chemical synthesis of the entire C3a yielded peptides of full biological activity.

C5a (M_r about 15,000) is derived from the alpha chain of C5 (Fernandez et al. 1978, Chenoweth et al. 1979). It contains mannose, galactose, glucosamine and sialic acid. After the removal of the carbohydrate side chains, the M_r of the remaining peptide of 77 amino acids is 8,500. The C-terminal arginine is essential for the biological activity also in this case. Chemotactic activity is displayed by the C-terminal pentapeptide (Met-Gln-Leu-Gly-Arg) and even by smaller peptides (Chenoweth et al. 1979). C5a has an optimum concentration range between $4x10^{-10}$ and $2x10^{-8}$ mol/l (Fernandez et al. 1978). C4a is released after cleavage of the α chain of C4 between amino acid residues 77 and 78. C4a is positively charged. No histidine or tryptophan is found in C3a. It has a M_r of 9,000. The biological activity of C4a, similar to that of C3a, was only recently discovered (Gorski et al. 1979).

After elucidating the primary stucture of human C4a anaphylatoxin, Moon and coworkers (1981) pointed out that there is a 30% homology between C5a and C4a. In spite of the structural similarities the three anaphylatoxins are immunologically distinct, giving no cross reaction with each other. This may be explained by the large number of residue substitutions in surface regions. On the other hand, immunoassays specific for C3a, C4a and C5a allow the monitoring of complement activation with a specific indicator (C4a) for the classical pathway.

7.3. CHEMOTACTIC PEPTIDES

In the immune system protecting processes against microbial invasion, the discrimination of self from nonself and the localization and degradation of nonself by phagocytic cell such as polymorphonuclear leukocytes (PMNs) and macrophages, cells with immune effector functions are of great importance. During this process immune effector cells accumulate at sites of inflammation. Local accumulation of immune effector cells is mediated by chemotaxis induced by chemotactic factors. An important consequence of the recognition of antigenic factors by affected cells is the production and/or release of such chemo-

tactic factors (see e.g., Zigmond and Hirsch 1973). These substances may be synthesized and released by immune effector cells, such as granulocytes, lymphocytes or mast cells. They may be produced from inactive prosubstances by the activation of certain enzyme cascade systems.

The interrelationship between biological activities of the complement, clotting, fibrinolytic, and kinin-forming systems has been well recognized (Ratnoff 1969). For example, enzymes from one system have been shown to activate enzymes from other systems. In addition, naturally occurring inhibitors of one pathway may function as inhibitors of other pathways. Products with chemotactic activity are derived from the kinin-forming system as well as from the fibrinolytic and clotting systems. Chemotactic factors can be produced by proteolytic cleveage of different complement proteins (C3, C4, C5) as it has already been mentioned in the section dealing with the complement systems.

In this section noncomplement-derived chemotactic peptides are briefly surveyed.

7.3.1. EOSINOPHIL CHEMOTACTIC FACTOR OF ANAPHYLAXIS

The eosinophil chemotactic factor of anaphylaxis, (ECF-A) was discovered in hypersensitivity reactions in guinea pig and human lung slices (Kay et al. 1971, Kay and Austen 1971, Goetzl and Austen 1975). Two acidic peptides with the sequence of Ala-Gly-Ser-Glu and Val-Gly-Ser-Glu, both displaying eosinophilotactic activity, have been identified. ECF-A was also found in human lung mast cells. Subcellular granules of rat peritoneal mast cells also contain ECF-A. ECF-A is released by IgE-mediated immune mechanisms (Wasserman et al. 1974). They stimulate the migration of eosinophils at concentrations of 5×10^{-8} to 10^{-6} mol/l.

The structural requirements of the tetrapeptide for eosinophilotactic activity have been widely studied (Goetzl and Austen 1975, 1976). The valyl-peptide was consistently more active than the alanyl-peptide, with a maximal biological activ-

ity occurring at concentrations of $3x10^{-7}$ and 10^{-6} mol/l, respectively. From the synthesized tetrapeptide analogs the leucyl-tetrapeptide achieved its biological activity at the concentration of 10^{-8} mol/l while the phenylalanyl-tetrapeptide only at the concentration of 10^{-4} mol/l. This suggests that any amino acid substitutions at the N-terminus decreases biological activity (Goetzl and Austen 1976). Inversion of the order of the internal residues, glycine and serine, does not alter the maximal eosinophilotactic activity of either the valyl- or the leucyl-tetrapeptides.

However, elimination of the glycine residue from either tetrapeptides produced tripeptides with effective biological activity at a concentration of 10^{-6} mol/l. This suggests that the distance of the N-terminal residue from the C-terminal one might play a role in the biological activity of eosinophilotactic peptides.

The tripeptide analogs usuallly inhibit the eosinophil chemotactic response to the intact tetrapeptides, e.g., the Val-Gly-Ser tripeptide caused a 50% inhibition of the eosinophil response elicited by the tetrapeptide at about an equimolar concentration (Goetzl and Austen 1976).

7.3.2. FORMYL-METHIONYL-LEUCYL-PHENYLALANIN (FOR-MET-LEU-PHE) CHEMOTACTIC PEPTIDES

The filtrates of various bacterial cultures were reported to be chemotactic for neutrophils in vitro. The highest concentrations of chemotactic substances were found in the log phase of bacterial growth.

Initially different chemotactic substances with unknown molecular weight and structure were described (Wilkinson 1974). Schiffmann's invenstigations resulted in a great progress recognizing that the N-terminal residue of bacterial chemotactic factors was formylated (Schiffmann et al. 1975a,b). He has also noticed that these substances are the N-terminal sequences of newly synthesized bacterial proteins. They were shown to be formyl-methionyl peptides displaying

Table 7.1. Chemotactic activity of synthetic peptides (Showell et al. 1976c)

	$ED_{50} \pm SE$
1. For-Met	$2.1 \pm 0.62 \times 10^{-3}$
2. For-Met-Leu	$4.0 \mp 0.45 \times 10^{-7}$
3. For-Met-Leu-Phe	$7.0 \mp 1.7 \times 10^{-11}$
4. For-Met-Leu-Glu	$1.3 \mp 0.38 \times 10^{-6}$
5. For-Met-Leu-Arg	$3.6 \mp 1.00 \times 10^{-7}$
6. For-Met-Leu-Leu	$4.8 \mp 1.30 \times 10^{-8}$
7. For-Met-Phe	$4.1 \mp 0{,}95 \times 10^{-7}$
8. For-Met-Phe-Leu	$5.4 \mp 1.90 \times 10^{-8}$
9. For-Met-Phe-Met	$1.5 \mp 0.33 \times 10^{-9}$
10. For-Met-Met	$8.8 \mp 2.30 \times 10^{-7}$
11. For-Met-Met-Met	$5.1 \mp 0.62 \times 10^{-9}$
12. For-Met-Met-Met-Met	$3.0 \mp 0.13 \times 10^{-10}$
13. For-Met-Met-Ala	$5.4 \mp 1.80 \times 10^{-7}$
14. For-Met-Met-Phe	$2.1 \mp 0.49 \times 10^{-10}$
15. For-Leu-Trp-Met	$2.5 \mp 1.50 \times 10^{-8}$
16. For-Leu-Trp-Met-Arg	$1.1 \mp 0.40 \times 10^{-6}$
17. Met-Leu-Phe	$6.7 \mp 1.90 \times 10^{-7}$
18. Met-Leu-Glu	$2.7 \mp 1.00 \times 10^{-3}$
19. Met-Leu-Leu	$2.1 \mp 0.24 \times 10^{-4}$
20. Met-Phe-Leu	$2.4 \mp 1.10 \times 10^{-4}$
21. Met-Met-Met	$1.0 \mp 0.39 \times 10^{-4}$
22. Met-Met-Met-Met	$1.3 \mp 0.29 \times 10^{-6}$
23. Met-Met-Ala	$1.9 \mp 0.39 \times 10^{-4}$
24. Met-Met-Phe	$9.0 \mp 3.90 \times 10^{-7}$

chemotactic activity at a concentration of 10^{-9} mol/l. These findings led to the synthesis of a series of peptide analogs and the investigation of their biological activity (Schiffmann et al. 1975a, Showell et al. 1976).

Chemotactic peptides not only induce cell migration (Zigmond and Hirsch 1973) but For-Met-Leu-Phe is also able to induce lysosomal enzyme secretion, aggregate cells, generate peroxide radicals, increase oxigen consumption, form methylated protein components, and to stimulate the mobilization of Ca^{2+} and chemoluminescence was also observed (Becker et al. 1974, Schiffmann et al. 1978). Altogether 24 analogs of formyl-methionyl-peptides were synthesized and their biological activity analyzed (Table 7.1; Showell et al. 1976).

As to their relative migratory activity, two of them, For-Met-Leu-Phe and For-Met-Met-Met proved to be truly chemotactic. The former peptide was found to be the most active peptide, having an ED_{50} of 7×10^{-11} mol/l for induced migration and 2.4×10^{-11} and 2.6×10^{-10} mol/l for lysozyme and β-gluconidase release, respectively. The Met-Leu-Glu tripeptide was 1,4 mil-

lion times less active in these respects than the For-Met-Leu-Phe- tetrapeptide.

Very small changes in the structure of peptides can induce significant changes in biological activity. The activity of a given peptide depends not only on its constituting amino acids, but also on the position of the amino acid in the peptide chain. For instance, if phenylalanine is in the C-terminal position of the tripeptide, its biological activity is higher than if it is the second amino acid from the N terminus.

Biological studies suggest, that the receptors of PMN cells are stereospecific (Showell et al. 1976). Specific receptors have been described on the surface of PMNs (Aswanikumar et al. 1977, Williams et al. 1977). Using radioactively labeled For-Met-Leu-Phe and For-Nle-Leu-Phe peptides, it was possible to demonstrate the receptors on the surface of cells. These studies have also enabled the investigators to study the mechanism of action of the For-Met-Leu-Phe peptide.

In a binding assay with radioactively labeled and fluorescent derivatives of the chemotactic peptide, For-Nle-Leu-Phe-Nle-Tyr-Lys, 120,000 binding sites/cell could be estimated at a peptide concentration of 1.3×10^{-9} mol/l. Neutrophils incubated with chemotactic peptide for 1 minute at 37°C, a diffuse and relatively homogenous membrane distribution could be observed (Niedel et al. 1979a,b). After two minutes, aggregates of peptides could be seen on the membrane surface. This phenomenon is similar to that seen on the surface of lymphocytes in the presence of different peptides. Chemotactic peptides also affect interreceptor relations on the cell surface, which are essential for the transduction of an extracellular signal from the cell surface receptors into the cells. At 3 minutes, the aggregates on the membrane increased in size and intensity, and were internalized into the cells and appeared within the endocytic vesicles.

According to these observations three steps can be distinguished in the interaction of chemotactic For-Met-Leu-Phe peptides and neutrophils (Antoni et al. 1980): (a) binding of the peptides to the plasma membrane, (b) aggregation of the

membrane-bound peptides on the cell surface and (c) internalization of the peptides.

7.4. IMMUNOMODULATING PEPTIDES

The term "immunomodulating substance" is applied to materials displaying either stimulating or inhibiting activities on the immune system, and to those amplifying immune responses elicited by other immunogenic agents (immunoadjuvant or simply adjuvant substances). Freund's adjuvant, the archetype and the most widely known member of the latter group of substances, is an emulsion of killed tubercle bacilli in paraffin oil whose adjuvant activity is attributable to a peptidoglycolipid fraction, called wax D. In experiments aimed at analyzing ingredients in an active wax D derived from human strains of *Mycobacterium tuberculosis* a lipid constituent mainly consisting of mycolic acids esterified to a polysaccharide and a nitrogen containing moiety has been identified. The chemical structure of the latter is closely related to the peptidoglycan which forms the backbone of mycobacteria' and other bacterial cell walls. In the last decade, a variety of microbial and animal organisms have been shown to produce peptides endowed with immunomodulating activities. Simple and complex peptides having a carbohydrate and/or a lipid moiety were equally shown to contribute to this group of substances. In the following paragraphs, immunomodulating peptides of microbial origin will be briefly surveyed, whereas similar peptides of animal origin will be discussed more exhaustively.

7.4.1. GLYCOPEPTIDES AND PEPTIDES OF MICROBIAL ORIGIN

7.4.1.1. MURAMYL PEPTIDES

Attempts at characterizing the ingredients responsible for the immunopotentiating activities of mycobacterial cells present in Freund's adjuvant, culminated in a breakthrough when water-soluble fractions with adjuvant activity could be isolated from Mycobacteria. Subsequent studies have led to the identification of N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyldipeptide, MDP) as the minimal structural requirement of adjuvant activity (Ellouz et al. 1974). Since then, muramylpeptides (MPs) and their several synthetic analogs have been extensively studied (review by Chedid and Lederer 1978, Adam and Lederer 1984).

As to the cardinal feature of MDP and its active synthetic analogs, their adjuvant activity on antibody production should be stressed, though they also enhance the nonspecific resistance of mice to various infectious agents. In vitro, MPs inhibit the growth of several tumor cell lines, or they may even kill tumor cells through activating macrophages. In vivo, some MDP derivatives retard the growth of transplantable tumors in mice, prevent metastases and increase survival time of the animals. An unfavorable feature of MDP is its pyrogenicity which is absent in some of its derivatives (Riveau et al. 1980).

Structure-activity studies have indicated that L-alanine can be replaced, without loss of activity, by various L-amino acids, and that D-glutamic acid residue is essential (Adam et al. 1976). Many MDP derivatives of lipophilic character proved to be more active than MDP itself in stimulating cell-mediated versus humoral immune responses (for references see Werner et al. 1986). Although the mechanism of action of MPs remains essentially unknown, it is now clear that the macrophages and the B cells are the main targets of such peptides.

It was recently established that MDP exerts a somnogenic activity when injected intracerebroventricularly (i.c.v.) at large doses in rabbits (Krueger et al. 1985). This is all the

more intriguing because "Sleep factor S", a substance found in human cerebrospinal fluid and urine, which induces slow wave sleep (SWS) following i.c.v. administration in several mammals, was demonstrated to yield muramic acid, alanine, glutamic acid and diaminopimelic acid upon acid hydrolysis suggesting an intriguing connections between the neuroendocrine and the immune systems. (For more details and references see Adam and Lederer 1984, Werner et al. 1986).

7.4.1.2. LIPOPEPTIDES

The dogma that the presence of a muramic acid moiety is essential for the immunopotentiating activities of peptidoglycan fragments from bacterial cell walls was refuted by the observation that chemical coupling between lauric acid and a biologically inactive tetrapeptide from a Streptomyces species yielded an agent with pronounced immunopotentiating activities. Studies with a synthetic variant of this lauryltetrapeptide revealed divergent activities of the molecule directed on cells of the mononuclear-phagocytic system, on T lymphocytes and NK cells. Furthermore, its adjuvant effect on delayed type hypersensitivity (DTH) and antibody production has also been established. This lauryltetrapeptide was also shown to enhance the resistance of mice to microbial infection and to inhibit the production of metastases in mice inoculated with various tumor cell lines. Some synthetic analogs of this peptide displayed more pronounced immunostimulating activities than the parent molecule. (see e.g., Specter et al. 1977). Phagocytic cells as well as T lymphocytes were identified as the main targets of immunomodulating lipopeptides, the B lymphocytes remaining largerly unaffected.

These observations indicate that peptides lacking the muramic acid moiety may also be capable of eliciting immunomodulating activities as significant as those of muramyl peptides, although the nature and spectrum of these activities are not strictly identical with the latter. Lipopeptides appear to be promising candidates for clinical evaluation of their thera-

peutic potential, especially in states associated with immune deficiency (1986 for more details and references see Werner et al. 1986).

7.4.1.3. CICLOSPORINE (FORMER NAME: CYCLOSPORIN A)

Ciclosporine, a cyclic undecapeptide isolated from a fungus species is a potent inhibitor of both humoral and cell-mediated immune response. It acts specifically by suppressing T lymphocyte functions, an activity mediated, at least in part, by inhibiting lymphokine secretion by T cells which are essential for the growth and differentiation of T and B cells as well as of macrophages. Ciclosporine also inhibits interleukin-2 (IL-2) gene expression in T cells. Its capability of preventing the rejection of allogenic organ transplants has been successfully utilized in humans in bone marrow and kidney transplantations. Ciclosporine proved to be an immunosuppressing agent far superior to those previously used in human medicine (for further references see Werner et al. 1986).

7.4.1.4. BESTATIN

Bestatin, isolated from a Streptomyces species consists of a leucine and a 2-hydroxy-3-amino-4-phenylbutyric acid molecule linked together by a single peptide bond. It is a powerful inhibitor of leucine aminopeptidease, an enzyme located on the cell surface of macrophages and lymphocytes. Bestatin stimulates cell-mediated immunity and, at higher doses, enhances antibody production. This molecule is claimed to induce IL-1 production and to augment IL-2 production by stimulated lymphocytes. Its reported antitumor effect appears to be mediated through the activation of cytolytic T cells and of NK cells. Bestatin is already in clinical use, but it is too early to draw conclusion about its overall efficacy (for further details and references see Werner et al. 1986).

7.4.2. PEPTIDES OF ANIMAL ORIGIN

7.4.2.1. THYMIC PEPTIDES

During the last two decades or so, it has successively become clear that, in contrast to what formerly was thought, the thymus is far for simply being an "infant gland", but it maintains physiologically important functions also in adults by functioning as an endocrine organ and producing a whole array of polypeptides variously referred to as thymic factors, thymic hormones or thymic peptides (Davies and Carter 1973). (Since thymus was also reported to contain oxytocin and neurophysin, its neuroendocrine organ character should also be considered, as proposed by Geenen et al. 1986.) Data accumulated so far strongly suggest that thymic factors are intimately related to the maturation, differentiation and the functions of T cells, thereby partaking in the physiological processes through which thymus-possessing organisms acquire their immunological competence. Accordingly, the main function of thymic factors is to regulate thymocyte differentiation, but not unconditionally cell division. The suggestion, that the thymic factors would simultaneously function as growth factors for thymocytes is not unequivocally documented. However, since the majority of thymus-controlled processes of lymphocyte maturation/differentiation is inherently associated with multiple divisions of the cells, furthermore, since some of the thymic factors were in fact shown to be endowed with the capability of stimulating lymphocyte proliferation in both thymus and peripheral lymphatic organs (Friedman 1975, Luft and Hall 1975), the growth factor-like character of thymic peptides can be accepted.

The practical importance of at least some of the thymic peptides should also be stressed. It has recently been recognized that, although the thymic factors have no direct antitumor effects on their own, some of them can substantially ameliorate secondary immunodeficiencies resulting from tumor chemotherapy, or from the presence of a tumor itself, and thus may be applied as valuable adjuvant therapeutical agents in

the treatment of various tumors and other conditions associated with immunodeficiency.

A list of the thymus-derived factors and some of their characteristics are presented in Table 7.2 (see also the review by Goldstein and associates 1981).

7.4.2.1.1. THYMOPOIETIN

Apart from being a powerful promoter of the differentiation of prothymocytes to thymocytes, thymopoietin (TP) also exerts a curare-like blocking effect on the neuromuscular transmission. (Goldstein and Hofmann 1969). As a matter of fact, the discovery and isolation of TP was accomplished in experiments that were designed on the assumption that autoimmune thymitis was central in myasthenia gravis, a human disease, and that an increased production or release of a thymus-derived substance(s) with curare-like activity would be responsible for the impaired neuromuscular transmission characteristic of this disease. (Goldstein 1966, Goldstein and Manganaro 1971, Goldstein and Whittingham 1966; in this context, the recent finding by Venkatasubramanian et al. 1986 that TP binds to the acethylcholine receptor deserves a special attention). This hypothesis was subsequently confirmed in a series of elegant experiments (reviewed by Goldstein 1975). Thymin was the name selected first to designate this factor, (Basch and Goldstein 1974), but it was later replaced for TP in order to avoid confusion with the pyrimidine base thymine. The first TP was isolated from calf thymus saline extract by Goldstein (1974). The extract was later shown to contain several closely related peptides, of which two termed TP I and TP II were the first to be sequenced (Schlesinger and Goldstein 1975; see also Goldstein and Lau 1980).

These sequence data were later revised and corrected by Audhya and associates (1981), who have simultaneously published the amino acid sequence of a novel peptide closely related to TP which was, however, isolated from spleen extract instead of thymus. This peptide was first termed TP III, but later re-

Table 7.2. Thymic peptides and some of their characteristics

Designation		Chemical nature	Sequenced (+) or not (-)	N° of residues	M_r	References
Thymosin fraction 5		Mixture of heat-stable acidic pps	-	?	1,000 1,500	Goldstein et al. 1977
Thymo-poietin	I.	pp	+	49	5,562	Schlesinger and Goldstein 1975
	II.		+	49	5,648	Audhya et al. 1981
Splenin		pp	+	49	5,662	Audhya et al. 1981
Thymosin alfa	1	pp	+	28	3,108	Goldstein et al. 1977 Low and Goldstein 1979 Low et al. 1979b
	2-6	pp	-	?	?	Goldstein et al. 1981
	7	pp	-	?	2,500	Low et al. 1979a
	8-10	pp	-	?	?	Goldstein et al. 1981
	11	pp	+	35	3,853	Caldarella et al. 1983
Thymosin beta	1	pp	+	74	8,451	Low and Goldstein 1979 Low et al. 1979b
	2	pp	-	?	?	Low et al. 1979a
	3	pp	-	50(?)	5,550(?)	Low et al. 1981
	4	pp	+	43	4,982	Low et al. 1981
	5-7	pp	-	?	?	Low et al. 1979a
	8	pp	+	39	4,518	Hannappel et al. 1982b
	9	pp	+	41	4,717	Hannappel et al. 1982b
	10	pp	+	42	4,842	Erickson-Viitanen et al. 1983
Thymosin gamma		mixture of pps	-	?	?	Goldstein et al. 1981
Serum thymic factor thymulin		op	+	9	857	Bach et al. 1977 Pleau et al. 1977 Dardanne et al. 1982
Thymic humoral factor		pp	-	31 or 27	3,200 or?	Kook et al. 1975 Trainin et al. 1975
Thymone	A	pp	-	68-71	7,291 7,677	Folkers et al. 1980a,b
	B	pp	-	?	?	Folkers et al. 1980a,b
Human serum factor		adenosine + op. (?)	-	4(?)	500(?)	Astaldi et al. 1976

Table 7.2. cont.

Lymphocyte stimulating hormone	r	pp	-	?	8,000	Hand et al. 1967
	h	pp	-	?	15,000	Robey et al. 1972 Robey 1975
Thymocyte specific growth factor		op	-	?	1,000(?)	Soder and Ernstrom 1981, 1984
Thymic factor X		pp	-	-	4,200	Aleksandrowicz and Skotnicki 1976
Thymic hypocalcemic factor	1	pp	-	?	68,000	Mizutani 1973
	2	pp	-	?	57,000	Mizutani et al. 1975
Homeostatic thymic hormone		gp	-	?	18,000 25,000	Comsa 1973
Thymostimulin		mixture of pps	-	?	12,000	Falchetti et al. 1977

Abbreviations: pp: polypeptide; op: oligopeptide; gp: glycopeptide; question marks indicate unavailability or uncertainty of data in question.

named splenin (SP) referring to its source (Audhya et al.1984). Sequences of the corresponding human peptides were recently reported by Audhya et al. (1987) that are shown in Fig.7.5 together with their bovine counterparts. The human and bovine peptides are composed of 48 and 49 amino acids, respectively. Although TP and SP are closely related in both species, the change at position 34 in the active-site region (see below) changes the receptor specificities and biological activities of the affected molecules. It was proposed, that Glu(1) as Gln(1) in serum thymic factor (STF), a nonapeptide (see later) might be linked to Arg (49) of TP and form a new 58 amino acid

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hSP: Gly-Leu-Pro-Lys-Glu-Val-Pro-Ala-Val-Leu-Thr-Lys-Gln-Lys-Leu-Lys-Ser-Glu-Leu-Val
hTP: -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
bTP: Pro-Glu-Phe-Leu - Asp - Ser -   -   -   - Glu -   -   -   -   -   -   -   -
bSP: Pro-Glu-Phe-Leu - Asp - Ser -   -   -   - Glu -   -   -   -   -   -   -   -

                                                30
hSP: Ala-Asn-Asn-Val-Thr-Leu-Pro-Ala-Gly-Glu-Met-Arg-Lys-Ala-Val-Tyr-Val-Glu-Leu-Tyr
hTP: -  Glu -   -   -   -   -   -   -   -   -   -   -   - Asp -   -   -   -   -   -
bTP: -   -   -   -   -   -   -   -   -   - Asn -   -   - Asp -   -   -   -   -   -
bSP: -   -   -   -   -   -   -   -   -   - Asn -   -   - Glu -   -   -   -   -   -

                                                48
hSP: Leu-Gln-Ser-Leu-Thr-Ala-Glu-His
hTP: -   - His -   -   - Leu -  49
bTP: -   -   -   -   -   - Leu-Lys-Arg
bSP: -   - His -   -   - Leu-Lys-Arg
```

Fig. 7.5. Primary structures of human (h) and bovine (b) thymopoietins (TP) and splenins (SP). The active cores of the individual molecules are boxed. Dashes indicate residues identical to those in hSP

peptide in the thymus (Folkers and Wann 1978). Cleavage between Arg(49) and Gln(50) in the latter molecule could liberate TP and Glu(1)-STF which cyclize to STF enzymatically and chemically. Alternatively, STF may be linked to thymone A (see later), because each of the nine amino acids in STF is also present, at least once in thymone A.

A 13 amino acid peptide with a sequence corresponding to residues 29 through 41 in TP was synthesized and shown to have Tp activity. Subsequently, a pentapeptide fragment corresponding to residues 32 through 36 in TP was also synthesized, and termed thymopentin or TP5 (see Fig.7.5). The latter, which displays full TP activity, is now regarded as the "active site" of the molecule (Goldstein et al. 1979). Structural requirements for biological activity of TP 5 analogs and the safety conditions needed to be observed during its clinical application were discussed by Kisfaludy et al. (1983), Friedman (1985) and Heavner and associates (1985).

In mammals, no B-cell-differentiating organ equivalent to the T-cell-differentiating thymus has been defined, and the existence of a mammalian B-cell-differentiating hormone equivalent to TP has not been unequivocally demonstrated. In birds, however, B-cell-differentiation occurs within the bursa of Fabricius, a dorsal diverticulum of the cloaca. Bursal extracts contain the low M_r inducing agent b u r s i n (formerly termed b u r s o p o i e t i n), which selectively induces avian B cells, but not avian T cells, from their precursors in vitro. Avian bursin was found to be an efficient B cell inducer also in the mouse. Recently, the tripeptide sequence Lys-His-GlyNH_2 was determined for bursin and shown that biological activity of the synthetic tripeptide was identical to that of the natural product (Audhya et al. 1986).

7.4.2.1.2. SPLENIN

Although not a thymic peptide, splenin is discussed here because of its extensive homology to TP (see Fig.7.5), its former name TP III, and also because it shares many, though not all of its biological properties with TP. The observation

that serum levels of TP in rats failed to decline after thymectomy led to the finding that the spleen and lymph nodes, but not other tissues, yield a product that reacts with the antibody raised against TP. This product was subsequently isolated both from bovine and human spleens and their primary sequences determined (Fig.7.5; Audhya et al. 1981, 1987). Due to its structural similarity to TP, the splenic product was first named TP III. However, when some characteristic features of the splenic material were recognized, its former name was replaced by splenin referring to its tissue of origin (Audhya et al. 1984). A synthetic pentapeptide called splenopentin (SP5) was derived from splenin in the same manner as TP5 from TP, and it similarly corresponds to residues at positions 32-36 in the native molecule: Arg-Lys-Glu-Val-Tyr.

As it was mentioned earlier, both native TP and synthetic TP5 affect neuromuscular transmission and induce phenotypic differentiation of T precursor cells in vitro, while inhibiting phenotypic transformation of B cells. Splenin and SP5, in contrast, do not affect neuromuscular transmission, and they induce both T and B cell precursors (Audhya et al. 1984).

7.4.2.1.3. THYMOSIN PEPTIDES

Thymosin is the generic name used to designate a whole array of thymus-derived polypeptides, partly with established structure, but the name is also used to designate chromatographic thymic fractions of varying purity with more or less defined chemical composition. Since the start of early pioneering research with thymosin, saline extract of bovine thymus glands and a multistep separation procedure has been used for the separation of thymosins. Purification procedures are regularly monitored by a family of assay systems aimed at measuring various lymphocyte functions including those associated with the proliferation of immature forms (Hooper et al. 1975, White and Goldstein 1975).

One of the most widely known thymus fractions obtained at the fifth step of a widely used purification scheme is called thymosin fraction 5 (TF5). This fraction is still heterogeneous in composition, but is largely free of unwanted contaminants, has a marked immunopotentiating effect even in humans, and is generally used as the starting material for further purification of its constituents. By using a separation procedure of high resolution power, TF5 could be resolved into 10-15 major components and 20 or so minor ones with M_r ranging from 1000 to 15,000. (Goldstein et al. 1977). As to the recognized biological activity of TF5, it induces T-cell differentiation and enhances immunological function in animal models, increases ACTH, β-endorphin and glucocorticoid release, stimulates the production of the migration inhibiting factor (MIF), interleukin-2 (IL-2), α and β interferons and other lymphokines.

Thymosin fraction 8 (TF8) is another thymic fraction, more extensively purified than TF5. Determination of its qualitative amino acid composition revealed approximately 108 constituent residues, indicating a M_r of about 12,000. This fraction is rich in acidic residues, primarily glutamic and aspartic acids. Preliminary data suggest the presence in TF8 of polypeptide subunits with M_r between 3,200 and 2,400. The finding, however, that the specific activity of TF8 in some of the assay systems was much lower than that of TF5 indicats that an essential cofactor(s) may have been lost, or that the molecule was modified by the purification steps between TF5 and TF8.

In order to avoid, or at least ameliorate the confusion experienced in the nomenclature of thymosin preparations obtained from TF5, it was proposed by Goldstein et al. (1977) that classification of the purified thymosins be based on the regions the separated peptides occupy during their migration on the isoelectric focusing pattern of TF5, and the regions be identified by the Greek letters α, β, and γ. Accordingly, the α-region contains peptides with isoelectric points below 5 (highly acidic), the β-region between 5.0 and 7.0 (acidic), and the γ-region above 7.0 (basic). As soon as the complete sequence of peptides separated within the same region becomes

established, they should be distinguished by subscript numbers, such as α_1, α_2; or β_1, β_2 etc. Biological activity of TF5 resides primarily in its acidic polypeptides, a considerable number of which have extensively characterized, or even sequenced (see Table 7.2).

(1) Thymosin α_1 (T α_1) is the first thymosin peptide that was purified to homogeneity from calf thymus (Goldstein et al. 1977) and sequenced (Low and Goldstein 1979). It is a 28 amino acid peptide of highly acidic character with a M_r of 3,108 and the following primary structure:
AcSer-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn.
Its N terminus is blocked by an acetyl group, and the molecule has no homology to any other thymic factors hitherto characterized. Apart from calf thymus, T α_1 was also isolated from human, pig, sheep and chinchilla, which all appear to have sequences identical to that of the calf thymus peptide. T α_1 and peptide T from the human immunodeficiency virus envelope are homologous (Ngujen and Scheving 1987).

T α_1 was chemically synthesized both by solution (Wang et al. 1979, Birr and Stolenwerk 1979) and solid phase procedures (Wong and Merrifield 1980) and the synthetic product displayed activities identical to natural T α_1. T α_1 has also been produced by recombinant DNA techniques in E. c o l i in its desacetyl form after synthesis of the entire gene for T α_1 (Wetzel et al. 1980). Evidence was also presented for a 16,000 M_r polypeptide synthesized in an in vitro translation system containing thymus mRNA (Freire 1981). Partial sequences of the 16,000 peptide were found to be characteristic of T α_2 indicating its thymic origin and also its probable synthesis from a longer peptide chain. This was indirectly supported by recent experiments designed to isolate thymic peptides under conditions which minimize the chances of proteolytic modification of peptide molecules in general (Hannappel et al. 1982a). Apart from being active in a variety of bioassays, T α_1 proved to be mitogenic to a remarkable extent (Low et al. 1979b). T α_1 induces enhancement of MIF, interferon, interleukin-3, and

lymphotoxine production, modulates TdT activity, increases viral, fungal and tumor immunity, and amplifies T cell immunity (in humans). Immunorestorative properties of synthetic $T\alpha_1$ is being currently evaluated in several institutions (for details see Schulof et al. 1985, Ohta et al. 1987).

Recently, two additional peptides have been isolated and partially sequenced from rat thymus extract (Haritos et al. 1985a,b, and Komiyama et al. 1986). The one containing 113 amino acid residues, is the putative precursor of $T\alpha_1$, hence its name prothymosin α (pTα). The other peptide first obtained as a byproduct of the procedure used for the isolation of $pT\alpha_1$ is a 105 amino acid peptide that was named parathymosin (para-T) because of its structural similarities to pTα. Partial amino acid sequences of the two N-terminally blocked peptides are shown in Fig.7.6. More recently, the gene for human pTα has been cloned and shown to encode a 110 amino acid mature protein whose deduced amino acid sequence is 90% homologous to the rat sequence and is also shown in Fig.7.6 (most of the uncertain Glx residues in the rat sequence are substituted for Glu residues in the human protein (Eschenfeldt and Berger 1986, Goodall et al. 1986). The human gene was shown to be polymorphic and induced upon growth stimulation. pTα was recently shown to be a nuclear protein (Gomez-Marquez and Segade 1988).

The precise functions of these two peptides remain unclear. Preliminary data suggest that pTα can protect mice against opportunistic infections more effectively than $T\alpha_1$. Para-T does not exhibit such an activity, but it appears to block the effects of pTα instead. pTα was shown to be restricted predominantly to the thymus and spleen, whereas para-T was found in significantly higher concentrations in the liver and kidney than in the thymus, a reason why liver tissue proved to be a better source for the isolation of this peptide. The fact that pTα is present in thymus and spleen, whereas the concentrations of para-T is higher in nonlymphoid tissues may provide an important clue to their biological functions. By all indications, para-T in the liver and thymus is encoded by a single gene (for references see Komiyama et al. 1986).

```
hpTα:   Ser-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-
                                                  10                                  20
rpTα:  AcSer - - - - - - - - - - - - - - - - - - - -
rpT :  AcSer-Lys-Ser-Glu - Glu-Ala-Ala-Ala - Leu-Ser-Ala - - - - - - - -

                                                  30                                  40
hpTα:   Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-Gly-Arg-Asp-Ala-Pro-Ala-Asn-Gly-Asn-Ala-Glu-Asn-
                                      Asp         30                  Asp             40
rpTα:   - - - - - - - - Asn - - - - - - - Asn - - - - Gln -
rpT :   Asp-Lys - - - Lys-Ala-Gly-Arg-Lys-Glu-Arg-Lys-Lys-Glu-Val-Val-Glu-Glu-Glu-

                                                  50                                  60
hpTα:   Glu-Glu-Asn-Gly-Glu-Gln-Glu-Ala-Asp-Asn-Glu-Val-Asp-Glu-Glu-Glu-Glu-Glu-Gly-Gly-
                                                  50                                  60
rpTα:   - - - - - - - - - - - - - - - - - - - - - -
rpT :    - Asn-Gly-Ala - Glu - Glu-Glu-Glu-Thr-Ala-Glu(Gly,Gly,Gly,Asx,Asx,Asx,Asx,

                                                  70
hpTα:   Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Gly-Asp-Gly-Glu-Glu-Glu          Asp-Gly-Asp-Glu-
                                                  70                                  80
rpTα:   Gly - - (Gly,Gly,Asx,Glx,Glx,Glx,Glx,Glx,Glx,Glx,Glx,Glx,Glx)Asn - - -
rpT :   Asx,Asx,Asx,Glx,Glx,Glx,Glx,Glx,Glx,Glx,Glx,Glx,Glx,Glx,Glx,Glx)Glu-Gly-Pro-

          80                                      90
hpTα:   Asp-Glu-Glu-Ala-Glu-Ser-Ala-Thr-Gly-Lys-Arg-Ala-Ala-Glu-Asp-Asp-Glu-Asp-Asp-Asp-
                                                  90                                  100
rpTα:    - - - - - - Ala-Pro - - - - Val - - - - - - - - -
rpT :   Val-Arg-Lys-Arg-Thr - Glu-Glu-Glu-Asp-Glu-Ala-Asp-Pro-Lys-Arg-Gln-Lys-Thr-Glu-

          100                                     110
hpTα:   Val-Asp-Thr-Lys-Lys-Gln     Lys-Thr-Asp-Glu-Asp-Asp
                                                  110         113
rpTα:    - Glu - - - - Lys - - - - - -
        Asp         105
rpT :   Asn-Gly-Ala-Ser-Ala
```

Fig. 7.6. cDNA-derived amino acid sequence of human prothymosin α (hpTα) and chemically determined partial amino acid sequences of rat prothymosin α (rpTα) and parathymosin (rpT). Residues in parentheses represent uncertainties and compositional data only. Dashes indicate residues identical to those in the hpTα sequence. Homologous sequences present in different regions of rpTα and rpT, respectively, are underlined. AcSer indicates a serine residue blocked by an acetyl group

(2) Thymosin α_7 ($T\alpha_7$) was partially purified, characterized and finally identified as a M_r 2,200-2,500 polypeptide with a highly acidic character (pI 3.5) containing an abundance of acidic residues, but not one with a basic character (Low et al. 1979a). The main biological function of this peptide seems to be the induction of suppressor cells in vitro, and expression of Lyt 1,2,3 positive cells.

(3) Thymosin α_{11}. Two peptides related to $T\alpha_1$ have been isolated from calf TF5 (Caldarella et al. 1983). One lacking 4 amino acids from the C terminal end of $T\alpha_1$ is designated des-(25-28)$T\alpha_1$. The other designated as $T\alpha_{11}$ has a 7 amino acid

extension at the C terminus of $T\alpha_1$ and an acetylated serine residue as the N-terminal amino acid. The sequence of calf thymus $T\alpha_{11}$ is as follows:
AcSer-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-Gly-Arg-Glu-Ala-Pro-Ala-Asn.

Accordingly, $T\alpha_{11}$ is a 35 amino acid peptide of M_r 3,853. The synthesis of this peptide and characterization of the product has recently been reported (Heimer et al. 1985). It can not be excluded that $T\alpha_{11}$ and $T\alpha_1$ may represent a proteolytically modified form of a larger thymic peptide. If so, $T\alpha_{11}$ is a larger fragment than $T\alpha_1$, closer in structure to the native peptide and displays also its biological activities. $T\alpha_{11}$ displays similar biological activity to $T\alpha_1$ in protecting mice against opportunistic infections with C a n d i d a a l b i c a n s.

(4) Apart from the highly purified and partly sequenced thymosin peptides, other peptides of the α region: $T\alpha_{2-6}$ and $T\alpha_{8-10}$ have also been purified to a significant extent from calf thymus extracts (Low et al. 1979a), but their sequences and exact chemical characteristics remain unknown for the present time.

(5) Thymosin β_1 ($T\beta_1$), also known as polypeptide β_1, was isolated by Low and Goldstein (1979) from calf thymus. It was identified as a 74 amino acid polypeptide of slightly acidic character with a M_r of 8,451. It has been sequenced by Low and Goldstein (1979). The sequence is as follows:
Met-Gln-Ile-Phe-Val-Lys-Thr-Leu-Thr-Gly-Lys-Thr-Ile-Thr-Leu-Gln-Val-Glu-Pro-Ser-Asp-Thr-Ile-Glu-Asn-Val-Lys-Ala-Lys-Ile-Gln-Asp-Lys-Glu-Gly-Ile-Pro-Pro-Asp-Gln-Gln-Arg-Leu-Ile-Phe-Ala-Gly-Lys-Gln-Leu-Glu-Asp-Gly-Arg-Thr-Leu-Ser-Asp-Tyr-Asn-Ile-Gln-Lys-Glu-Ser-Thr-Leu-His-Leu-Val-Leu-Arg-Leu-Arg.
It is identical to the N-terminal 74 amino acids of ubiquitin (UB) and to a partial sequence of A24, a conjugated chromosomal protein (Olson et al. 1976) consisting of histone 2A (H2A) and UB (Andersen et al. 1981). The only difference between the

primary structure of T β_1 and UB is that the latter has a two glycine extension at the C-terminal end of the T β_1 molecule. UB was coisolated with thymopoietin by Goldstein et al. (1975) and was sequenced shortly thereafter (Schlesinger et al. 1975). Human and bovine UBs proved to be identical. UB's (57-74) and (59-63) sequences are biologically fully active, as is a synthetic pentapeptide, called UB5, corresponding to the sequence 59 through 63 in the UB molecule. UB induces non-specific differentiation of both T and B lymphocytes in vitro via β-adrenergic receptors and adenyl cyclase activation. On the other hand, polypeptide β_1 proved to be inactive in most lymphocyte assays used to monitor the activities of thymic peptides, and thus it does not seem to contribute to the activities of TF5, but it might exert a modulatory action on the activities of other thymic factors.

Accordingly, T β_1 is thought to be present in calf thymus extract as a nonspecific tissue product of nuclear protein origin. UB is found in all eukaryotic cells, either in a free form covalently joined to cytoplasmic and nuclear proteins. Its amino acid sequence is conserved to an extent unparalleled among known proteins. In the cytoplasm, ubiquitin is involved in ATP-dependent nonlysosomal proteolysis, whereas in the nucleus it is conjugated to histone H2A and may play a role in the regulation of chromatin structure and/or transcriptional activity. (Readers interested in more detail on molecular and gene structure, phylogenetic representation and possible physiological roles of UB are advised to read the recent publications by Finley and Varshavsky 1985, Lund et al. 1985, Wiborg et al. 1985, and Viestra et al. 1986).

(6) Thymosin β_3 and β_4 (T β_3 and T β_4) were also prepared from TF5 (Low et al. 1979b). Both were shown to be moderately acidic polypeptides. Calf thymus T β_3 was partially, whereas T β_4 was fully sequenced (Low et al. 1981). T β_4 proved to be a 43 amino acid peptide with an identical primary structure in all mammals hitherto studied:

AcSer-Asp-Lys-Pro-Asp-Met-Ala-Glu-Ile-Glu-Lys-Phe-Asp-Lys-Ser-
Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-Ser-
Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-Gly-Glu-Ser. $T\beta_4$ has a M_r of 4,982 and an acidic character. On the other hand, $T\beta_3$ possesses approximately 50 amino acid residues and a M_r of about 5,000. The two peptides appear to share identical sequences through most of their N-terminal parts but differ in their C-terminal ends. $T\beta_3$ and β_4 may possibly act on stem cells to form prothymocytes (Low et al. 1979a,b). $T\beta_4$ induces TdT in bone marrow cells from normal and athymic mice both in vivo and in vitro and it does the same in thymocytes of immunosuppressed mice in vivo. It stimulates the release of LH-RH and LH (Rebar et al. 1981). However, $T\beta_4$ originally isolated from calf TF5 was subsequently shown to be ubiqutous both in terms of the vertebrate phylogenetic scale and of tissue occurrence, and thus it occurs in relatively large amounts in tissues other than the thymus. In addition, it was also demonstrated that $T\beta_4$ is actively synthesized by cells unrelated to the reticulo-endothelial system. These findings brought the proposed role of $T\beta_4$ as a thymic hormone in question. In a recent paper, the product formed in an appropriate in vitro system by rat spleen mRNA was shown to be identical to $T\beta_4$ with no evidence for a signal or leading sequence for the formation of a larger precursor polypeptide (Wodnar-Filipowicz et al. 1984). $T\beta_4$ was shown to be synthesized as a M_r 5,100 peptide containing 44 amino acid residues. Removal of the initiator methionyl residue and acetylation of the N-terminal serine residue would yield mature $T\beta_4$. The absence of a signal sequence makes it highly unlikely that $T\beta_4$ is a secreted peptide.

(7) Thymosin β_8 ($T\beta_8$) and β_9 ($T\beta_9$) have been sequenced by Hannappel et al.(1982b). Calf thymus $T\beta_8$ was identified as a 39 amino acid polypeptide:
AcAla-Asp-Lys-Pro-Asp-Leu-Gly-Glu-Ile-Asn-Ser-Phe-Asp-Lys-Ala-
Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Thr-Leu-Pro-Thr-
Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln, with an acetylated alanine at its N terminus and a M_r of 4,518. 31 residues of this peptide are identical to those of $T\beta_4$.

$T\beta_9$ is identical to $T\beta_8$ molecule with the exception that the latter is a 41 amino acid peptide of M_r 4,117 having an Ala-Lys dipeptide extension at the C terminus of $T\beta_8$. Its 32 residues are identical to those of $T\beta_4$. The suspicion was raised that $T\beta_8$ might be an artifact being generated from $T\beta_9$ during separation. The β_4 and β_9 peptides may have closely related functions in principle, though no biological activity was reported for $T\beta_8$ and $T\beta_9$ up to the present.

(8) Thymosin β_{10} ($T\beta_{10}$) is another member of a family of closely related peptides including $T\beta_4$ and $T\beta_9$ which occurs together with $T\beta_4$ in tissues of various mammalian species including rat, mouse, cat and man. Mammalian $T\beta_{10}$ was identified as a 42 amino acid peptide of M_r 4,842 the primary structure of which was determined by Erickson-Viitanen and associates (1983). However, sequence analysis of a cloned cDNA for rat spleen $T\alpha_{10}$ has indicated that it is, in fact, a 43 amino acid peptide having an additional arginine residue at position 39:
AcAla-Asp-Lys-Pro-Asp-Met-Gly-Glu-Ile-Ala-Ser-Phe-Asp-Lys-Ala-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Thr-Leu-Pro-Thr-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Arg-Ser-Glu-Ile-Ser (Goodal and Horecker 1987).

If its structure is compared to those of $T\beta_4$ and $T\beta_9$, its 86% homology with the former and 75% homology with the latter becomes evident. Like $T\beta_4$ and $T\beta_9$, $T\beta_{10}$ has its N-terminal residue in an acylated form. It occurs together with $T\beta_4$ in a variety of tissues including the spleen, liver and the thymus and also in sevaral cultured cell lines. In the spleen of the rat, mouse, cat and man this peptide accounts for approximately 0.02% by weight of the total protein. In the calf, $T\beta_{10}$ is replaced by $T\beta_9$, a peptide homologous to $T\beta_{10}$ (see the previous section).

(9) Besides the β peptides already mentioned, $T\beta_2$ and $T\beta_{5-7}$ were also extensively purified, but their sequences and characteristics remain largely unknown at present (Low et al. 1979a).

7.4.2.1.4. SERUM THYMIC FACTOR

Serum thymic factor (STF), or facteur thymicque serique (FTS) was first isolated from pig blood by Bach and associates (Bach et al. 1971, 1972, Bach and Dardenne 1973, Bach et al. 1975, 1977), who have also demonstrated the capacity of this factor to induce theta antigen on theta negative rosette forming spleen cells (Bach et al. 1977; see also White and Burton 1979).

Porcine STF was identified as a moderately basic nonapeptide (Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn) having a M_r of 857 (Pleau et al. 1977). There is no homology between STF and that of other sequenced thymic factors summarized in Table 7.2. STF was shown to stimulate DNA synthesis in prothymocytes (Incefy et al. 1980) and to act through high affinity binding sites on the plasma membrane (Gastinel et al. 1982). The suggested participation of STF in the construction of a larger molecule consisting of STF and thymopoietin was mentioned earlier in this section. Thymic origin of STF was demonstrated by its rapid reappearance after thymus grafting in thymectomized animals. STF was shown to lose its biological activity in some in vitro assay systems after treatment with metal ion chelating agent. However, the biological activity could be restored by addition of Zn. These observations were interpreted to mean that STF exists in two forms: one lacks Zn and is biologically inactive, while the other one contains Zn and is biologically active, for which the name "thymulin" (FTS-Zn) was proposed (Dardenne et al. 1982). The presence of Zn in the native material was convincingly demonstrated by chemical and histochemical methods.

STF enhances the generation of effector cytotoxic T cells both in vitro and in vivo and inhibits contact sensitivity in normal mice. A brief but comprehensive review on thymulin has been recently published by Bach and Dardenne (1985) discussing structural aspects, physiological functions and properties, as well as the clinical application of this immunomodulator peptide (for recent data on the regulation of thymulin secretion in humans see Cohen et al. 1986).

7.4.2.1.5. THYMIC HUMORAL FACTOR

A lymphopoietic activity termed thymic humoral factor (THF) has been partially purified from both mouse and calf thymus (Trainin et al. 1975). THF is capable of alleviating wasting syndrome, the consequence of neonatal thymectomy. It was first identified as a trypsin- and heat-sensitive, moderately acidic (pI 5.7) N-terminal blocked, 31 amino acid polypeptide with a M_r of 3,220 (Trainin et al. 1975), though Kook et al. (1975) assumed, that THF contains 27 amino acid residues only. Its sequence is unknown at present, and its exclusive thymus origin has recently been questioned.

THF restores the ability of spleen cells from neonatally thymectomized donors to induce graft versus host reaction in vivo and enhances the ability of normal spleen cells to respond to PHA and Con A (for recent data on biological properties and clinical application of THF see Trainin et al. 1985).

7.4.2.1.6. THYMONE

Folkers and associates (1980a,b) reported the isolation of two polypeptides from bovine thymus with characteristics distinct from those of other thymic factors hitherto identified. The authors did not follow either the purification scheme or the classification principles proposed by Goldstein and coworkers (1977) and termed the two peptides thymone A and thymone B, respectively.

Thymone A was isolated from bovine thymus and purified to homogeneity according to electrophoretic criteria (Folkers et al. 1980b). It was identified as a polypeptide rich in glutamic and aspartic acid, and especially in lysine and arginine, but extremly poor in aromatic residues. According to the molar ratio of its constituent amino acids thymone A may have approximately 68-71 amino acid residues, and a M_r between 7,291 and 7,677. The size of the molecule as well as the abundant presence of lysine and arginine strongly suggest that thymone A might be a pro-molecule. Available data support the sugges-

tion that thymone A might be a proform of serum thymic factor or, after being linked to thymosin α_1 at its N-terminal end, it may become part of a pro-thymosin α_1 molecule (Folkers et al. 1980b).

As to the biological activity of thymone A, it stimulates ^{3}H-TdR incorporation into splenocytes of thymectomized mice, and also the generation of cAMP, but not cGMP. Trypsin digestion results in a complete loss of its biological activity.

While attempting to purify thymone A from a bovine thymus extract, Folkers and associates isolated and characterized an additional thymic peptide, named thymone B (Folkers et al. 1980a). Judged from the molar ratio of its constituent amino acids, but also from chromatographic criteria, thymone B appears to be a much smaller peptide than thymone A. Available data also indicate that thymone B is a molecule distinct from other thymic factors hitherto identified.

Like thymone A, thymone B stimulates ^{3}H-TdR incorporation into splenocytes of thymectomized mice, but, unlike thymone A, it stimulates the generation of cGMP, but not cAMP.

7.4.2.1.7. HUMAN SERUM FACTOR

Astaldi and associates (1976, 1980) reported the isolation of a small molecular weight component (M_r 500) with a pronounced lymphopoietic activity from human sera. The chemical nature of this factor, called human serum factor (SF) is uncertain at present. It appears to contain adenosine and also some unidentified small molecular weight moiety, perhaps tetrapeptide, thereby resembling transfer factor. Others suggest that SF may be a split product of STF (Bach et al. 1979). Whatever is the case, the exact chemical nature, the the thymus origin and the biological activity of SF remain to be clarified.

7.4.2.1.8. LYMPHOCYTE-STIMULATING HORMONE

A lymphopoietic substance capable of stimulating antibody production in mice was isolated from beef thymus by Hand et al. (1967), who termed it lymphocyte-stimulating hormone (LSH). In 1972, isolation of a second thymic factor with biological activities similar to those of LSH was reported by Robey et al. (1972). In order to distinguish these two factors, they were designated as LSHh and LSHr, where h and r stand for Hand and Robey, respectively, the persons who first undertook the isolation of the two substances. LSHh is a 15,000 molecular weight heat-labile polypeptide with a relatively weak lymphopoietic activity. On the other hand, LSHr is a 80,000 molecular weight heat-stable protein with a pronounced lymphopoietic activity (Luckey and Venugopal 1975). LSHr may be associated with cell-mediated immunity, whereas LSHh with humoral immunity. PAG electrophoretic analysis of LSHr in human sera revealed the presence of two basic protein components with different electrophoretic mobility. The fast moving component was assumed to be identical to LSHr itself, whereas the slow-moving one has tentatively been termed GEM 126, referring to its gel electrophoretic mobility (Luckey and Venogoupal 1975). The author of this chapter is unaware of further characterization of these peptides.

7.4.2.1.9. THYMOCYTE-SPECIFIC GROWTH FACTOR

In 1981, Soder and Ernstrom (1981) partially purified a thymocyte-specific growth factor from calf thymus which stimulated the proliferation of thymocytes in vitro without affecting the proliferation of peripheral lymphocytes, or that of the erythroid and granuloid precursor cells in the bone marrow. The target cells belong, by all probability, to the rapidly changing population of immature thymocytes. The responsible molecule appears to be an acidic, hydrophylic, low molecular weight compound (M_r 1,000) which is apparently devoid of a reactive N-terminal amino group. Its peptide

character is indicated by the observation that after its acid hydrolysis, ninhydrin positive spots appear on the electrophoretogram. Its amino acid composition has recently been reported (Soder and Ernstrom 1984).The purified material, which appeared homogenous on thin layer chromatography and HPLC, appears to be an N-terminal blocked acidic peptide having no basic amino acid residues at all. Available data indicate that this thymic factor is not identical with any other thymic substances characterized so far.

7.4.2.1.10. THYMIC FACTOR X

Thymic factor X (TFX) is a calf thymus-derived polypeptide purified to homogeneity which has a M_r of 4,200. Its amino acid composition has been reported (Aleksandrowicz and Skotnicki 1976) without sequence data. TFX was reported to restore the azathioprine sensitivity of spleen rosette-forming cells from adult thymectomized mice in vitro, and to increase blood T cell number and return delayed hypersensitivity in vivo. The effect of TFX on human T cell and neutrophil mobility in vitro is discussed in a recent paper by Smogorsewska and associates (1985).

7.4.2.1.11. THYMOSTIMULIN

Thymostimulin (TS) was first described by Falchetti and associates (1977). It is a partially purified, heterogenous thymus fraction with pronounced immunostimulatory effects in man. TS was shown to consist of a mixture of polypeptides with M_r lower than 12,000 but with unknown structures at present. The peptides in TS were demonstrated to induce markers and specific functions of T lymphocytes both in immunosuppressed animals and in immunodeficient patients. TS also stimulates interferon production in mice following challange with poly(I): poly(C). By using TS preparations, efficient treatment of disease states associated with severe immunodeficiency

has recently been reported (Hogarty et al. 1984, Rimoldi et al. 1984). In a more recent paper, Yagi and associates (1985) discuss the beneficial effect of TS on growth and metastases of Lewis lung carcinoma, whereas Tovo and coworkers (1986) the thymostimulin therapy of patients with measles meningoencephalitis.

7.4.2.1.12. THYMIC HYPOCALCEMIC FACTOR

In 1973, Mizutani described a component in thymus extract with pronounced hypocalcemic and lymphopoietic activity. It was termed the thymic hypocalcemic factor (Mizutani 1973). Subsequently, two factors with a hypocalcemia-inducing potential were separated from thymic extract, that were termed TP1 and TP2, respectively (Mizutani et al. 1975). TP1 and TP2 were assumed to be polypeptides of M_r 68,000, and 57,000, respectively. The qualitative amino acid composition, and several physico-chemical parameters of TP1 and TP2 were determined, and found to be different from those of other identified thymic peptides. Although their sequences remain undetermined at present, the two factors are clearly not identical with calcitonin, also a hormone with serum calcium-lowering activity.

7.4.2.1.13. HOMEOSTATIC THYMIC HORMONE

The discovery and preliminary characterization of homeostatic thymic hormone (HTH), a heat-labile glycopeptide of thymus origin with a M_r between 1,800-2,500 was first reported by Comsa (1973). HTH was shown to have the capability of counteracting the thymoprivic state in laboratory experiments. Further purification of HTH has recently been shown to yield 2 polypeptide chains, HTHα and HTHβ, which were subsequently identified by sequence analysis as histones H2A and H2B, respectively, without evidence of subtypes, proteolytic processing or other peptide fragment (Reichart et al. 1985a,b).

This finding gives full support for the suggestion of a new histone function indicating that, apart from being nucleosome components histones may have other unexpected roles (see also ubiquitin in section 7.4.2.1.3). Besides suppressing consequences of thymectomy, HTH was demonstrated to antagonize the effect of several hormones (thyroxine, ACTH, desoxycorticosterone, TSH, and GnRHs) but to be synergistic with growth hormone. HTH-like activity has been reported to be present in lymph nodes, spleen and urine, while disappearing after thymectomy (for more detail and references see Reichart et al. 1985a,b).

7.4.2.1.14. HUMAN THYMUS-SPECIFIC PROTEIN

Human thymus-specific protein described by Tallberg and associates (1968) is only mentioned here for the sake of completeness. It is assumed to be a thymus factor with an immunogenic property which has, however, not been further characterized.

Concluding remarks on thymic peptides

The seemingly immense diversity of thymic factors is puzzling, and their relationship is also a matter of some confusion. It is likely that not all thymic factors hitherto described are involved in T cell differentiation, or that some of them may show no activity whatsoever on lymphocytes. The exclusive thymus origin of each thymic factor can also be seriously questioned: much of the thymic peptides seem to be widely distributed in various cells and tissues of vertebrate species (see e.g., Horecker and Morgan 1984). According to some researchers (see e.g., Low et al. 1979b), thymosin α_1 is the only authentic thymic hormone, whereas other peptides in thymosin F5 would belong to the family of lymphokines. In addition, presently there are too many differences in techniques used for purification, for judging the state of purity, for assessing biological activity of thymic peptides, as well as

in the animal strains used and in their breeding conditions, and all these together do not allow us to make clear distinctions among the many putative thymic factors. Until these conditions are standardized, doubts continue to be raised concerning the mere existence, and the separate entity of thymic peptides, and their interrelations. Despite all these uncertainties, thymus peptides continue to play an increasingly significant role in understanding both the development of immunocompetence and the molecular basis of immune mechanisms in adult organisms. Attempts aimed at practical applications of thymus peptides in fighting immunodeficienies of various origins may also register remarkable successes. For instance, synthetic thymosin α_1 produced by solid phase procedures is currently undergoing phase I and phase II clinical trials in cancer patients, and clinical studies are also in progress using chemically synthesized thymulin and the active pentapeptides moiety of thymopoietin (TP5). A brief, but excellent review on thymus peptides has been published by Oates and Goldstein (1984) whereas a novel aspect concerning the mechanism of action of thymic peptides is raised in a recent paper by Zozulya and associates (1985) demonstrating the interaction of thymic peptides with opiate receptors.

7.4.2.2. LYMPHOKINES AND MONOKINES: THE INTERLEUKINS

It is now an established knowledge that cultured lymphocytes and macrophages, but also other cells involved in immune responses, release a number of factors of polypeptide character into their culture media which specifically affect lymphoid cells undergoing an immune response, and occasionally other cells as well. Lymphokine and monokine are the generic terms introduced to designate these factors of lymphocyte and macrophage origin, respectively. The main function of these factors is to modulate the differentiation and functional activities of cells involved in immune responses, first and foremost, T cells. Since these processes are inherently associated with stimulation of the proliferation of the affected cells, these factors administer growth factor functions as

well. A special group of lymphokines and monokines are termed interleukins (ILs) referring to their ability to transmit messages between leukocytes (for clinical significance of lymphokines and monokines see the reviews by Dexter and Moore 1986, Platzer et al. 1986, Billingham 1987, Dinarello and Mier 1987, Fauci 1987).

(1) Interleukin-1

Over the past 10-20 years, it has become gradually clear that the host response to bacterial infections, inflammation, immunogenic agents etc. is manifested in a whole array of biochemical and clinical alterations collectively called acute pahse response. More recent evidence lend a strong support to the notion that individual components of the acute phase response and also other immune responses including delayed type hypersensitivity, are elicited by host-derived substances (e.g., lymphokines) rather than by the attacking microorganisms themselves, by their toxins, or by other inflammatory or immunogenic agents. Depending on the bioassay used, many seemingly different agents were described, each with its own particular acronym. These include the fever-inducing substances variously called endogenous pyrogen (EP), granuloid or leukocyte pyrogen (LP); the activated macrophage-derived agent termed lymphocyte activating factor (LAF) which stimulates lymphocyte proliferation upon antigen or mitogen stimulation; the substance(s) mediating various components of acute phase reaction, such as neutrophilia, hypozincemia, increased hepatic synthesis of acute phase proteins which were thought to be mediated by a protein called leukocyte endogenous mediator (LEM); the so called synovial factor (SF); the mononuclear cell factor (MCF); catabolin and the proteolysis-inducing factor (PIF) both having proteolytic enzyme stimulating activity; or the epidermal cell-derived thymocyte-activating factor (ETAF), etc. Whilst it was formerly thought that all these factors represent separate entities, more recent evidence lends strong support to the assumption that various components of the acute phase reaction and other immune responses are mediated by a much smaller number of closely related polypep-

tides, than formerly thought, whereby all of a sudden, the huge list of reported lymphokines has began to shrink. Thus, the current opinion is that all the postulated agents listed above represent a restricted number of molecules belonging to the IL-1 family of proteins (Dinarello 1984a).

IL-1 is a monokine most abundantly produced by mononuclear phagocytes: blood monocytes, fixed macrophages in lung, liver and spleen belonging to the RES, tissue macrophages lining the body cavities and joint spaces, bone marrow and lymph-node macrophages and the macrophages located under the intestinal epithelium. However, it is clear that IL-1 or a similar mediator(s) can also be produced by other cell types, such as renal mesangial cells, astrocytes, skin Langerhans' cells (ETAF) and even B cells (for more details and references on ETAF see Sauder 1984). IL-1 was also demonstrated in brain cells of rats with experimental allergic encephalitis, and in dendritic cells from synovial tissue of patients with rheumatoid arthritis (for references see Mayernik et al. 1984 and Duff 1984). In the central nervous system, IL-1 is produced by ameboid microglia (Giulian et al. 1986).

Some confusion existed over the molecular characteristics of IL-1 polypeptides. Human monocyte-derived IL-1 appearently occurs in two sizes: an about M_r 15,000-17,000 and a M_r 35,000 species. Each yields two peaks on chromatofocusing with pIs of about 5 and 7, respectively, which makes at least four distinct molecular species. Molecules greater than 50,000 daltons with some IL-1 activities (Lachman et al. 1980, Lachman and Metzgar 1980) may either be polymers of the smaller M_r species or its complexed form with a carrier protein. On the other hand, smaller molecules with M_r of 2,000-4,000 found in human urine and having thymocyte-activating properties but no pyrogenic activity, may be fragments of the M_r 15,000-17,000 IL-1 lacking the full complement of active sites. A M_r 15,000 polypeptide isolated from macrophage and originally termed mitogenic protein (MP) (Beller and Unanue 1979) was later identified as IL-1 by Oppenheimer and coworkers (1979). Murine, and also human IL-1 (see e.g., Krakauer 1985) have been purified to a significant extent and some of its basic structure-func-

tion relationships were elucidated. However, it proved to be difficult to prepare sufficient amounts of IL-1 for direct and detailed sequence and structure studies.

Under such circumstances, it was a major breakthrough when Lomedico and associates (1984) and Auron and coworkers (1984) succeeded in cloning and expressing murine and human monocyte IL-1 cDNA, respectively. Shortly thereafter, three additional human IL-1 cDNAs were cloned and expressed in E. c o l i. Two of them termed IL-1 α and IL-1 β cDNA, respectively, were prepared from human peripheral blood cells and were shown to be distinct, but distantly related molecules (March et al. 1985) sharing a common cell surface receptor (Dower et al. 1986b). The third cloned and characterized human IL-1 cDNA derived from induced HL-60 cells, a human monocyte-like cell line was reported by Furutani and associates (1985), who have also characterized a rabbit IL-1 cDNA derived from induced rabbit alveolar macrophages. Sequence analysis of the mentioned human IL-1 cDNAs revealed that cDNAs for IL-1 α and IL-1 β (March et al. 1985) form analog pairs with cDNAs described by Furutani and coworkers (1985) and Auron and associates (1984), respectively. The paired cDNAs are practically identical exhibiting more than 98% homology (amino acid level), whereas cross homology between these pairs is much less extensive, except a short N-terminal stretch. Amino acid sequences of IL-1 precursors deduced from human (IL-1 α and IL-1 β), murine and rabbit cDNAs are shown in Fig.7.7. The primary translational product of the two human IL-1 genes reported by March et al. (1985) consists of 271 (IL-1α) and 269 amino acids (IL-1β) with M s 30,606 and 30,749, respectively. Expression in E. c o l i of the C-terminal 154 or 153 amino acids of the two precursors produced IL-1 biological activity (Hopp et al. 1986). The human, and the rabbit IL-1 cDNAs simultaneously reported by

Fig. 7.7. cDNA-derived amino acid sequences (partly confirmed by amino acid analysis) of the primary translation products of human (hα), murine (mα) and rabbit (rα) interleukin-1α (IL-1α), and that of the human IL-1β (hβ) precursors. Dashes indicate identical residues to the human IL-1α sequence. Deletions (empty spaces) are introduced for maximizing homology. Underlined residues indicate probable N termini of the mature proteins, except rα whose N-terminal residue is unknown presently

```
                                                  10                                                  20
hα :Met-Ala-Lys-Val-Pro-Asp-Met-Phe-Glu-Asp-Leu-Lys-Asn-Cys-Tyr-Ser-Glu-Asn-Glu-Glu-
mα : -   -   -   -   -   -   -  Leu  -   -   -   -   -   -   -   -   -   -   -  Asp-
rα : -   -   -   -   -   -   -  Leu  -   -   -   -   -   -  Phe  -   -   -   -   -
hβ : -   -  Glu  -   -  Glu-Leu-Ala-Ser-Glu-Met-Met-Ala-Tyr  -   -  Gly  -   -  Asp

                                                  30                                                  40
Asp-Ser-Ser-Ser-Ile-Asp-His-Leu-Ser-Leu-Asn-Gln-Lys      Ser-Phe-Tyr-His-Val-Ser-Tyr-
Tyr  -   -  Ala  -   -   -   -   -   -   -   -   -   -   -   -   -  Asp-Ala  -   -
Tyr  -   -  Ala  -   -   -   -   -   -   -   -   -   -   -   -   -  Asp-Ala  -   -
 -  Leu-Phe-Phe-Glu-Ala-Asp-Gly-Pro-Lys-Gln-Met  -  Cys  -   -  Gln Asp-Leu-Asp-Leu-

                                                  50                                                  60
Gly-Pro-Leu-His-Glu-Gly-Cys-Met-Asp-Gln-Ser-Val-Ser-Leu-Ser-Ile-Ser-Glu-Thr-Ser-
 -  Ser  -   -   -  Thr  -  Ile  -   -  Phe  -   -   -  Arg-Thr  -   -   -   -
Glu  -   -   -   -  Asp  -   -  Asn-Lys-Val  -   -   -   -  Thr  -   -   -   -
Cys  -   -                  -  Gly-Gly-Ile-Gln  -  Arg  -   -  Asp-His-His-

                                                  70                                                  80
Lys-Thr-Ser-Lys-Leu-Thr-Phe-Lys-Glu-Ser-Met-Val-Val-Val      Ala-Thr          Asn-Gly-Lys-Val
 -  Met  -  Asn-Phe  -   -   -   -   -  Arg  -  Thr  -  Ser  -   -  Ser-Ser  -   -   -  Ile-
Val-Ser-Pro-Asn  -   -   -  Gln  -  Asn-Val  -  Ala  -  Thr  -  Ser              -   -  Ile-
Tyr-Ser          Lys-Gly  -  Arg-Gln-Ala-Ala-Ser  -   -  Val  -  Met           Asp  -

                                                  90                                                 100
Leu-Lys-Lys-Arg-Arg-Leu-Ser-Leu-Ser-Gln-Ser-Ile-Thr-Asp-Asp-Asp-Leu-Glu-Ala-Ile-
 -   -   -   -   -   -   -   -  Phe  -  Glu-Thr-Phe  -  Glu  -   -   -  Gln-Ser  -
 -   -   -   -   -   -   -   -  Asn  -  Pro  -   -  Asp-Val  -   -   -  Thr-Asn-
 -  Arg-Lys-Met-Leu-Val-Pro-Cys-Pro  -  Thr-Phe-Gln-Glu-Asn  -   -  Ser-Thr-Phe-

                                                 110                                                 120
Ala-Asn-Asp-Ser      Glu-Glu-Glu-Ile-Ile-Lys-Pro-Arg-Ser-Ala-Pro-Phe-Ser-Phe-Leu-Ser-
Ile-His  -  Leu          -   -  Thr  -  Gln  -   -   -   -   -  Tyr-Thr-Tyr-Gln  -
Val-Ser  -  Pro      -   -  Gly  -   -   -   -   -   -  Val  -  Tyr-Thr  -  Gln-Arg-
Phe-Pro-Phe-Ile-Phe  -   -   -  Pro  -          Phe-Phe-Asp-Thr-Trp-Asp-Asn-Glu-Ala-

                                                 130                                                 140
Asn-Val-Lys-Tyr-Asn-Phe-Met-Arg-Ile-Ile-Lys-Tyr-Glu-Phe-Ile-Leu-Asn-Asp-Ala-Leu-
Asp-Leu-Arg  -  Lys-Leu  -  Lys-Leu-Val-Arg-Gln-Lys  -  Val-Met  -   -  Ser  -
 -  Met-Arg  -  Lys-Tyr-Leu  -   -   -   -  Gln  -   -  Thr  -   -   -   -   -
Tyr  -  His-Asp-Ala-Pro-Val-Arg-Ser-Leu          Asn-Cys-Thr  -  Arg  -  Ser-Gln-

                                                 150                                                 160
Asn-Gln-Ser-Ile-Ile-Arg-Ala-Asn-Asp      Gln-Tyr-Leu-Thr-Ala-Ala-Ala-Leu-His          Asn-Leu
 -   -  Ile  -  Tyr-Gln-Asp-Val  -  Lys-His  -   -  Ser-Thr-Thr-Trp  -  Asn          Asp  -
 -   -   -  Leu-Val  -  Asp-Ile-Ser-Asp  -   -   -  Arg  -   -  Pro  -  Gln           -   -
Gln-Lys  -  Leu-Val-Met-Ser-Gly-Pro      Tyr-Glu  -  Lys  -  Leu-His  -  Gln-Gly-Gln-Asp-Met-

                                                 170                                                 180
Asp-Glu-Ala-Val-Lys-Phe-Asp-Met-Gly-Ala-Tyr-Lys-Ser-Ser-Lys-Asp-Asp-Ala-Lys-Ile-
Gln-Gln-Glu  -   -   -   -   -   -  Tyr  -   -  Ser  -  Gly-Gly  -   -  Ser  -  Tyr-
Gly-Asp      -   -   -   -   -   -   -  Val  -  Met-Thr  -  Glu       -  Ser-Ile-Leu-
Glu-Gln-Glu  -  Val  -  Ser  -  Ser-Phe-Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Ile-Leu-

                                                 190                                                 200
Thr-Val-Ile-Leu-Arg-Ile-Ser-Lys-Thr-Gln-Leu-Tyr-Val-Thr-Ala-Gln      Asp-Glu-Asp-Gln-
Pro  -  Thr  -  Lys  -   -  Asp-Ser  -   -  Phe  -  Ser  -   -          Gly  -   -   -
Pro  -  Thr  -   -   -   -  Gln  -  Pro  -  Phe  -  Ser  -   -          Asn  -   -  Glu-
Pro  -  Ala  -  Gly-Leu-Lys-Glu-Lys-Asn  -   -  Leu-Ser-Cys-Val-Leu-Lys-Asp  -  Lys-

                                                 210                                                 220
Pro-Val-Leu-Leu-Lys-Glu-Met-Pro-Glu-Ile-Pro-Lys-Thr-Ile-Thr          Gly-Ser-Glu-Thr-Asn-
 -   -   -   -   -   -  Leu  -   -  Thr  -   -  Leu  -   -                -   -   -   -  Asp-
 -   -   -   -   -   -   -   -   -  Thr  -  Arg-Ile  -   -           Asp  -   -  Ser-Asp-
 -  Thr  -  Gln-Leu  -  Ser-Val-Asp      -   -  Asn-Tyr-Pro-Lys-Lys-Lys-Met  -  Lys-Arg-

                                                 230                                                 240
Leu-Leu-Phe-Phe-Trp-Glu-Thr-His-Gly-Thr-Lys-Asn-Tyr-Phe-Thr-Ser-Val-Ala-His-Pro-
 -  Ile  -   -   -  Lys-Ser-Thr-Asn-Ser  -   -   -   -   -   -  Ala  -  Tyr  -
Ile  -   -   -   -   -   -  Gln  -  Asn  -   -   -   -  Lys  -  Ala  -  Asn  -
Phe-Val  -  Asn-Lys-Ile-Glu-Ile-Asn-Asn  -  Leu-Glu  -  Glu  -  Ala-Gln-Phe  -

                                                 250                                                 260
Asn-Leu-Phe-Ile-Ala-Thr-Lys-Gln          Asp-Tyr-Trp-Val-Cys-Leu          Ala-Gly-Gly-Pro-Pro-Ser
Glu  -   -   -   -   -   -  Glu          Gln-Ser-Arg  -  His  -            -  Arg  -  Leu  -   -
Gln  -   -   -   -   -   -  Pro          Glu-His-Leu  -  His-Met            -  Arg  -  Leu  -   -
 -  Trp-Tyr  -  Ser  -  Ser  -  Ala-Glu-Asn-Met-Pro  -  Pro  -  Gly-Gly-Thr-Lys  -  Gly-Gln-Asp

                                 270 271
Ile-Thr-Asp-Phe-Gln-Ile-Leu-Glu-Asn-Gln-Ala(154/153)
Met  -   -   -   -   -  Ser(270) 156
Met  -   -   -   -   -  Ser(267)  -
 -   -   -   -  Thr-Met-Gln-Phe-Val-Ser-Ser(269) 153
```

Furutani et al. (1985) encode 271 and 267 amino acid proteins with calculated M_rs of 30,621 and 30,289, respectively.The length and the structure as well as the biological and biochemical properties of the mature proteins encoded by these human and rabbit IL-1 genes, of which the human gene appears to be identical to the gene for human IL-1 , remain to be elucidated. Finally, the murine preIL-1 gene reported by Lomedico et al. (1984) is initially translated as a 270 amino acid protein of M_r 33,000. The mature murine protein corresponds to the 156 amino acid long C-terminal segment of this precursor and has a calculated M_r of 17,992 and a very high biological activity. In a few cases,the predicted amino acid sequences of mature IL-1s were confirmed by direct protein analysis of the molecules expressed in, and secreted by, E. c o l i and mammalian expression systems (e.g., Rosenwasser et al. 1986). The organization and the nucleotide sequence of the genes for human IL-1 α and IL-1 β has been described by Furutani and coworkers (1986) and Clark and associates (1986), respectively. The human IL-1α and genes were assigned to the long arm of chromosome 2 (Webb et al. 1986) where they are tandemly organized (D'Eustachio et al. 1987).

All IL-1 precursors shown in Fig.7.7 have one pair or more cysteine residues,a structural condition required for the formation of intramolecular disulfide bonds. However, of the C-terminal fragments of IL-1 cDNAs corresponding to mature IL-1 molecules, only that designated as IL-1β in Fig.7.7 possesses cysteine residues in a number sufficient for disulfide bond formation. In contrast to most secreted proteins, preIL-1 molecules hitherto recognized do not contain typical signal sequences, indicating that these preIL-1s are released from their cellular sources in a manner distinct from other secreted proteins. Although, there are one or more possible N-glycosylation sites in all preIL-1s shown in Fig.7.7, no firm evidence is available indicating that they or the mature proteins could be glycoproteins. Interspecies sequence comparison reveals that, while human IL-1α is rather closely related to the murine sequence (about 62% homology), human IL-1β is

only distantly related both to human IL-1α (about 26% homology) and the murine protein (about 30% homology). There is a significantly higher homology between IL-1α and IL-1β at the nucleic acid level (about 45% homology). On the other hand, while there is a considerable homology between the rabbit and the human IL-1α sequences, the rabbit precursor displays much less homology either with the human IL-1β or the murine proteins. It is interesting to note that, of the residues common both in the human and the murine proteins, 47% occurs in the portions of the primary translation products that preceed the mature M_r 17,500 IL-1 segments. Consequently, these regions in the molecules are more homologous (about 72%) than are the mature IL-1 segments (about 54%). Such a degree of homology would not be expected from a nonfunctional portion of a protein. Therefore, it is likely that the N-terminal halves of the precursors are the material carriers of some as yet unidentified biological function. This is consistent with several reported biological activities associated with high M_r forms of IL-1 (for references see March et al. 1985) which might easily be identical to the conserved N-terminal segments of the IL-1 precursors.(Since the submissions of this manuscript, sequences and characteristics of additional IL-1 (α and β) species of human and murine origin have been published by Cameron et al. 1986, Matsushiam et al. 1986, Rimsky et al. 1986, Telford et al. 1986, Wingfield et al. 1986).

Data presented above give strong support to the notion that mature IL-1s, including human varieties, are proteolytically cleaved from larger precursors yielding forms of about M_r 17,500 (Sahasrabuddhe et al. 1985). On the other hand, in the light of recently collected data, it is highly plausible that, despite the similar spectrum of bioactivities, there are biochemically distinct types of IL-1s forming a rather extended family of molecules (for more detail and references see Allison 1985, Billiau et al. 1985, Cameron et al. 1985, Dinarello 1985, Ihrie and Wood 1985, Arai et al. 1986, Hopp et al. 1986, Kilian et al. 1986, Oppenheimer et al. 1986).

Finally, three additional comments should be made on the biochemistry of IL-1 molecules. A lymphokine, called

osteoclast-activating factor (OAF), a potent calcium releaser in fetal rat long bones, has been purified from stimulated human peripheral mononuclear cells. By comparing its isoelectric point, biological activity and the N-terminal 41 residues with those of hIL-1β, the identity of the two molecules has been established (Dewhirst et al. 1985). Accordingly, hIL-1β is the major protein with OAF activity produced by lectin-stimulated peripheral blood mononuclear cells. On the other hand, it was demonstrated that IL-1β and catabolin, an approximately M_r 22,000 protein produced by stimulated pig mononuclear leukocytes, share many similarities in their biological actions. For instance, both purified catabolin and recombinant hIL-1β induce demineralization (loss of calcium) in bone cultures and, when added to cartilage culture, both induce the degradation of proteoglycan. On this basis, the identity of hIL-1β and (pig) catabolin has been proposed (Saklatvala et al. 1984), and its suggested production by synovial tissue in rheumatoid arthritis may be a central factor in the destruction of joints characteristic of this disease. Due to the significant homology found between the 38 N-terminal amino acid sequence of the M_r 22,000 interferon-inducing factor, a monokine, and that of human and murine IL-1 described by Auron et al. (1984) and Lomedico et al. (1984), this interferon-inducing factor was suggested to be identical with, or closely related to, IL-1s and to be a new member of the IL-1 family of molecules (for more details tails and references see Billiau et al. 1985 and Cameron et al. 1985).

Apart from being an important mediator of acute phase recations including its role as endogenous pyrogen, IL-1 stimulates a large number of T cell functions, such as proliferation, lymphokine production, antigen specific cytotoxicity, helper activity, and the appearance of cell surface differentiation markers. IL-1α and IL-1β bind to the same receptor on T cells (Kilian et al. 1986). In addition, IL-1 may be an important signal in both T and B cell activation, and also in chronic inflammation. As to the latter, IL-1 and/or similar molecules function as endogenous pyrogen, stimulate the pro-

duction of collagenase by fibroblasts and by rheumatoid synovial cells and stimulate the release of acute phase reactants by the liver. However, more recently, separation of pyrogenic activity from IL-1 production has been reported (Allison 1985). IL-1, or a very similar molecule stimulates fibroblast proliferation and therefore may contribute to the fibrosis-accompanying chronic inflammatory diseases, although other macrophage-derived growth factors may also contribute to this (for references see Schmidt 1984). In this context it may be of interest to mention that IL-1s may be homologous to fibroblast growth factors and tumor necrosis factors (see e.g., Hopp et al. 1986). It was reported that, in response to IL-1, osteoblasts also proliferate and generate a short-range signal for osteoclast activation (for references see Duff 1984). On the other hand, IL-1 from different sources of varoius species was reported to inhibit the proliferation of various types of cells (Gaffney and Tsai 1986, Hanazawa et al. 1986, and Tsai et al. 1986). Macrophages may be stimulated to produce IL-1 via CSF secretion by T lymphocytes. Direct stimuli for IL-1 production include many microbial products or their analogs. Vitamin D_3 and interferon-γ can augment this process and hypoxia may stimulate IL-1-producing cells in the lung. While lymphocyte-mediated IL-1 production in delayed-type hypersensitivity has previously been demonstrated, recent observations provide evidence of cell-mediated suppression of this IL-1 release in desensitized animals, indicating that IL-1 itself may have a major effect on the balance between positive and suppressed immunity. Furthermore, as a stimulator of acute phase response, it seems that brain-mediated as well as direct hepatic effects of IL-1 may be important. Patients with rheumatic diseases show varying acute phase responses to different stimuli and disease activity may influence IL-1 responsiveness. In active rheumatoid arthritis, the high levels of synovial fluid IL-1, a characteristic feature of cartilage destruction, synovial proliferation, adjacent osteopenia and muscle wasting, and the systemic changes associated with the acute phase response, such as lymphocyte activation and fever, all suggest that overproduction of IL-1 might be a key patho-

genic factor. Available evidence indicates that IL-1 markedly enhances the production of IL-2 induced by phytohemaglutinin (PHA) or lectin-stimulated macrophage depleted T cells. Since IL-2 is directly mitogenic for T cells, the question of whether the mitogenic effect of IL-1 is direct or is mediated via the induction of IL-2 remains unanswered at the present time. Evidence has been presented that the interplay between IL-1 and IL-2 is a critical requirement for T cells to produce specific effector clones by proliferation. T cells may stimulate macrophages to release IL-1 by producing CSF. Then, the IL-1 thus produced may initiate IL-2 production through the interaction with a specific antigeen and, following an interaction with another specific antigen, IL-1 may also participate in the initial activation of antigen specific IL-2 target cells, a process inevitable for the appearance of IL-2 receptors on these cells. Subsequently, IL-2 may bring about the full activation of the partially activated cells, thereby allowing T cells to express their effector function. IL-1 activity may be assessed by measuring ^{3}H-TdR incorporation by responding cells, e.g., mouse thymocytes (Lachamn et al. 1980). It is one of the factors lacking antigen specificity and exhibiting helper T cell-replacing properties (Aarden 1979, Mizel 1979). IL-1 is also needed for the production of IL-2 by T cells, which are helpers for cytotoxic T cell generation, in response to mitogenic or strong antigenic stimulus in the presence of IL-1.

As to the in vivo role of IL-1, one should remember that this molecule is a potent inducer of several metabolic and immunological changes which can be considered components of the response to inflammation. Some of these responses have a beneficial role in the resolution of many disease states and probably can be utilized in the medical practice in the future (Allison 1985). Other IL-1 induced responses, however, may be harmful by promoting continued host processes of chronic inflammation, thereby contributing to the aggravation of some of the symptomes associated with chronic inflammatory processes (Dinarello 1984b, Kampschmidt 1984, Pujol and Loyau 1987).

That IL-1 may have a role in normal physiology is indicated by the finding that it can be detected in the plasma of healthy women and that IL-1 plasma levels vary with the menstrual cycle (for references see Duff 1985). Thus, IL-1 appears to be a protein, or more correctly, a family of related peptides with local and distant actions on many different tissues. Disregulation of IL-1 production and its effects may contribute to the pathology of several types of diseases. (for additional data on IL-1 see Dinarello 1984a,c, 1987, Duff 1985, Kampschmidt 1984, Farrar 1985, Feldman 1985, Lachman 1985, DeChiara et al. 1986, Dinarello and Mier 1986, Dower et al. 1986a, Kasahara 1986, Kilian et al. 1986, Oppenheimer et al. 1986, Wingfield et al. 1986).

(2) Interleukin-2

It has been widely accepted that regulation of cellular immunity requires a close interaction, or rather, a direct contact between a number of cells. In recent years, however, it became gradually clear that, at leats some of these direct interactions can be replaced with specific soluble mediators. These include a number of T cell-derived factors termed lymphokines. Comparison of various lymphokines indicated that they consist of a restricted number of closely related molecules. One of them, variously called T cell growth factor (TCGF), thymocyte stimulating factor, thymocyte mitogenic factor, killer cell helper factor or costimulator is now designated IL-2, referring to the recently emerged assumption that distinct biological activities that can be inferred from the various names listed above probably reside in the same molecule, or in molecules whose numbers are much more restricted than was formerly thought. IL-2 is a factor involved in cell-mediated and humoral immune responses, and it is also able to restore lost immunogenicity.

Previous studies suggested that human and rat IL-2 might be polypeptides of M_r about 15,000 and a pI of approximately 6.6, whereas murine IL-2 appeared to be a protein or glycoprotein having a M_r of about 30,000 and a pI ranging between 4.3-4.9 (Aarden et al. 1979). The IL-2 produced by mitogen-stimulated

murine lymphomas was reported to be similar, both chemically and biologically, to that produced by normal splenic T cells. Exogenous IL-2 was demonstrated to be stable in tissue culture in the presence of nonresponding cells, but was actively removed from the medium if lymphocytes were stimulated by a "first signal", e.g., concanavalin-A (Con-A). In general, activated T cells, but not resting ones, readily absorb IL-2 (Smith et al. 1979).

Earlier characterization of human IL-2 indicated that the molecule was heterogenous with respect to size and charge, and these heterogeneities were attributed to various degree of glycosylation of the molecule. Microheterogeneity in the amino acid sequence was ruled out by the isolation of a cDNA clone encoding human Il-2 from a cDNA library prepared from partially purified IL-2 mRNA which showed a single amino acid sequence and a single copy of the structural gene (Devos et al. 1983, Taniguchi et al. 1983). Shortly thereafter, Robb and associates (1984) reported the chemical determination of the amino acid sequence of human IL-2 and its posttranslational modification. The directly determined sequence confirmed the sequence deduced from cDNA. Almost simultaneously, the organization and structure of mouse IL-2 gene was also reported, and the amino acid sequence was deduced from the gene sequence (Fuse et al. 1984, Kashima et al. 1985). Human and murine IL-2 exhibits 76% homology. The directly determined amino acid sequence of human IL-2 (Robb et al. 1984) and the deduced amino acid sequence of the murine species (Kashima et al. 1985) are shown in Fig.7.8. Both the human and murine genes have 21 amino acid long signal peptides. The murine peptide has 149 amino acid residues, whereas the human species consists of only 133 residues and has a M_r of 15,820. The presence of a disulfide bridge between cystein residues at positions 58 and 115 in the human peptide has been established (Robb et al. 1984). According to a recent report (Reichert et al. 1987) there is a sequence homology between AIDS virus envelope protein and IL-2.

```
                +                                  10
h: Ala-Pro-Thr-Ser-Ser-Ser-Thr-Lys-Lys      Thr             Gln-Leu-Gln-Leu
                +                                  10                                  20
m: -   -   -   -   -   -   -   Ser-Ser-Ser   -   Ala-Glu-Ala   -   Gln   -   Gln-Gln-Gln-

                                                                   20
h:                      Glu-His                 Leu-Leu-Leu-Asp-Leu-Gln-Met-Ile-Leu-Asp-
                                                30                                  40
m: Gln-Gln-Gln-Gln-Gln-Gln   -   Leu-Glu-Gln   -   -   Met   -   -   -   Glu-Leu   -   Ser-

                30                                 40
h: Gly-Ile-Asn-Asn-Tyr-Lys-Asn-Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr-Met-
                                                50                                  60
m: Arg-Met-Glu   -   -   Arg   -   Leu   -   -   Pro   -   -   -   -   -   -   -   -   Leu-

                50                                 60
h: Pro-Lys-Lys-Ala-Thr-Glu-Leu-Lys-His-Leu-Gln-Cys-Leu-Glu-Glu-Glu-Leu-Lys-Pro-Leu-
                                                70                                  80
m:   -   -   Gln   -   -   -   -   -   Asp   -   -   -   -   -   Asp   -   -   Gly   -   -

                70                                 80
h: Glu-Glu-Val-Leu-Asn-Leu-Ala-Gln-Ser-Lys-Asn-Phe-His-Leu      Arg-Pro-Arg-Asp-Leu-
                                                90                                  100
m: Arg-His   -   -   Asp   -   Thr   -   -   -   Ser   -   Gln   -   Glu-Asp-Ala-Glu-Asn-Phe-

                90                                 100
h: Ile-Ser-Asn-Ile-Asn-Val-Ile-Val-Leu-Glu-Leu-Lys-Gly-Ser-Glu-Thr-Thr-Phe-Met-Cys-
                                                110                                 120
m:   -   -   -   -   Arg   -   Thr   -   Val-Lys   -   -   -   -   Asp-Asn   -   -   Glu   -

                110                                120
h: Glu-Tyr-Ala-Asp-Glu-Thr-Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys-
                                                130                                 140
m: Gln Phe-Asp   -   -   Ser   -   -   Val   -   Asp   -   -   Arg   -   -   -   Ala   -   -

                130        133
h: Gln-Ser-Ile-Ile-Ser-Thr-Leu-Thr
                                   149
m:   -   -   -   -   -   -   Ser-Pro-Gln
```

Fig. 7.8. Amino acid sequence of the mature human (h) interleukin-2 and cDNA-derived primary structure of the murine (m) protein. Alignment was made for maximizing homology. Dashes indicate residues identical to those in the human sequence. Crossmarked residue represents a possible glycosylation site

The presence of twelve glutamines in sequence (residues at positions 15 through 26) in murine IL-2 may account for the observation that, while human IL-2 acts on mouse cells, mouse IL-2 does not act on human cells (for more details see Feldmann 1985). Using various methods, natural human IL-2 could be separated into multiple molecular forms. For the most part, this heterogeneity was attributed to a variation in glycosylation of the threonine residue in position 3 of the polypeptide chain (see Fig.7.8). Since the various molecular forms have nearly identical specific activities in the in vitro T cell proliferation assay, the glycosylation does not seem to play a major role in biological activity of the molecule. By using human-mouse somatic hybrids and Southern filter hybridization

of cell hybrid DNA, the 4,930 base pair long human gene for IL-2 was assigned to chromosome 4 (Shows et al. 1984). Each species of mammals so far tested had only a single copy of IL-2 gene.

By using a combination of molecular cloning and more conservative techniques, Clark and associates (1984) produced, sequenced and expressed a human TCGF/IL-2 cDNA clone prepared from both peripheral blood lymphocytes and a leukemic T cell line, i.e., Jurkat. Deduced amino acid sequence of this TCGF/IL-2, which was also confirmed by partial sequence analysis of the protein, proved to be identical with the chemically determined sequence reported by Robb and coworkers (1984). Comparison of amino acid sequences of TCGF/IL-2 prepared from normal blood lymphocytes and the Jurkat leukemic T cell line revealed the identity of the two sequences despite observed difference in a single nucleotide of corresponding cDNAs.

More recently, Kato et al. (1985a) have reported the purification and partial sequence analysis of a human IL-2 species derived from peripheral blood leukocytes. In this case IL-2 activity could be resolved by column chromatography into three peaks of activity, termed IL-2A,B and C, with pIs 7.2, 6.6 and 7.9, respectively. All three peaks of activity were further purified by HPLC and resolved into two apparently homogenous peaks each with identical M_r: A-1 and A-2 (M_r 17,000), B-1 and B-2 (M_r 17,500) and C-1 and C-2 (M_r 14,400). The analysis of amino acid composition and N-terminal amino acid sequences revealed the identity of these molecular species with those predicted from IL-2 cDNA sequences derived from Jurkat and peripheral blood leukocytes. Kato and associates (1985b) have also purified a human recombinant IL-2 (rIL-2) produced in E. c o l i. Several chemical properties of this rIL-2 were consistent with those deduced from the cDNA sequence. Besides the molecular form with the N-terminal Ala residue, the purified preparation contained another species having an additional Met residue at the N terminus. Ultracentrifugal analysis showed that rIL-2 existed as a monomeric form in 0.1 M NaCl. Lymphokines with TCGF activity that are molecularly and/or

functionally distinct from IL-2 have been recently reported from a number of laboratories (Kupper et al. 1986, Milstone and Parker 1986, Smith and Rennick 1986). The molecular characteristics of the IL-2 system is discussed in a recent review by Taniguchi and associates (1986).

The proliferogenic activity, and thus the growth factor character of IL-2 is stressed, when IL-2 is defined as a class of molecules which stimulate the continuous proliferation of T cells in culture, an effect of paramount importance in T cell growth and regulation (Aarden 1979, Farrar et al. 1986, Stern and Smith 1986). IL-2 participates in cell mediated immune response by eliciting strong CTL responses. IL-2 is constantly required for the proliferation of long term CTL lines (Gillis et al. 1978). Beside enhancing T cell proliferation, IL-2 also stimulates the generation of helper and cytotoxic T cells, and sustains cell proliferation in antigen-specific T helper and cytotoxic cell lines. The IL-2-induced proliferative effects on cytotoxic/helper T cell lines implies that IL-2 directly interacts with T cells (Watson 1979, Mochizuki et al. 1980). It is involved in the amplification of the production of cytotoxic T cells, in the stimulation of lectin-induced mitogenic response in thymocyte culture at limitating cell density, and in the maintanance of the proliferation of antigen activated T cells. Briefly, IL-2 is produced by T cells which are helpers for CTL generation in response to a mitogenic or a strong antigenic stimulus in the presence of IL-1. Murine, rat and human lymphocytes can all be induced to secrete IL-2 activity, previously defined as a T cell growth promoting activity (Gillis et al. 1980). IL-2 may act directly on the proliferation of T cells in culture that were previously activated by antigens. Thus, IL-2 would stimulate the growth of T cells in culture by exerting an antigen nonspecific, or hormonal effect on T cells. Ia or Ia-like antigens seem to play an important role in T cell proliferation (Paetkau et al. 1980). It appears that the antigen stimulates the initial activation of T cells and confers specificity on the cells as a first signal, whereas IL-2, while not involved in the activation process, is required as a second signal to cause the clonal expansion of

activated T cells (Ruscetti et al. 1978, Bonnard et al. 1979, Kurnik et al. 1979). Production of IL-2 by macrophage-depleted T cell populations can be markedly enhanced in the presence of mitogens. Since IL-2 is directly mitogenic for T cells, it is not clear as to whether the mitogenic effect of IL-1 is direct, or is mediated via the induction of IL-2. By using IL-2, antigen-specific cytotoxic, suppressor and helper T cells may be grown for extended periods without loosing specificity (helper, cytotoxic, or killer activity). It is now evident that the IL-2/T cell ratio is important in maintaining the growth of T cells (Ruscetti et al. 1980). Beside T cells, IL-2 also stimulates the proliferation of other types of cells (Benveniste and Merrill 1986, Lehmann et.al. 1986) and exerts a wound-healing promoting activity as well (Barbul et al. 1986). The capability of IL-2 to rapidly induce phosphorylation of cellular proteins (Ishi et al. 1986, Kohno et al. 1986) may be associated with its proliferogenic activity.

It appears that the IL-2-releasing T cells belong to a functionally distinct T cell subset: removal of Ly cells from the population completely abrogates IL-2 release. For the production of IL-2, mature T cells are required. IL-2 is only generated after mitogen and antigen stimulation. Its generation also requires the participation of adherent cells, probably macrophages, but the latter can be replaced by macrophage-derived IL-2 (Larsson et al. 1980, Pilarski et al. 1980, Smith et al. 1980). One of the systems most widely used for studying the effects of IL-2 consists of mitogen-stimulated murine splenocytes. Mitogen-stimulated murine T cell lymphomas, LBRM-33 and RBL-31 proved to be especially rich sources of IL-2 (Watson et al. 1981). TCGF/IL-2 activity, however, can also be studied in lectin-stimulated human peripheral blood lymphocytes (Ruscetti et al. 1980).

As to the mechanism of action of IL-2, it might utilize a common mechanism via the clonal expansion of antigen or mitogen activated T cells (Watson et al. 1981). T cells may release CSF that stimulates macrophages to release IL-1. IL-1, in conjunction with a specific antigen initiates IL-2 production and may, in addition, participate (in concert with anoth-

er specific antigen) in the initial activation of the antigen-specific IL-2 target cells, an event required for the appearance of IL-2 receptors on these cells. IL-2 may then convert these partially activated cells to a fully activated state, at which the T cells express their effector function, e.g., cytotoxicity.

LBRM-derived IL-2 seems to exert a helper T cell-replacing activity. On the other hand, mitogen activated murine splenocytes appear to secrete both IL-2 and another class of T cell-replacing factor (TRF),two distinct entities, although the cellular requirements for the production of both seem to be similar (Metcalf et al. 1980). TRF may exhibit an antigen specific activity and may be secreted by T cells, but bind cytophillically to another cell type in the cooperating system to provide helper activity (Watson et al. 1981). (TRF is discussed more exhaustively in section 7.4.2.2.4.). It is disturbing that most of the presently available IL-2 preparations are contaminated with IL-2 inducible immune interferon, and it is almost impossible to discount the role of interferon production by T cells. It is, therefore, hard to distinguish between IL-2, which induces interferon and restores immunogenicity, and the interferon itself. Due to the multitude of T cell-derived lymphokines and to the presently poor status of their purification, it is evident that only the molecular characterization of various lymphokine activities will allow the distinction of their biological activities.

Initiation of DNA synthesis and cellular proliferation has no effect on the releaese of TCGF (Ruscetti et al. 1980). However, the inhibition of protein synthesis eliminates TCGF production. Thus, TCGF/IL-2 production can occur in non-dividing cells, probably in terminally differentiated T cells, without the need for proliferation, but requires the binding of a stimulatory agent, and also protein synthesis. It is not known, whether the cells involved in the production of TCGF/IL-2 proliferate in response to TCGF/IL-2 or not.

Cells that respond to IL-2 possess a surface receptor glycoprotein specific for this lymphokine. Recently, Cosman and associates (1984) and Waldmann and coworkers (1985) re-

ported the cloning, sequencing and expression of human IL-2 receptor, together with several important characteristics of this membrane receptor protein. Analytical data revealed that the receptor, which could easily be composed of 50% carbohydrate, is a rather extraordinary molecule if its carbohydrate content is compared to those of other glycoprotein receptor molecules hitherto characterized. IL-2 was reported to regulate the expression of its own receptor and the synthesis of interferon-γ by human T lymphocytes (Smith and Cantrell 1985). The gene encoding human IL-2 receptor was assigned to chromosome 10 (Leonard 1985). Molecular cloning and structure analysis of the genes for both human and mouse IL-2 receptors has been reported also from other laboratories (Ishida et al. 1985, Miller et al. 1985, Shimizu et al. 1985). It should be mentioned, however, that despite abundance of data available on IL-2 receptor,including those reported on purification of the receptor from both normal and transformed lymphocytes (Urdal et al. 1984) and the isolation of the gene (cDNA) encoding receptor protein (Cosman et al. 1984, Nikaido et al. 1984, Leonard et al. 1985), structural data hitherto reported have been rather contradictory. This may indicate that the functional IL-2 receptor remains to be identified in the future (for a recent review on IL-2 receptor see Waldmann et al. 1985).

Although, the physiological role of IL-2 is far from clear at present, this molecule has become a valuable tool for the selection and generation of cloned lines of antigen-specific T cells. Recent availability of recombinant human IL-2 in large quantities has allowed in vivo testing of this molecule. It was found that IL-2 had profound effects, leading to a transient decrease in T cells and an increase in monocytes, a marked and prolonged eosinophilia and a marked gain in weight (for references see Feldman 1985). This phase I studies precede a prospective trial of IL-2 in cancer patients based on its beneficial effect on metastatic tumors in mice (Greenberg and Cheever 1984). IL-2 may also prove to be a useful agent for treating patients with acquired immunodeficiency syndrome (Siegel et al. 1986). Regarding the pivotal role that IL-2 has

been shown to play in T-cell proliferation, this lymphokine may also manipulate host response as a natural mediator of immunity. Results obtained in in vivo experiments suggest that IL-2 may have significant therapeutic potential for in vivo stimulation of T-cell responses that are relatively deficient in the production of endogenous IL-2 (Cheever et al. 1984). A somewhat different type of potential clinical use of IL-2 is suggested by several recent reports indicating that systemic administration of IL-2-activated lymphocytes may be a powerful tool for the immunotherapy of cancer and its metastases (Herberman 1984, Mazumder and Rosenberg 1984, Miyasaka et al. 1984, Rosenberg 1984, Rosenberg et al. 1985). (For a report on a recent meeting on lymphokines see Feldman 1985, while for recent reviews on IL-2 see Mertelsmann and Welte 1984, Paetkau 1985, Smith 1985, Watson et al. 1985, Borradon et al. 1987, Fletcher and Goldstein 1987.)

(3) Interleukin-3

Because interleukin-3 (IL-3) mainly acts as a mediator in the regulation of growth and differentiation of hemopoietic cells and possesses many biological properties characteristic of growth factors, IL-3 is discussed with the growth factors in Chapter 8 of this volume.

(4) Additional interleukins, B-cell growth and differentiation factors. The term interleukin merely refers to endogenous substances that carry messages between leukocytes. In the last few years, there has been a disturbing confusion in the interleukin terminology that especially concerned IL-4, IL-5 and IL-6, respectively (Sanderson and Klaus 1986, Smith 1986; see also an Editorial Note in the journal: Immunology Today 7:68, 1986). Fortunately, however, most of the difficulties of the nomenclature of ILs has been resolved for today, as it can be inferred from the forthcoming discussion.

The complexity of lymphokine regulation of B cell functions stands in sharp contrast to that of T cells, whose growth and differentiation appears to be regulated primarily by antigen recognized in association with molecules of the major histo-

compability complex (MHC) and a single T-cell product, IL-2.

A major goal of B-cell physiology is the development of long-term cultures of normal B-cell lines which can ultimately be cloned. This goal has precipitated the search for growth factors which can maintain B-cell proliferation, as IL-2 can T cell proliferation. Results collected initially, have demonstrated the existence of T cell-derived B-cell growth factor(s) (BCGF) also termed B-cell stimulatory fator(s) (BSF, Kishimoto 1985) which are able to promote the proliferation of activated B cells (Howard and Paul 1983). By using various culture and biochemical methods for producing and characterizing BCGF it was first suggested that human BCGF and IL-2 were biochemically similar but were indeed separate molecules (for details and references see Kehri et al. 1984). However, it became clear soon that more than a single BCGF exists. Until recently, macrophage- and T cell-derived soluble factors acting on B cells were functionally divided into two groups: one included BCGFs thought to be involved in B-cell proliferation, and the other comprised B cell differentiation factors (BCDFs) responsible for the maturation of activated B cells into immunoglobulin-secreting cells. However, this classification needs to be reexamined in the light of recent findings indicating that one and the same molecule may have both BCGF and BCDF activities and may simultaneously affect B cells, T cells and mast cells, respectively (see e.g., O'Garra et al. 1987).

Swain and coworkers (1983) reported a Dennert line C.C3.11.75-derived growth factor, termed (DL)BCGF which maintained the growth of the BCL1 tumor line in vitro and displayed characteristics that were different from those of BCGF previously reported by Howard and associates (1982). The latter was prepared from the murine thymoma EL4 and termed (EL4)BCGF. Besides physicochemical and biological differences, the two BCGFs also differ in their apparent molecular weight: while (EL4)BCGF has a M_r of about 18,000, (DL)BCGF has a M_r somewhere between 50,000-70,000. Due to these differences, Swain et al. (1983) termed (EL4)BCGF as BCGF I and (DL)BCGF as BCGF II. Furthermore, Yoshizaki et al. (1983) described two distinct forms of human BCGF which showed a synergistic effect

on anti-Ig-induced B cell proliferation. One of them was obtained from PHA-stimulated human T cells having a M_r of 17,000, whereas the other was isolated from supernatant of an IL-2-dependent T cell line and had a M_r of 50,000. A 18,000-20,000 M_r BCGF produced by a human T-T hybridoma was reported by Butler et al. (1983) whose similarity to murine BCGF I was stressed by the authors. More recently, an increasing number of publications provide evidence of the existence of B cell-derived BCGFs, indicating an immense complexity of the regulation of B cell activation (for more details see Brooks et al. 1984, Gordon et al. 1984, Nakajima et al. 1985). Partial and more extensive purification and characterization of both murine and human BSFs/BCGFs has recently been reported from several laboratories (Hirano et al. 1985, Mehta et al. 1985, and Ohara et al. 1985) and data on the mechanism of action of these factors have also been published (Rabin et al. 1985).

It follows from the foregoing discussion that the field of factors involved in the regulation of B cell growth and functions, and especially the nomenclature presently is in a rather confused state. This is due to a significant extent to the fact that the purity of the previously described factors, that were isolated from complex T cell supernatants was in many cases suspect and, with the exception of IL-2 and interferon-γ, the cloned gene products exhibiting functional activities did not, until recently, exist. The situation was somewhat simplified by Ohara and associates (1985) who have purified to homogeneity a T cell-derived protein of M_r 20,000, termed B cell-stimulatory factor-1 (BSF-1) that was active on several functional tests in vitro. This protein was shown to exhibit both BCGF and BCDF activities and the ability to induce expression of class II MHC molecules on resting B cells (for references see Cambier 1986). This was the first suggestion that the same or very closely related lymphokines may mediate multiple activities.

(a) Interleukin-4. The recent cloning of a mouse (Lee et al. 1986, Noma et al. 1986) and a human gene (Yokota et al. 1986) both encoding proteins of M_r 20,000 which function in

```
                                                10                  20
h: Met-Gly-Leu-Thr-Ser-Gln-Leu-Leu-Pro-Pro-Leu-Phe-Phe-Leu-Leu-Ala-Cys-Ala-Gly-Asn-
                                                10                  20
m:  -   -   -  Asn-Pro  -   -  Val-Val-Ile  -  Leu  -  Phe  -  Glu  -  Thr-Arg-Ser
    +                                       30
h: Phe-Val-His-Gly-His-Lys-Cys-Asp      Ile-Thr-Leu-Gln-Glu-Ile-Ile-Lys-Thr-Leu-Asn-
    +                                   30
m: His-Ile  -   -          -   -  Lys-Asn-His  -  Arg  -   -   -  Gly-Ile  -   -
    40                                  50
h: Ser-Leu-Thr      Glu-Gln-Lys-Thr-Leu-Cys-Thr-Glu-Leu-Thr-Val-Thr-Asp-Ile-Phe-Ala-
        40                              50
m: Glu-Val  -  Gly  -      Gly  -  Pro  -   -   - Met-Asp  -  Pro-Asn-Val-Leu-Thr-
        60                          70
h: Ala-Ser-Lys-Asn-Thr-Thr-Glu-Lys-Glu-Thr-Phe-Cys-Arg-Ala-Ala-Thr-Val-Leu-Arg-Gln-
            60                          70
m:  -  Thr  -   -   -   -   -  Ser  -  Leu-Val  -   -   -  Ser-Lys  -   -   -  Ile-
        80                          90
h: Phe-Tyr-Ser-His-His-Glu-Lys-Asp-Thr-Arg-Cys-Leu-Gly-Ala-Thr-Ala-Gln-Gln-Phe-Met-
            80                          90
m:  -   -  Leu-Lys  -  Gly  -      -  Pro  -   -  Lys-Lys-Asn-Ser-Ser-Val-Leu-Met-
       100                                      110
h: Arg-His-Lys-Gln      Leu-Ile-Arg      Phe-Leu-Lys-Arg      Leu-Asp-Arg-Asn-Leu-Trp-
                   100                                      110
m: Glu-Leu      -  Arg  -  Phe  -  Ala  -          -  Cys  -   -  Ser
                   120                                  130
h: Gly-Leu-Ala-Gly-Leu-Asn-Ser-Cys-Pro-Val-Lys-Glu-Ala-Asn-Gln-Ser          Thr
                                                        120
m:                 Ser-Ile  -   -  Thr-Met              Asn-Glu  -  Lys-Ser  -  Ser-
                                  140                                   150        153
h: Leu-Glu-Asn-Phe-Leu-Glu-Arg-leu-Lys-Thr-Ile-Met-Arg-Glu-Lys-Tyr-Ser-Lys-Cys-Ser-Ser
                                  130                                   140
m:  -  Lys-Asp  -   -   -  Ser  -   -  Ser  -   -  Gln-Met-Asp  -   -
```

Fig.7.9. cDNA-derived amino acid sequences of the precursors of human (h) and murine (m) IgG1-inducing factors/interleukin-4. Dashes indicate residues identical to those of the human sequence. Probable N-termini of the mature proteins are indicated by crossmarked residues. Underlined residues represent possible glycosylation sites. Alignment was made for maximizing homology

several assays used for studying factors acting on B cells should be regarded as a major breakthrough in the field of research concerned with BCGF/BCDF. Deduced amino acid sequences of proteins encoded by a murine and a human cDNA, respectively, are shown in Fig.7.9. The murine sequences separately published by Lee and associates (1986) and Noma and coworkers (1986) proved to be identical and to correspond to that shown in the figure. The murine cDNA encodes a protein consisting of 140 amino acid residues. The mature protein, termed IgG1-inducing factor, but also named IL-4, probably has 120 amino acid residues and begins with a histidine residue at position 21 of the encoded precursor. There are three potential glycosylation sites at positions 61-63, 91-93 and 117-119. Although the biological activities of this molecule and those of IL-2 and IL-3 are similar, there is no significant sequence homology between IL-4 and either IL-2 or IL-3.

The corresponding human cDNA encodes a 153 amino acid precursor, containing two potential glycosylation sites at positions 62-64 and 129-131. The mature protein probably has 133 amino acid residues with a phenylalanyl residue at position 21 of the precursor as its N terminus. The mouse and human polypeptides share extensive homology with the exception of about 40 amino acids near the middle portion of the molecules.

Murine IgG1-inducing factor/IL-4 displays both growth and differentiation factor activities and affects B cells, T cells and mast cells. As a major effect it induces Ia expression on resting B cells and enhances IgG, and IgE production by B cells, two characteristic properties of BSF-1 (Roehm et al. 1984, Vitetta et al. 1985, Coffman and Carty 1986). On the other hand, supernatants of COS 7 monkey cells transfected with a human IL-4 cDNA clone stimulated the proliferation of human helper T cell clones (see also Zlotnik et al. 1987) and that of anti-IgM-activated human B cells, in the mouse assay system which are two additional characteristics of BSF-1. These results indicate that the human protein is structurally and functionally homologous to mouse BSF-1 and thus the names IgG1-inducing factor, IL-4, BSF-1 and BCGF I are synonyms for the same or closely related mediator molecules (for reviews see O'Garra et al. 1987, Paul 1987, Paul and Ohara 1987).

(b) Interleukin-5. It has been well established both in murine and human systems that T cells elaborate nonspecific soluble factors which mediate T cell helper function for Ig production by normal B cells or B cell lines (for references see Kehri et al. 1984). These factors have been referred to as T cell-replacing factor (TRF). Although many properties of TRFs have been characterized previously (see e.g., Watson et al. 1979) their detailed chemical characteristics are emerging only just now. This is supported by the cloning of a cDNA for human (Azuma et al. 1986) and murine (Kinashi et al. 1986) TRF, respectively. Predicted amino acid sequences of putative mature TRFs from these two species are shown in Fig.7.10 as-

suming that the putative signal sequences comprising 22 (human) and 21 (mouse) hydrophobic amino acids, respectively, are cleaved off the N-terminal end of the encoded 134 (human) and 133 amino acid (mouse) precursors. Thus, both types of mature TRFs would have 112 amino acid residues and a M_r of about 12,800. The human and the murine peptide has two and three possible glycosylation sites, respectively. Of these, two are in identical position (residues 25-27 and 68-70), whilst the third site located at positions 54 through 56 in the murine sequence is lacking from the human peptide. Of the three cysteine residues in murine TRF, the C-terminal two are conserved in the human molecule. The nucleotide and amino acid sequences of the coding regions of human and murine TRF cDNAs are 77% and 70% homologous, respectively. The homology of human and murine TRF is more marked in the C-terminal half (80%) than in the N-terminal half (66%) of the putative mature peptides.

TRF induces IgM secretion from BCL1 leukemic B cell line, and secondary IgG synthesis in antigen primed B cells. Due to this property, TRF derived from B151K12 cells, a murine T cell hybridoma cell line, was first classified as a BCDF. However, purified TRF also has BCGF II activity, stimulating the proliferation of B cells. Systematic comparative studies on the biological activities and cDNA-derived amino acid sequences of

```
                                        10                                  20
h: Glu-Ile-Pro-Thr-Ser-Ala-Leu-Val-Lys-Glu-Thr-Leu-Ala-Leu-Leu-Ser-Thr-His-Arg-Thr-
m:  -   -   - Met  -  Thr-Val  -   -   -   -   - Thr-Gln  -   - Ala  -   - Ala
                                        30                                  40
h: Leu-Leu-Ile-Ala-Asn-Glu-Thr-Leu-Arg-Ile-Pro-Val-Pro-Val-His-Lys-Asn-His-Gln-Leu-
m:  -   - Thr-Ser  -   -   - Met  -  Leu  -   -   - Thr  -   -   -   -   -   -
                                        50                                  60
h: Cys-Thr-Glu-Glu-Ile-Phe-Gln-Gly-Ile-Gly-Thr-Leu-Glu-Ser-Gln-Thr-Val-Gln-Gly-Gly-
m:  - Ile-Gly  -   -   -   -   - Leu-Asp-Ile  - Lys-Asn  -   -   - Arg  -   -
                                        70                                  80
h: Thr-Val-Glu-Arg-Leu-Phe-Lys-Asn-Leu-Ser-Leu-Ile-Lys-Lys-Tyr-Ile-Asp-Gly-Gln-Lys-
m:  -   -   - Met  -   - Gln  -   -   -   -   -   -   -   -   -   - Arg  -   -
                                        90                                 100
h: Lys-Lys-Cys-Gly-Glu-Glu-Arg-Arg-Arg-Val-Asn-Gln-Phe-Leu-Asp-Tyr-Leu-Gln-Glu-Phe-
m: Glu  -   -   -   -   -   -   -   -   - Thr-Arg  -   -   -   -   -   -   -   -   -
                                       110    112
h: Leu-Gly-Val-Met-Asn-Thr-Gly-Trp-Ile-Ile-Glu-Ser
m:  -   -   -   - Ser  -   -   - Ala-Met  - Gly
```

Fig. 7.10. cDNA-derived amino acid sequences of the mature forms of human (h) and murine (m) T-cell-replacing factor/interleukin-5. Dashes indicate identical residues to the human protein. Underlined residues represent possible glycosylation sites

differently named molecules from murine and human species finally led to the recognition that TRF, BCGF II and eosinophil differentitation factor (EDF) are one and the same molecules (see e.g., Kinashi et al. 1986, Sanderson et al. 1986, Campbell et al. 1987). On the other hand, because TRF was claimed to be the fifth structurally identified interleukin, the term IL-5 was also proposed to designate this molecule (Kinashi et al. 1986; for information on the structure of the human IL-5 gene see Tanabe et al. 1987).

(c) Interleukin-6. Another human factor with interleukin characteristics, termed BCDF/BSF-2 has been purified to homogeneity from the culture supernatant of TCL-Nal cells, a human T cell line, and its N-terminal 12 amino acids sequenced (Hirano et al. 1985). Subsequently, a cDNA encoding the precursor of this protein was cloned and its nucleotide sequence determined in the same laboratory (Hirano et al. 1986). Knowing the N-terminal sequence of the purified protein, the amino acid sequence of the putative mature protein could be deduced from the precursor sequence.

BSF-2 is initially made as a 212 amino acid precursor and is subsequently processed into a mature form by cleaving off the N-terminal 28 amino acids from the precursor. Hydrophobic amino acids are abundant in the cleaved off region that seems to be a typical signal peptide. Thus, the putative mature protein probably consists of 184 amino acids ordered in the following sequence:

Pro-Val-Pro-Pro-Gly-Glu-Asp-Ser-Lys-Asp-Val-Ala-Ala-Pro-His-
Arg-Asn-Pro-Leu-Thr-Ser-Ser-Glu-Arg-Ile-Asp-Lys-Asn-Ile-Arg-
Tyr-Ile-Leu-Asp-Gly-Ile-Ser-Ala-Leu-Arg-Lys-Glu-Thr-Cys-Asn-
Lys-Ser-Asn-Met-Cys-Glu-Ser-Ser-Lys-Glu-Ala-Leu-Ala-Glu-Asn-
Asn-Leu-Asn-Leu-Pro-Lys-Met-Ala-Glu-Lys-Asp-Gly-Cys-Phe-Gln-
Ser-Gly-Phe-Asn-Glu-Glu-Thr-Cys-Leu-Val-Lys-Ile-Ile-Thr-Gly-
Leu-Leu-Glu-Phe-Glu-Val-Tyr-Leu-Glu-Tyr-Leu-Gln-Asn-Arg-Phe-
Glu-Ser-Ser-Glu-Glu-Gln-Ala-Arg-Ala-Val-Asn-Met-Ser-Thr-Lys-
Val-Leu-Ile-Asn-Phe-Leu-Gln-Lys-Lys-Ala-Lys-Asn-Leu-Asp-Ala-
Ile-Thr-Thr-Pro-Asp-Pro-Thr-Thr-Asn-Ala-Ser-Leu-Leu-Thr-Lys-

Leu-Gln-Ala-Gln-Asn-Gln-Trp-Leu-Gln-Asp-Met-Thr-Thr-His-Leu-Ile-Leu-Arg-Ser-Phe-Lys-Glu-Phe-Leu-Gln-Ser-Ser-Leu-Arg-Ala-Leu-Arg-Gln-Met (underlined residues indicate possible glycosylation sites). Partial amino acid sequence of the purified protein agrees well with that deduced from the cDNA. There are two potential glycosylation sites (positions 45-47 and 144-146) in human BSF-2, but it is not determined yet wheter BSF-2 is a glycoprotein. The calculated M_r of the protein is 20,718. Human G-CSF showed a similarity to BSF-2 with an overall sequence homology of about 25%. Cysteine residues at positions 44, 50, 73 and 83 in BSF-2 matches those at positions 39, 45, 67 and 77 in G-CSF. This indicates that BSF-2 is distantly related to G-CSF.

On the other hand, the structural identity of human BSF-2 interferonβ_2 (Sehgal and May 1987, Seghal et al. 1987), HPl an, hybridoma/plasmacytoma growth factor and the fibroblast-derived 26 kD protein (Van Damme et al. 1987) has recently been demonstrated. In addition BSF-2 was also shown to share identity with monocyte-derived hepatocyte (Gauldie et al. 1987) and B cell growth factors (Tosato et al. 1988). To resolve prescribing nomenclature confusion, the term IL-6 was proposed to designate this multinamed molecule (Van Damme et al. 1987). As its major biological activity, BSF-2 induces the final maturation of B cells into immunoglobulin-secreting cells, pointing to its BCDF character, but it also exerts a growth-modulating effect on B cells representing a BCGF activity (Poupart et al. 1987; for other activities of BSF-2 see Andus et al. 1987). T and B cell-derived BCGFs render the activated B cells susceptible to lineage-specific growth factors (see Chapter 8). Other molecules described as human BCGFs include low (M_r~12,000) and high molecular weight (M_r~50,000-60,000) T cell-derived factors. Of these, the gene for a human BCGF related to the low molecular weight species has been molecularly cloned and sequenced from lectin-stimulated T cells (Sharma et al. 1987). The cDNA-derived amino acid sequence of the 106 amino acid mature protein encoded by this gene is as follows:

Gly-Gln-Glu-Phe-Gln-Asn-Ile-Leu-Tyr-Leu-Asp-Cys-Gly-Gly-Ser-Tyr-Thr-Thr-Asp-Ile-Asn-Leu-Ile-Lys-Tyr-Gln-Asp-Trp-Ile-His-Leu-Lys-Gly-Arg-Leu-Leu-Leu-Tyr-Glu-Val-Tyr-Leu-Ile-Asn-Asn-Gln-Pro-Lys-Asn-Leu-Cys-Ser-His-Phe-Ser-Phe-Pro-Thr-Thr-Tyr-Ile-Lys-Lys-Glu-Arg-Leu-Trp-Leu-Gly-Pro-Val-Ala-His-Thr-Tyr-Asn-Pro-Ser-Thr-Leu-Gly-Gly-Arg-Gly-Gly-Trp-Ile-Thr-Arg-Gly-Gln-Glu-Phe-Lys-Thr-Ser-Leu-Ala-Asn-Met-Val-Glu-Pro-Cys-Leu-Tyr. It remains to be determined if the high molecular weight BCGFs are equivalent to IL-5 or are precursors for the low molecular weight class (for references see O'Garra et al. 1988).

The observations discussed above convincingly demonstrate that many B cell-modulatory activities previously ascribed to a multitude of molecular species can be mediated by much less lymphokines than formerly thought, and that a single lymphokine interacting with B cells in distinct differentiative states may have different regulatory effects. This, of course, has far reaching implications suggesting that a ligand interacting with its receptor on cells in different states results in the activation or inactivation of different gene sets. How this may happen at the molecular level remains to be elucidated. The availability of cloned gene products described here and that of isolated B cell populations in defined differentiative states will certainly help in answering these questions.

Recently collected data indicate that B cell activation occurs as follows. Normal B cell can be trigerred to the point of activation without entrance into the S phase by low concentration of anti-Ig antibody. Among others, these activated cells express receptors for BCGF. In the presence of BCGF, the B cells undergo early to late G1 transition, express transferrin receptors and enter the S phase without terminal differentiation. Besides BCGF, IL-1 and interferon can costimulate with anti-Ig, although to a lesser degree. If T cell-derived BCDF is supplied to proliferating B cells, the latter will differentiate into Ig-producing cells. IL-1 can enhance the differentiation of B cells induced by BCDF. The role of IL-2 in the human B cell activation pathway remains unresolved, al-

though activated B cells express IL-2 receptors (for more details and references on B cell activation see Kehri et al. 1984; the attention of interested readers is directed to Vol. 78 of Immunological Reviews (1984) which is devoted to the discussion of the most varied aspects of B cell growth and differentiation factors. Further valuable readings concerning this topic can be found in the following papers and reviews: Kishimoto 1985, Muraguchi et al. 1985, Anderson and Melchers 1986, Arai et al. 1986, Cambier 1986, Dorshkind 1986, Fernandez-Botran et al. 1986, Hamaoka and Ono 1986, Jurgensen et al. 1986, Mosman et al. 1986, Muraguchi et al. 1986, Rabin et al. 1986, Romagnani et al. 1986, Sahaskabuddhe et al. 1986, O'Garra et al. 1988 (see also colony-stimulating factors in Chapter 8).

7.4.2.3. TUFTSIN

In 1967, a research team working at Tufts University in Boston, demonstrated the existence of leukokinin, a leukophilic immunoglobulin of the G class, which binds to, and stimulates the phagocytic activity of granulocytes, monocytes and of tissue macrophages (for references see Werner et al. 1986). Subsequently, it was shown that a tetrapeptide, Thr-Lys-Pro-Arg, corresponding to residues 289-292 of the heavy chain of leukokinin was responsible for the phagocyte-stimulating activity of this particular IgG species (Nishioka et al. 1972). The tetrapeptide was named tuftsin, referring to Tufts University, the place of its discovery. Two enzymes, tuftsin endopeptidase (in the spleen) and leukokinase (on the outer side of the plasma membrane of phagocytic cells) are involved in the liberation of tuftsin from the parent carrier IgG (Najjar 1979, Fridkin and Gottlieb 1981). The biologically active conformation of tuftsin has been reported by Nikiforovich et al. (1984).

In vivo, tuftsin stimulates all functions of phagocytic cells such as pino- and phagocytosis, motility (chemotactic activity), antigen processing, bactericidal and tumoricidal activities, and it modulates the humoral and cell-mediated im-

mune responses. Tuftsin was shown to enhance natural cytotoxicity against tumor cells, an effect mediated by macrophages and monocytes as well as by NK cells. In the mouse, tuftsin enhances the resistance against experimental microbial infections and delays mortality in several tumor transplantation models. Upon repeated administration, tuftsin exerts a restorative activity on some immunodepressed functions of aged mice (Nishioka 1978, 1979, Catane et al. 1981, Najjar and Fridkin 1983, Bump and Najjar 1984, Bump et al. 1985). On the other hand, this peptide stimulates the growth of HL60 leukemic cells in vitro (Bump and Najjar 1988).

Several analogs of tuftsin have been synthesized and studied for their biological activities (Nozaki et al. 1977, Kavai et al. 1981, Gottlieb et al. 1982, Werner et al. 1986). Specific binding sites were shown to exist for tuftsin on polymorphonuclear cells, monocytes and macrophages, respectively. Attachment of the peptide to these sites was shown to affect intracellular calcium and cyclic nucleotide levels (Nair et al. 1978, Stabinsky et al. 1980, Fridkin and Gottlieb 1981). It is interesting to note that neuropeptides such as substance P, neurotensin and kentsin (Thr-Trp-Arg) compete with tuftsin for binding sites and that intracerebroventricular administration of tuftsin into rats exerts an analgesic effect, additional indications of mutual interactions between the immune and the neuroendocrine systems.

Oligopeptides with structures closely related to that of tuftsin but having antituftsin activity have also been described (for more information see Werner et al. 1986). A tripeptide, Thr-Lys-Pro (TKP) deserves a special attention; while not exerting antituftsin activities, it directly inhibits various macrophage functions, i.e. exerts activities which are opposite to those of tuftsin. These two peptides with closely related chemical structure may be involved in modulating macrophage-directed immune functions. Due to the several valuable activities of tuftsin in vivo, preliminary clinical studies are under way with this peptide (for more details see Werner et al. 1986).

7.4.2.4. FIBRIN(OGEN) DEGRADATION PRODUCTS

Small peptides released by plasmin from circulating fibrinogen that are called micromolecular fibrinogen degradation products (FDP) were shown to exert a variety of immunomodulating activities directed on various immune effector cells. Some of these products have been sequenced, such has the pentapeptides Ala-Arg-Pro-Ala-Lys, and Thr-Ser-Glu-Val-Lys (Gerdin et al. 1980), and were shown to be vasoactive and to suppress in vitro response of murine spleen lymphocytes to lipopolysaccharide and concanavalin. In addition, the first pentapeptide was as active as tuftsin in stimulating phagocytosis in murine peritoneal macrophages (for further readings see Girmann et al. 1976, Plow et al. 1982).

7.4.2.5. β_2 MICROGLOBULIN FRAGMENTS

The observation that cell-mediated immune functions are often impaired in uremic patients led to the isolation and identification by Abiko and coworkers of several oligopeptides from uremic hemodialysates (for references see Chapter 12). These peptides proved to be the fragments of β_2 microglobulin and carriers of various immunomodulating activities, predominantly of inhibitory type (for a detailed analysis of the activities of these uremic peptides see Rola-Pleszczynski et al. 1985).

7.4.2.6. COLOSTRUM- AND MILK-DERIVED PEPTIDES

Studies on ovine colostral immunoglobulins led to the isolation of a proline-rich polypeptide (PRP) fraction of M_r 6,000 which increased the permeability of skin vessels and exerted immunomodulating activities (Wieczorek et al. 1979). From a chymotrypsin digest of PRP a nonapeptide Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro possessing activities qualitatively simlar to those of PRP was isolated (Staroscik et al. 1983). Both PRP and the nonapeptide appear to interact

with T cell precursors, T-helper and T-suppressor cells, and their effects resemble those of some thymic peptides, though there is only a low degree of amino acid sequence homology with some of the β-thymosin peptides.

Purified fractions from delipidated human milk casein of M_r between 2,000 and 600 were shown to stimulate both phagocytosis and antibody production (Jolles et al. 1981). A number of tripeptides, for instance Gly-Leu-Phe, and a hexapeptide, Val-Glu-Pro-Ile-Pro-Tyr isolated from human casein digest also exert phagocyte stimulating activities and the hexapeptide increases the survival time of mice inoculated with a lethal dose of *Klebsiella pneumoniae* (for more details and references see Werner et al. 1986).

7.4.2.7. NEUROPEPTIDES

Data accumulated during the last decade have convincingly demonstrated a vital link between the immune system and the central nervous system (CNS), and the communication of these two systems with each other. Neuroendocrine hormones of peptide character seem to act as signals from the CNS to the immune system. It is noteworthy that, while there is still some controversy concerning the functions of most neuropeptides as neurotransmitters, their interaction with lymphoid cells is an established fact. An impressive number of evidence is now available suggesting an interaction with the immune system of the following neuropeptides: endorphin, enkephalin, angiotensin, substance P, somatostatin and the intestinal vasocative peptide (VIP). Various forms of these interactions and the involved neuropeptides are briefly reviewed by Werner and associates (1986) who also provide further references for interested readers. Studies aimed at providing a deeper insight into, and clarifying the detailed mechanism of the interactions between the CNS/neuroendocrine system and the immune system doubtlessly represent an intriguing and potentially fruitful area of future research.

7.4.2.8. TRANSFER FACTOR

Transfer factor (TF), also termed dialyzable lymphocyte/leukocyte extract (DLE) represents a biological activity obtainable from lymphocytes of immune donors (Lawrence 1974). It has the capability of ameliorating immunodeficiencies of various origin by enhancing cell-mediated immunity (CMI). Although biological activity of TF is thought to necessitate the simultaneous presence of a nucleotide and a peptide component in the lymphocyte dialyzate, the exact chemical character of TF remains unclear at present. A major impeding factor in its use in clinical trials has been the unvailability of sufficient amounts of specific material with standardized activity. This has been partially overcome by elaborating methods permitting the in vitro production of specific TF, designated TFd (Viza et al. 1975) in sufficiently large quantities. Clinical trials with varieties of TFd, such as TFdL, TFdH etc. with specific activity against pathological antigens have given encouraging results in a number of diseases including transitional cell carcinoma of the bladder, viral hepatitis, Hodgkin's disease, herpes infections, etc. (for more details and references see Fudenberg 1985, Nkrumah et al. 1985, Pizza et al. 1985, Shaw et al. 1985, Viza et al. 1985, Zielinski et al. 1985).

Important lymphokines and monokines not mentioned in this Chapter (e.g., interferon-γ, lymphotoxin, tumor necrosis factor, IL-3 etc.) are discussed as growth-inhibitory or antitumor agents in Chapter 8.

7.4.2.9. IMMUNOSUPPRESSIVE FACTORS

Although substances with immunosuppressive activities have been mentioned amongst the previously discussed immunomodulating agents, this section is exclusively devoted to a short survey of additional immunosuppressive materials.

Apart from containing several well characterized substances carrying various biological activities (for more information

see Chapter 8), mouse submaxillary gland extracts have also been shown to induce morphological alterations in the thymus and peripheral lymphoid tissues, and to suppress some immune reactions including antibody production against sheep red blood cells (SRBC) (Koch et al. 1984). Fractionation of mouse submaxillary gland extracts revealed that the profound inhibitory effect on the immune response of mice to SRBC was attributed to epidermal growth factor (EGF)-like materials. In fact, Koch and associates (1984) have prepared three EGF-like substances termed EGFa, EGFb and EGFc, respectively, from mouse gland extracts. EGFa was identified as EGF(1-53), the natural peptide, whereas EGFb and EGFc were shown to differ from EGFa: EGFb lacks the N-terminal asparagine residue, and EGFc has a β-aspartyl instead of an asparaginyl residue. All three peptides display regular EGF activity, but only the natural peptide has full immunosuppressive activity, similar activity of EGFb and EGFc being negligible. The natural peptide, if enzymatically shortened by 2-5 amino acids at its C terminus, loses its immunosuppressive activity suggesting that, in contrast to regular EGF activity, intact N and C terminals are required for the immunosuppressive activity (for more information references see Koch et al 1984).

Crude fractions of urine from pregnant women were found to exert immunosuppressive activities. An immunosuppressive glycopeptide of M_r 85,000 purified to homogeneity from pregnant urine inhibited the activities of human T cells and monocytes at 10^{-9} to 10^{-11} mol/l concentrations in vitro. The material in question was shown to be nontoxic and to block the early events required for normal T cell proliferation in vitro. Referring to its tissue source, the authors (Muchmore and Decker 1985) proposed the name uromodulin to designate this glycoprotein. Uromodulin, which also is a potent IL-1 inhibitor (Brown et al. 1986), was demonstrated to be distinct from other known pregnancy associated proteins such as pregnancy associated plasma protein-A (PAPP-A) or α_2 -pregnancy-associated glycoprotein (α_2-PAG). A new group of immunosuppressive proteins termed trophoblast antigen-1 (TA-1) has been recently described. Although none of the AT-1s have yet been

purified to homogeneity, preliminary data indicate that they share functional similarities with uromodulin (for more information and references see Muchmore and Decker 1985).

Soluble immune response suppressor (SIRS) is a member of the so-called non-antigen-specific suppressor factors, and presently is the most extensively characterized immunosuppressive material (Webb 1986). In mice, SIRS is the product of a specific subset of T cells bearing the Ly^{1-2+} phenotype. It consists of a number of polypeptides with a minimum M_r of 1,000 that are characterized to a significant extent (for details and references see Webb 1986). SIRS has the capability of inhibiting the appearance of antibody forming cells in culture and it also inhibits the growth of a variety of lymphoid cell lines and other cells predominantly of hematopoietic lineage. SIRS activity has also been detected in mitogen-stimulated human peripheral lymphocytes and in various human biological fluids (for further information on SIRS see Webb 1986).

A partially purified and characterized human T cell suppressor factor (HTsF) derived from tonsil cells (Steele et al. 1985), a leukemia cell-derived factor inhibiting lymphocyte activation and function (Chiao et al. 1986), lymphocyte suppressive peptides derived from the α-chain of fibrinogen (Plow and Edgington 1986), and a suppressor lymphokine that inhibits IL-2 activity (hence its name contra-IL-2) (Maki et al.1986), are only mentioned here as additional examples of recently emerging immunosuppressive materials with poorly characterized chemistry at present.

Without going into a lengthy discussion of endogenous immunosuppressive factors, we can conclude that various immune responses are obviously subjected to an extremely complex regulation in which, apart from the generally more extensively characterized stimulatory factors, generally less characterized substances with immunosuppressive activities also play a part, the final outcome being determined by the proper interplay between these immunomodulating substances (for a recent survey of immunosuppressive factors see Webb 1986).

7.5. PERFORIN

Although the proteins discussed below are lymphocyte-derived substances, they cannot be regarded as lymphokines in the ordinary sense. Instead of functioning as molecular messages operating between cells undergoing an immune response, these proteins are involved in the effector functions of cytotoxic T lymphocytes (CTTL), ultimately in killing cells that are destined to be eliminated from the organism for any reason.

Cells attacked and lysed by CTTLs and NK cells carry tubular structures on their surface. Lysis of the target cells occurs through a contact-dependent mechanism. The cytolytic functions of effector lymphocytes has been localized to the granules of these cells: isolated granules from CTTLs are cytotoxic in the presence of Ca^{2+} and they produce circular lesions on target membranes accompanied by changes in the electrical properties of the membranes. The same results can be achieved by solubilized and partially purified granule fractions. Attempts to isolate substances responsible for the lytic function resulted in partial purification of proteins of M_r 27,000, 29,000 and about 70,000, respectively. They induce formation of large aqueous channels, or pores in planar lipid bilayers including the cell membranes. This is the reason why the name pore-forming protein (PFP) or perforin was coined to designate these proteins. At 37 °C, and in the presence of Ca^{2+}, PFPs that form the lesions on target cell membranes polymerize into a supramolecular tubular complex of M_r 1,000,000, termed polyperforin, which resembles the ring-shaped lesions seen on the membrane of target cells attacked and lysed by CTTLs and NK cells. Isolated granules lyse different cells, including erythrocytes, without specificity, whereas purified proteins lyse a variety of tumor cells. Thus,the activity of these proteins may play a crucial role in T cell-mediated cytolysis also in vivo. Molecular characterization of these proteins and the analysis of their biological properties are in progress in several laboratories. Structural, immunological and functional similarities between the 9th component of the complement (C9)

and one of the perforins (perforin 1) have recently been published (Young et al. 1986a; for further readings on perforin see Masson and Tschopp 1985, Ludwig et al.1986, Young et al. 1986b.)

7.6. ANTIBIOTIC PEPTIDES IN GRANULOCYTES: THE DEFENSINS

Granulocytes possess two major defense mechanisms against invading microorganisms. One of these operates through the production of "reactive oxygen intermediates" (ROI). The latter acts either directly or in concert with other granulocyte components, such as myeloperoxidase, to destroy ingested microbes.

Apart from this oxidative/peroxidative mechanism, granulocytes also possess ROI-independent microbicidal mechanisms. Due to the latter, granulocytes retain substantial efficacy against certain microbes even under conditions which preclude the generation of ROIs.

The molecular mechanism and the effector molecules of the O_2-independent antimicrobial activity in polymorphonucleated neutrophilic leukocytes (PMN) are far from being fully defined. Antimicrobial peptides abundantly present in rabbit PMNs are among the best-characterized effector molecules of the ROI-independent defense system, the human counterparts, however, remained unknown until recently. A crude mixture of proteins with bactericidal activity in vitro has been prepared from rabbit granulocytes and named "phagocytin" by Hirsch (1956). Phagocytin was subsequently shown to consist of a whole array of arginine- and cysteine-rich small proteins, "lysosomal cationic peptides", displaying specificity in their antibacterial activity. Six of these rabbit PMN-derived "lysosomal cationic peptides" have been recently purified to homogeneity and sequenced (Selsted et al. 1985a). Shortly thereafter, or rather simultaneously, sequencing of three human counterparts of the rabbit peptides was also reported (Selsted et al. 1985b). They were given the generic name "defensins", referring to their antimicrobial activity. It is es-

tablished knowledge that human PMNs also contain a group of 25,000-28,000 molecular weight, chymotrypsin-like cationic proteins (CLCP) displaying microbicidal activity against Gram-positive and Gram-negative bacteria (for references see Ganz et al. 1985). In rabbit PMNs the presence of another antimicrobial constituent of M_r about 50,000-58,000 has also been described with a reported specific activity against certain Gram-negative bacteria which, due to its distinct cationic character (pI 9.6) and to its biological activity, was termed bactericidal/permeability increasing protein (BPI) (for references see Ganz et al. 1985).

Lysozyme (muramidase) and (apo)lactoferrin, two additional components in the granules of PMNs, may also contribute to the microbicidal activity of granulocytes by acting directly on the cell wall of bacteria, though lactoferrin may additionally facilitate the generation of ROIs, too (for references see Ganz et al. 1985).

By applying separation techniques with high resolution power, at least 20 molecular species with bactericidal activity have been separated from prefractionated crude human granulocyte extracts (Modrzakowski et al. 1979). Of these, the most active fraction termed "Valley AB" had a M_r of about 37,000, but its relationship to BPI remains to be established.

7.6.1. DEFENSINS

Defensin is the generic name used to designate small, cationic polypeptides displaying antimicrobial/antifungal activity that were purified to homogeneity from both human and rabbit PMNs. Presently, the primary structures of three human (Selsted et al. 1985b) and six rabbit (Selsted et al. 1985a) defensins have been established (Fig.7.11). The human peptides contain 29 or 30 amino acid residues, whereas the rabbit ones contain 33 or 34. Both groups of peptides are rich in cysteine (6 residues/molecule). While each human defensin contains 4 arginine residues/molecule, the rabbit peptides vary

in their arginine content from 5 (NP-5 in Fig.7.11) to 10 (NP-1 in Fig.7.11) residues/molecule. With the sole exception of NP-3b, none of the presently known defensins contains lysine. The presence of one tryptophan and three tyrosines in each human defensin contrasts with the virtual absence of these residues in the rabbit peptides.

With the alignment shown in Fig.7.11 complete conservation of 11 residues, including all the six cysteinyl residues can be seen in all human and rabbit peptides. The absence of free sulfhydryl groups both in the human and rabbit peptides indicates the presence of three disulfide bridges in each peptide.

As to the biological activity of human defensins, the HNP 1-3 mixture was shown to efficiently kill *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *E. coli* in vitro, if tested in nutrient-containing phosphate buffer, but it was practically ineffective in nutrient-free buffer. In contrast, the HNP 1-3 mixture present in nutrient-free buffer was shown to be highly effective against *Cryptococcus neoformans*. Apart from its antibacterial and antifungal activity, HNP 1-3 mixture directly inactivates type 1 *Herpes simplex* virus. HNP-1 and HNP-2 applied alone proved to be as effective as the HNP 1-3 mixture, whereas HNP-3 was found to be generally less effective in killing bacteria, with some exceptions.

```
                                                  10                                  20
HNP-1:      Ala-Cys-Tyr-Cys-Arg-Ile-Pro-Ala-Cys-Ile-Ala-Gly-Glu-Arg-Arg-Tyr-Gly-Thr-Cys-Ile-
                                                  10                                  20
NP-1 : Val-Val - Ala - - Arg-Ala-Leu - Leu-Pro-Arg - - - Ala - Phe - Arg-
NP-3a: Gly-Ile - Ala - - Arg-Arg-Phe - Pro-Asn-Ser - - Phe-Ser - Tyr - Arg
NP-3b: Gly-Arg - Val - - Lys-Gln-Leu - Ser-Tyr-Arg - - - Ile - Asp - Lys-
NP-4 : - Ser - Thr - - Arg-Phe-Ser - Gly-Phe-Gly - - Ala-Ser - Ser - Thr-
NP-5 : - Phe - Thr - - Gly-Phe-Leu - Gly-Ser-Gly - - Ala-Ser - Ser - Thr-

                                                  30
HNP-1: Tyr-Gln-Gly-Arg-Leu-Trp-Ala-Phe-Cys-Cys
                                                  30          33
NP-1 : Ile-Arg - - Ile-His-Pro-Leu - - Arg-Arg 34
NP-3a: Val-Asn - Ala-Arg-Tyr-Val-Arg - - Ser - Arg
NP-3b: Ile-Arg - Val-Arg-Phe-Pro - - - Pro-Arg
NP-4 : Val-Asn - Val-Arg-His-Thr-Leu - - Arg-Arg
NP-5 : Ile-Asn - Val-Arg-His-Thr-Leu - - Arg-Arg
```

Fig. 7.11. Primary structures of human (HNP) and rabbit (NP) defensins. Alignment was made to show the structural homology conferred by the 11 residues common to both groups (HNP and NP) of peptides. Dashes indicate residues identical to those in HNP-1

All three HNPs were shown to be localized in azurophil granules in PMNs. The defensin system described here may operate in concert with, or independently from, ROI-dependent microbicidal processes to enable human neutrophils, the key effector cells in host defense against microbial infections, to destroy potential pathogens.

7.7. NEUTROPHIL-ACTIVATING FACTOR

Of the numerous reported mononuclear phagocyte-derived factors with a neutrophil-stimulating activity, the one recently described by Walz et al. (1987) is the first whose primary structure has been, if only partially, identified. Of the assumed 50 amino acids of the approximately M_r 6,000 protein, termed neutrophil-activating factor (NAF), sequence of the N-terminal 32 residues have been determined and found to be as follows:

Ser-Ala-lys-Glu-Leu-Arg-Cys-Gln-Cys-Ile-Lys-Thr-Tyr-Ser-Lys-Pro-Phe-His-Pro-Lys-Phe-Ile-Lys-Glu-Leu-Arg-Val-Ile-Glu-Ser-Gly-Pro.

NAF was shown to elicit a release of granule enzymes from, and the production of superoxide and H_2O_2 in, neutrophils. Its activity profile resembles that of the chemotactic peptides C5a and formyl-Met-Leu-Phe, but is mediated by a novel separate surface receptor. A computer search showed partial homology with connective tissue-activating peptide CTAP III, but no significant sequence homology to C5a, interleukins 1α and 1β, tumor necrosis factor or to granulocyte and granulocyte-macrophage colony-stimulating factors. There is a possibility that neutrophil activating activities published thus far may all be identical with NAF (for more details and references see Walz et al. 1987).

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CHAPTER 8

ENDOGENOUS PEPTIDES IN THE INTEGRATION OF CELL MULTIPLICATION

J. MENYHART

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INTRODUCTION

Growth is generally defined as an increase in the mass of an organism, or of its tissues. The cellular event underlying this process is an increase either in the size (hypertrophy) or in the number (hyperplasia) of constituent cells. From a molecular point of view, the growth of animal cells is a result of both temporaly and spatially ordered sequence of cellular events culminating in DNA replication, and ultimately in mitotic division (meiotic division is not considered here). The growth-related molecular events include a whole array of synthetic and catabolic processes precisely coordinated at each phase of the cell cycle. Their regulation is accomplished to a significant extent through the action and interaction of specific molecules capable of transfering information as chemical messengers. These particular information-carrying molecules can be divided into two principal categories: positive growth factors (GFs) or growth stimulators, and negative GFs or growth inhibitors.

As to the positive GFs or simply GFs, there is a rather large number of substances with widely different chemical structure, which are known to stimulate growth or proliferation either universally, or in a cell-specific manner. Among them are known hormones, certain prostaglandins, as well as hormone-like substances specifically concerned with growth regulation. They are released from different types of cells and may reach their target cells through different routes including local diffusion (paracrine action), systemic transportation (hormonal action) and self-activation (autocrine action). They can contribute to the initiation of both hyper-

trophic and hyperplastic growth responses. At the cellular level, growth stimulators involved in hyperplastic responses may act either by reducing the rate of the genetically programmed cell death, or by increasing the mitotic rate in responsive cell populations. The latter types of growth stimulators are referred to as mitogens, which in turn can be grouped into at least three broad categories depending on their point of attack within the cell cycle. It is now clear that the metabolic events by which quiescent cells are transformed into proliferating ones can roughly be divided into three distinct classes. The introductory phase includes metabolic events which renders the quiescent cells ready, competent or committed in other words, to enter the S phase, without initiating DNA synthesis. Mitogens which induce metabolic events underlying the committed or competent state of cells are termed commitment or competence factors. The next class of metabolic events transfer cells from their committed state into the S phase. Mitogens which initiate DNA synthesis in competent cells are referred to as initiating factors. The third class of the growth-related events enable the cells that underwent DNA replication to proceed through mitosis. Mitogens that initiate mitosis in cells with duplicated DNA are called progression factors or cycle completion/termination factors.

Negative GFs or growth inhibitors constitute the second major category of GFs. Mostly for technical reasons, growth inhibitors in general are less well defined than are the growth-promoting factors. Nevertheless, circumstantial evidences do suggest that growth inhibitors are as numerous as growth stimulators and probably play just as important a role as the stimulators in vivo (Marx 1986, Wang and Hsu 1986).

A substantial proportion of GFs, positive or negative, are polypeptides in nature. The positive polypeptide growth factors play an additional role, distinct from their growth stimulatory one. In tissue culture experiments, it was repeatedly demonstrated that deprivation of cultures of some polypeptide GFs often led not only to the cessation of growth but also to the death of cells as well. Accordingly, some polypeptide GFs seem to be essential also for the survival of

cells. Such names as survival or maintenance factor refer to this capability of these GFs.

Several observations indicate that the first interaction of polypeptide GFs with their target cells is their binding to specific membrane structures, or cell surface receptors. In this regard, they resemble a spectrum of substances, including polypeptide hormones, perhaps the best known category of receptor active substances. The formation of receptor-ligand complexes almost instantaneously triggers the stimulation of a multitude of intracellular events, including transport processes, bidirectional ion fluxes, phospholipase A activity, changes in membrane composition and intracellular nucleotide concentrations, etc. In general, these early events are followed by long-term responses lasting for hours, or even for days, which are probably related to specific changes in protein synthesis. However, neither the mechanism by which short- and long-term responses are accomplished, nor the exact relation between the two classes of temporal responses have satisfactorily been elucidated until recently. Polypeptide GFs may interact with the cell membrane, and this interaction per se may generate a single signal responsible for both rapid and delayed responses. It is also conceivable, however, that one single specific event among the individual rapid responses serves as a signal for the subsequent slow responses. As a third alternative, ligand-receptor interaction may generate two independent signal-events: one for the rapid responses, and another for the long-term ones. Finally, the ligand-receptor interaction may generate a signal for the rapid events only, and subsequent internalization of the ligand-receptor complex may generate a signal for the delayed effects through dissociated receptors, or ligands, or their active fragments.

It has only recently been recognized that internalization by absorptive pinocytes, a process termed receptor-mediated endocytosis might play a role of paramount importance in the mechanism of action of the effector molecules. However, this seems to be a rather general phenomenon, and at present it is far from clear whether receptor-mediated endocytosis is merely

a function by which polypeptide GFs are degraded by lysosomal hydrolytic enzymes after the fusion of the internalized ligand-receptor complexes with lysosomes, or whether the process has a functional significance.

Bradshaw and Rubin (1980) have classified the potential roles of internalization in hormonal actions as follows: (1) interaction of the hormone, receptor or fragment(s) therefrom with intracellular receptors; (2) processing of the hormone or receptor to generate a "second messenger"; (3) direct action of the receptor after translocation to intracellular organelles; (4) degradation; (5) regulation of cell sensitivity to hormones by decreasing the number of surface receptors ("down regulation"). Mutatis mutandis, these apply to the polypeptide GFs as well. It is worthwhile to mention that intracellular receptors identified by binding assays were found to be associated with components of the nucleus, Golgi membranes and other cellular organelles.

A significant number of polypeptide GFs have been extensively characterized in terms of chemical structure and mechanism of action. However, the existence of some growth factors has only been postulated on the basis of their observed biological actions without identification of a specific molecule, its source, or the physiologically relevant target tissues. It may well turn out that some of the seemingly distinct growth related biological activities are, in fact, mediated by the same, presently unidentified molecular entity. Precisely because classification of polypeptide GFs on a chemical basis is hardly possible at present, they are grouped and discussed in this chapter according to their biological sources, or assumed sources (for a recent review see Deuel 1987).

8.1. GROWTH STIMULATORY PEPTIDES: THE GROWTH FACTORS

8.1.1. TISSUE-DERIVED GROWTH FACTORS

8.1.1.1. GROWTH FACTORS FROM THE SALIVARY GLAND

8.1.1.1.1. EPIDERMAL GROWTH FACTOR

Epidermal growth factor (EGF) was first isolated from the submaxillary gland of male mice by Cohen (1962), a Nobel laureate in 1986. The mouse peptide (mEGF) is a single chain polypeptide having 53 amino acid residues (M_r 6,045) but no free sulfhydryl groups or carbohydrate moieties (Taylor et al. 1972). mEGF whose primary structure is shown in Fig.8.1 (Savage et al. 1972, 1973) has three intramolecular disulfide bonds spanning between the cysteine residues at positions 6 and 20, 14 and 31, 23 and 42, respectively. Of the α-, β- and γ-mEGFs described by DiAugustino et al.(1985), the α-form corresponds to the 53 amino acid natural peptide, the β-form to the desasparaginyl variety of the latter, whereas the γ-form remains unidentified for the time present. Size heterogeneity including high-molecular-weight forms of EGF has been recognized (see e.g., Pesonen et al. 1987). EGF isolated from rat submaxillary gland (rEGF) has a M_r of 6,000 and an amino acid composition resembling those of the mouse and human peptides, but it is not identical to either (Moore 1978). The mEGF-like activity prepared from human urine and termed human EGF (hEGF; M_r 5,700-5,300) was found to be closely related to, but not fully identical with, mEGF (Cohen and Carpenter 1975). The high molecular weight form (M_r 30,000) of the latter detected in various body fluids (Gregory et al. 1977, Hirata and Orth 1979, Savage et al. 1986, Mount et al. 1987) appears to be a precursor for the low molecular weight species. β-urogastrone, a gastric antisecretory peptide isolated from human urine (Gregory 1975) is structurally and functionally related to mEGF (70% sequence homology) indicating that both peptides are members of a family of polypeptides that display some interspecies sctructural variations but are probably near identical in their active site regions responsible for receptor binding and biological activity (for canine EGF/urogastrone see Kobayashi et al. 1985). By all indications hEGF and β-urogastrone are identical entities (Fig.8.1). The terms EGF-2 (EGF minus 2) or EGF(1-51) and EGF-5 or EGF(1-48) refer to mEGF species

```
                                    10                                  20
h:Asn-Ser-Asp-Ser-Glu-Cys-Pro-Leu-Ser-His-Asp-Gly-Tyr-Cys-Leu-Gly-Asp-Gly-Val-Cys-
m: -   -   Tyr-Pro-Gly -   -   Ser -   Tyr -   -   -   -   -   Asn-Gly -   -   -
                                    30                                  40
h:Met-Tyr-Ile-Glu-Ala-Leu-Asp-Lys-Tyr-Ala-Cys-Asn-Cys-Val-Val-Gly-Tyr-Ile-Gly-Glu-
m: -   His -   -   Ser -   -   Ser -   Thr -   -   -   -   -   Ile -   -   Ser -   Asp-
                                    50          53
h:Arg-Cys-Gln-Tyr-Arg-Asp-Leu-Lys-Trp-Trp-Glu-Leu-Arg
m: -   -   -   Thr -   -   -   Arg -   -   -   -   -   -
```

Fig. 8.1. Amino acid sequences of human (h) and murine (m) epidermal growth factors. In the murine peptide, only the amino acid substitutions are indicated. Hyphens indicate identities

from the C-termini of which a dipeptide or a pentapeptide sequence is cleaved by an acidic protease or a mild tryptic digestion, respectively. On the other hand CNBr-EGF represents a C-terminal 21 amino acid sequence cleaved from natural EGF by cyanogen bromide treatment.

In tissue extracts at neutral pH EGF is present in a complexed form (M_r 70,000) consisting of two molecules of EGF and two molecules of binding protein of M_r 29,000 (Taylor et al. 1972, 1974; for sequence data see Lundgren et al. 1984). Similarly to the γ-subunit of nerve growth factor (NGF; see the next section) EGF-binding protein possesses arginine esterase activity, but the two proteins cannot be substituted for one another in binding to EGF or NGF. The M_r 9,000 mEGF precursor (preEGF) isolated by Frey and his associates (1970) could be converted to a M_r 6,000 EGF by arginine esterase present in the aforementioned EGF complex indicating a role for the binding protein in generating EGF from its precursor. This particular protein also enhances mitogenic properties of mEGF in culture (Barnes and Colowick 1976, Lembach 1976). Although mouse preproEGF mRNAs reported from two laboratories (Gray et al. 1983, Scott et al. 1983b) somewhat differed in length, both mRNA species were found to be of unusual length and had sequences amino-terminal to those coding for the mature protein that might produce eight additional mEGF-like peptides (Fig.8.2). This indicates the enormous potential of the precursor to generate peptides related to the 53 amino acid mature protein (Burmeister et al. 1984; for recent information on the domain structure of mEGF and on a cDNA encoding human EGF precursor see Mayo 1985, Bell et al. 1986a). It was unexpectedly found that the cell surface receptor for EGF and the

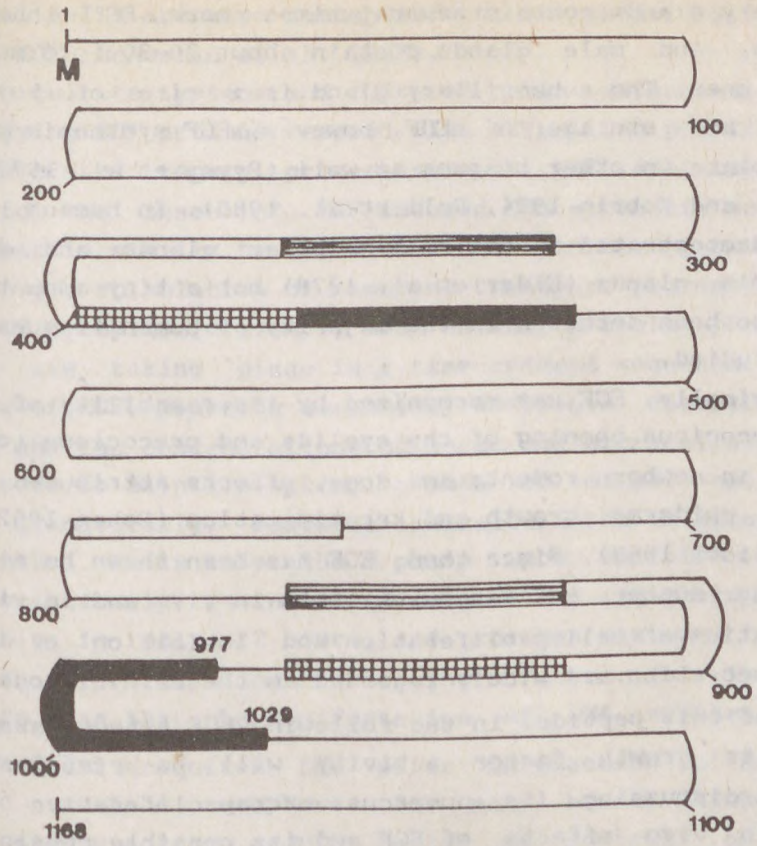


Fig. 8.2. Schematic drawing of the predicted structure of preproEGF (Gray et al. 1983). The segment at positions 977 through 1029 corresponds to the position of the mature EGF molecule, whereas the remaining 8 segments show homology with EGF. M indicates the initiator methinonine residue

EGF precursor are structurally related suggesting that unprocessed EGF may perform receptor function as well (Pfeffer and Ullrich 1985). The genes for human and mouse EGF precursors have been assigned to chromosomes 4 and 1, respectively (Zabel et al. 1985). EGF is also related, both structurally and functionally, to α-transforming growth factor.

Although the concentrations of EGF in various body fluids display interspecies differences, saliva as well urine contain EGF in highest concentrations virtually in all species. It is worth mentioning that the known mitogenic effect of human milk is also due, at least in part, to its EGF content (Petrides et al. 1985). From several EGF-containing mouse tissues the sub-

maxillary glands contain several times more EGF than other tissues, and male glands contain about 20-30 fold more than female ones. The submaxillary gland is a site of both synthesis and storage of mEGF, however, EGF synthesis probably takes place in other tissues as well (Byyny et al. 1972, 1974, Kudlow and Kobrin 1984, Rall et al. 1985). In humans, EGF was first demonstrated in the submandibular glands and duodenal Brunner's glands (Elder et al. 1978) but a tiny amount of EGF has also been detected in the majority of human tissues hitherto studied.

Originally, EGF was recognized by its capability of inducing precocious opening of the eyelids and precocious tooth eruption in newborn rodents and dogs, effects attributed to enhanced epidermal growth and keratinization (Cohen 1962, Cohen and Elliott 1963). Since then, EGF has been shown to elicit a striking number of responses both in vivo and in vitro but stimulation of cell proliferation and inhibition of gastric acid secretion are widely regarded as the main biological actions of this peptide. In the following EGF effects associated with its growth factor activity will be briefly surveyed without discussing its numerous extraproliferative actions (for in vivo effects of EGF and its possible physiological roles see Gregory 1985, Tsutsumi et al. 1986).

Mainly the cells of ecto- and endodermal origin, especially epidermal, epithelial and mesenchymal cells are the target cells for EGF (for details see Carpenter 1981). This relatively wide spectrum of EGF-responsive cells is consistent with the recognized wide tissue distribution of EGF receptors. As to its in vivo effects, EGF increases the mitotic rate and induces marked hyperplasia and hypertrophy in numerous in vivo proliferation assays. It may protect immature fetuses from developing distress syndrome by acting on the proliferation of respiratory epithelial cells and on surfactant production. The mitogenic activity of EGF is strongly potentiated by insulin and it also acts in synergism with platelet-derived growth factor (PDGF) on BALB/c-T3T cells, a mouse fibroblastic cell line (for references see Goustin et al. 1986). Capillary en-

dothelial cells are also susceptible to the proliferative effect of EGF (McAuslan et al. 1985).

Organ culture systems and cultured cells extensively used for studying EGF actions respond with stimulated proliferation and accelerated repair processes upon EGF administration. Prior to the onset of EGF-induced DNA synthesis molecular events partly similar to those elicited by other growth factors (e.g., stimulation of membrane transport processes and a number of cytoplasmic events, most notably protein phosphorylation) are taking place in a time-ordered sequence. In the presence of EGF, membrane morphology undergoes characteristic changes and the concentrations of serum and extracellular calcium needed to maintain optimal growth of normal cells decrease dramatically, features characteristic of tumor cells G_1 -arrested cells enter the S phase upon addition of EGF. Characteristically, an increased rate of DNA synthesis becomes detectable in only 12 hours, and reaches maximum in about 20 hours after addition of the growth factor. This is interpreted to mean that the manifestation of DNA synthesis-simulating effect requires the prior interaction of EGF with specific surface receptors for many hours which makes EGF a slowly-acting growth factor in contrast to other quick-acting ones. Various cells are differentially susceptible to DNA synthesis-promoting effect of EGF. For stimulating DNA synthesis EGF usually requires the presence of serum indicating that EGF has to interact with some serum factor(s), probably PDGF, to elicit its effect.

The ultimate step in EGF-induced proliferation response is mitotic division of affected cells. Due to its mitogenic effect EGF markedly increases maximum saturation density in a variety of cultured cells. Furthermore, human fibroblasts grown in the continuous presence of EGF form multilayers, like tumor cells do, instead of a tightly packed confluent monolayer characteristic of normal cells. However, unlike tumor cells that form randomly oriented multilayers, EGF-induced multilayers are arrenged in a formation resembling that of cells within tissues. Although not carcinogenic on its own, EGF enhances carcinogenic potential of methylcholantrene and

acts as a tumor promoter. Exogenous tumor promoters, such as 12-O-tetradecanoyl phorbol-13-acetate (TPA) were shown to affect EGF receptors in more than one way (Davis and Czech 1984). EGF and TPA stimulate DNA synthesis synergistically. Intriguing new data suggest a possible collaboration of growth factors and/or their receptors with viral and cellular (proto-)oncogenes in malignant transformation (reviewed by Heldin and Westermark 1984, Goustin et al. 1986). An EGF-like molecule may play a role also in the induction of vaccinia virus-induced benign hyperplasia. This virus encodes a 47 residue protein, termed vaccinia-derived growth factor (VDGF; Brown et al 1985) or vaccinia virus growth factor (VVGF; Stroobant et al. 1985, King et al. 1986) which shows a significant sequence homology to both EGF and α-TGF. It is important to note that EGF, under specific conditions and on certain cells, mediats inhibitory signals (Bravo et al. 1985, Korc et al. 1986, Lombardero et al. 1986) instead of mitogenic ones and induces the expression of c-myc and c-fos proto-oncogen, (Bravo et al. 1985, Ran et al. 1986, Paulsson et al. 1987). However, this EGF-induced expression of proto-oncogenes does not seem to be related to the mitogenic or inhibitory character of the response elicited by this growth factor.

The EGF-induced mitogenic response requires its prior interaction with specific high affinity surface receptors. The extensive knowledge recently acquired on EGF receptors has made this membrane protein the best known growth factor receptor hitherto characterized, and has also provided new insights into many intriguing aspects of signal transduction both in normal and transformed cells. From a methodological point of view, research on EGF receptor has been greatly facilitated by using membrane preparations from A341 cells, a human epidermoid carcinoma cell line renowned for its strikingly high number of EGF receptors ($2-3 \times 10^6$/cell), some 20-30 fold more than are present in most other EGF responsive cells (Chinkers et al. 1979, Haigler et al. 1979a,b, Akiyama et al. 1985). Extensive studies mainly on this membrane preparations have finally led to identification of the EGF receptor as an integral

membrane glycoprotein with a M_r of 170,000, of which the carbohydrate moiety represents somewhat more than 30% (Cohen and Carpenter 1975, Cohen et al. 1982). As a major breakthrough, a cDNA-derived amino acid sequence as well as the domain structure of the 1,186 residue EGF receptor have been established and the gene coding for the human receptor mapped to chromosome 7 (Xu et al. 1984, Merlino et al. 1985, Ullrich et al. 1985). The C-terminal region responsible for tyrosine kinase activity is homologous to the gene product of v-erb B, an oncogenic retrovirus, and also to other tyrosine kinases including the src gene family, another group of retroviruses. The c-erb B-related "neu" proto-oncogene was also shown to encode an EGF receptor-related protein (Bargmann et al. 1986). These homologies provided intriguing new insigts into the possible cooperation between growth regulating receptor proteins and products of various oncogenes associated with malignant transformation (Xu et al. 1984; for references see also Goustin et al. 1986). Besides tyrosine kinase activity, autophosphorylation of the tyrosyl residue at position 1,173 has also been observed as a response of the receptor to EGF. Furthermore, EGF receptor has several sites for serine and threonine phosphorylation and recent data indicate that EGF receptor is a substrate for the calcium/phospholipid-dependent diacylglycerol-activated serine/threonine-specific protein kinase, also known as protein kinase C, which may be responsible for phosphorylating serine and threonine residues of the EGF receptor. The manifestation of DNA synthesis-stimulating effect requires the prior interaction of EGF with its receptors for many hours and the occupancy of about 25% of available binding sites. It is a striking finding that functionally active EGF receptors can be spontaneously transferred to receptor-negative cells without using any fusogenic agent (Das 1982, Das et al. 1984). The apparent loss of receptors ("down regulation") following exposure of susceptible cells to EGF is probably due to internalization of the ligand/receptor complexes, or to the formation of nondissociable EGF/receptor complexes, that are subsequently degraded through lysosomal and/or extralysosomal mechanisms (Burwen et al. 1984, Miskimins and Shimizu 1984).

The exact character of EGF-induced mitogenic signal(s) remains to be clarified. Recent data suggest a direct interaction of the receptor with the genome implicating the receptor itself as the second messenger for EGF-induced mitogenesis (Mroczowski et al. 1984). It is interesting to note that EGF stimulates the synthesis of its own receptor (Earp et al. 1986). Receptor crossover i.e., the interaction of one growth factor with the receptor of another one may be important physiological means for modulating EGF receptor activity. Such interactions were already established between insulin and the receptor of insulin-like growth factor II (IGF-II) the PDGF and EGF receptor and between fibroblast growth factor (FGF) and the vasopressin receptor.

Due to its mitogenic and other valuable biological properties, EGF or its analogs may become useful therapeutic agents in the future. For example this peptide may be used for promoting wound healing, controlling gastric acid secretion and curing ulcer disease. In the presence of EGF, epidermal cells in culture can be used for producing epidermal sheets of extensive area that is suitable for grafting onto patients who have suffered extensive burning. EGF or its receptor may be used as markers for certain neoplastic diseases. However, practical utilization of the most intriguing properties of this peptide or its receptor, namely their possible participation in neoplastic transformation (reviewed by Stoschek and King 1986) cannot even be predicted at present (for further information and references on EGF and EGF receptor see Gill et al. 1985, Carpenter and Zendegui 1986, Cohen 1986, 1987a,b, Zoon et al. 1986, Carpenter 1987, Gill et al. 1987).

8.1.1.1.2. NERVE GROWTH FACTOR

Nerve growth factor (NGF) was discovered by Levi-Montalcini (1952, 1965), a 1986 Nobel laureate, as a product of spontaneously arising mouse sarcomas. It is most abundantly present in the submaxillary glands of adult male mice and the glands and venoms of poisonous snakes.

NGF occurs in multiple molecular forms. Mouse salivary gland NGF (mNGF) was first purified by Cohen (1960). Treatment of animals with antisera raised against Cohen's NGF preparation resulted in a selective destruction of sympathetic chain ganglia, a procedure called immuno-sympathectomy (Levi-Montalcini and Booker 1960). 7S NGF (S refers to sedimentation coefficient) is a high molecular weight complex consisting of three polypeptide chains with a M_r 26,500 each, called α-, β - and γ-subunit, respectively, that are associated with noncovalent forces. At concentrations eliciting biological responses, the complex is fully dissociated. Corresponding to its proposed $\alpha_2\beta\gamma_2$ stochiometry, M_r of the 7S complex was estimated to be about 130,000-140,000. The exact role of zinc detected within the complex remains to be determined (see e.g., Young and Koroly 1980). Only the β-subunit possesses nerve growth-promoting capability. This is why the β-subunit itself is often called NGF instead of the whole 7S complex. The α-subunit has only recently been identified as a serine protease (Isackson and Bradshaw 1984), whereas the arginylesteropeptidase activity of the γ-subunit is known for years (Greene et al. 1968, 1969). The latter also shares many properties with EGF-binding protein including a near 75% sequence homology, but their complex-forming ability proved to be distinctly specific. Both enzymic molecules may have a role in processing precursor molecules of their own growth factor.

The method most widely used for preparing biologically active NGF (Bocchini and Angeletti 1969) yields 2.5S NGF, a form which is higly active in producing neurite outgrowth in vitro, and is closely related to the β-subunit of 7S NGF. It has a homodimeric structure of M_r 26,500 consisting of two noncovalently linked 120 amino acid monomeric subunits having a M_r of 13,250 and three disulfide bonds each (disulfide-linked cysteine residues are localized at positions 15 and 80, 58 and 108, 70 and 110, respectively). The monomer seems to be the physiologically significant form. The amino acid sequences of human (Ullrich et al. 1983), mouse (Angeletti and Bradshaw 1971, Scott et al. 1983a), bovine and chicken 2.5S NGFs (Ebendal et al. 1986, Goedert 1986, Meier et al. 1986) have been

determined, partly by direct amino acid analysis, partly as cDNA-derived sequences (Fig.8.3). The human gene has also been structurally analysed (Ullrich et al. 1983) and assigned to chromosome 1 (Francke et al. 1983). The mammalian peptides are about 90% homologous between each other, while homology is less extensive in case of the chicken molecule (for more details and references see Calissano et al. 1984, Levi et al. 1985, Guerina et al. 1986).

In fact, the mouse structure shown in Fig.8.3 represents the β-subunit directly isolated from the 7S complex. In contrast to this, the murine 2.5S NGF prepared according to Bocchini and Angeletti (1969) is devoid of the N-terminal octapeptide sequence and the C-terminal arginine residue and thus it contains 111 amino acids only. This product does not combine with the γ-subunit but still combines with the α-moiety and maintains full biological activity in some assay systems (Moore et al. 1974). 2.5S mNGF is structurally related to

```
                                                  10                                                  20
h: Ser-Ser-Ser-His-Pro-Ile-Phe-His-Arg-Gly-Glu-Phe-Ser-Val-Cys-Asp-Ser-Val-Ser-Val-
m:  -   -  Thr  -   -  Val  -   -  Met  -   -   -   -   -   -   -   -   -   -   -
b:  -   -   -   -   -  Val  -   -   -   -   -   -   -   -   -   -   -   -   -   -
c: Thr-Ala      -   -      Leu  -   -   -   -   -   -   -   -   -   -   -   -  Met-
                                                  30                                                  40
h: Trp-Val-Gly-Asp-Lys-Thr-Thr-Ala-Thr-Asp-Ile-Lys-Gly-Lys-Glu-Val-Met-Val-Leu-Gly-
m:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -  Thr  -   -  Ala-
b:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
c:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -  Thr  -   -   -
                                                  50                                                  60
h: Glu-Val-Asn-Ile-Asn-Asn-Ser-Val-Phe-Lys-Gln-Tyr-Phe-Phe-Glu-Thr-Lys-Cys-Arg-Asp-
m:  -   -   -   -   -   -   -   -   -  Arg  -   -   -   -   -   -   -   -   -  Ala
b:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
c:  -   -   -   -   -   -  Asn  -   -   -   -   -   -   -   -   -   -   -   -   -
                                                  70                                                  80
h: Pro-Asn-Pro-Val-Asp-Ser-Gly-Cys-Arg-Gly-Ile-Asp-Ser-Lys-His-Trp-Asn-Ser-Tyr-Cys-
m: Ser  -   -   -  Glu  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
b:  -   -   -   -   -   -   -   -   -   -   -  Ala  -   -   -   -   -   -   -   -
c:  -  Arg  -   -  Ser  -   -   -   -   -   -  Ala  -   -   -   -   -   -   -   -
                                                  90                                                 100
h: Thr-Thr-Thr-His-Thr-Phe-Val-Lys-Ala-Leu-Thr-Met-Asp-Gly-Lys-Gln-Ala-Ala-Trp-Arg-
m:  -   -   -   -   -   -   -   -   -   -   -  Thr  -  Glu  -   -   -   -   -   -
b:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
c:  -   -   -   -   -   -   -   -   -   -   -  Glu  -   -   -   -   -   -   -   -
                                                 110                                                 120
h: Phe-Ile-Arg-Ile-Asp-Thr-Ala-Cys-Val-Cys-Val-Leu-Ser-Arg-Lys-Ala-Val-Arg-Arg-Ala-
m:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -  Thr  -   -  Gly
b:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   - Ile-Gly-Gln  -   -
c:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   - Ser-Gly  -      Pro
```

Fig.8.3. Amino acid sequences of the monomeric form of human (h), mouse (m), bovine (b) and chicken (c) nerve growth factors (β-subunit). The h, b and c sequences are deduced from cDNAs. Dashes indicate residues identical to those in the human peptide. Deletions (empty spaces) in the c sequence are introduced for maximizing homology

proinsulin (Frazier et al. 1972) and the two share other similarities, too (Bradshaw 1978) suggesting a common evolutionary origin. Of the several snake venom NGFs, cobra venom NGF shares numerous structural and functional properties with murine 2.5S NGF, including a more than 60% sequence homology indicating a remarkable evolutionary conservation of this form of NGF. On the other hand, NGFs from other snakes and from crotalids significantly differ not only from mNGF but also from cobra venom NGF (for more details see James and Bradshaw 1984). A number of cultured cells were shown to secrete NGF in a substantial amount (Bradshaw and Young 1976, Lillien and Claude 1985, Furukawa et al. 1986), but cell-secreted NGF has not been sufficiently purified thus far.

The sexually dimorphic character of mouse salivary glands is manifested in a much higher level of NGF in the male than in the female glands and saliva, but not in serum. There is a striking correlation between NGF/NGFmRNA and adrenergic innervation density in various peripheral organs with particularly high NGF mRNA levels in the iris, heart, vas deferens and the spleen capsule of the mouse (Heumann and Schwab 1985).

Besides β-NGF, the mouse α-subunit (Thomas et al. 1981) and a substantial segment of the mouse γ-subunit (Isakson and Bradshaw 1984) have also been sequenced. These studies revealed, quite unexpectedly, that striking sequence homologies exist between these subunits (Fig.8.4) and that the α-subunit is a member of the serine protease family which is present in an inactive form in the 7S complex.

The numerous actions of NGF observed both in vivo and in vitro (reviewed by Greene 1984) can be roughly classified as trophic and differentiative effects, but NGF may also be viewed, if only in a restricted sense, as a mitogenic agent. Sympathetic and sensory nerve cells (ganglia) are the main target cells of this peptide maintaining their NGF-responsiveness throughout the entire life span, similarly to some neoplastic and chromaffin cells. Susceptibility to the action of NGF is maximal during early embryonic life and progressively restricting in the adult. The number of histamine-releasing mast cells sharply increases upon NGF ad-

```
                                                  10                                  20
a: Ile-Val-Gly-Gly-Phe-Lys-Cys-Glu-Lys-Asn-Ser-Gln-Pro-Thr-His-Val-Ala-Val-Tyr-Arg-
b:x-------------------------------------------------------------------------------
                                                  30                                  40
a: Tyr-Thr-Gln-Tyr-Leu-Cys-Gly-Gly-Val-Leu-Leu-Asp-Pro-Asn-Trp-Val-Leu-Thr-Ala-Ala-
b: ---------------------------------------- - - - - - - - - -
                                                  50                                  60
a: His-Gly-Tyr-Asp-Asp-Asn-Tyr-Lys-Val-Trp-Leu-Gly-Lys-Asn-Asn-Leu-Phe-Lys-Asp-Glu-
b:  - - - Asn - Lys - Gln - - - - - - - Phe-Leu-Glu - -
                                                  70                                  80
a: Phe-Ser-Ala-Gln-His-Arg-Phe-Val-Ser-Lys-Ala-Ile-Pro-His-Pro-Gly-Phe-Asn-Met-Ser-
b:  - - - - - - - Leu - - - - - - - - - - - - - - - -
                                                  90                                 100
a: Leu-Met-Arg                  Phe-Leu-Glu-Tyr-Asp-Tyr-Ser-Asn-Asp-Leu-Met-Leu-Leu-
b:  - - - Glu-His-Phe-Thr-Gln-Pro - Asp - - - - - - - - (-------
                                                 110                                 120
a: Arg-Leu-Ser-Lys-Pro-Ala-Asp-Ile-Thr-Asp-Thr-Val-Lys-Pro-Ile-Thr-Leu-Pro-Thr-Glu-
b: ---) - - - - - - - - - - Val - - - - - - - - - -
                                                 130                                 140
a: Glu-Pro-Lys-Leu-Gly-Ser-Thr-Cys-Leu-Ala-Ser-Gly-Thr-Gly-Ser-Ile-Thr-Pro-Thr-Lys-
b:  - - - - - - - - - - - - - - - - - - (----------------) -
                                                 150                                 160
a: Phe-Gln-Phe-Thr-Asp-Asp-Leu-Tyr-Cys-Val-Asn-Leu-Lys-Leu-Leu-Pro-Asn-Glu-Asp-Cys-
b:    x----Ile-Pro - - - Gln - - - - - - - - - - - - - -
                                                 170                                 180
a: Ala-Lys-Ala-His-Ile-Glu-Lys-Val-Thr-Asp-Ala-Met-Leu-Cys-Ala-Gly-Glu-Met-Asp-Gly-
b: Asp - - - Glu-Met - - - - - - - - - - - - - - - - -
                                                 190                                 200
a: Gly-Lys-Asp-Thr-Cys-Lys-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Ile-Cys-Asp-Gly-Val-Leu-Gln-
b:  - Ser-Tyr - - Glu-His - - - - - - - - - - (--------------
                                                 210                                 220
a: Gly-Ile-Thr-Ser-Trp-Gly-His-Thr-Pro-Cys-Gly-Glu-Pro-Asp-Met-Pro-Gly-Val-Tyr-Thr-
b: ---------------------------------------------------------------------------
                                                 230                 237
a: Lys-Leu-Asn-Lys-Phe-Thr-Ser-Trp-Ile-Lys-Asp-Thr-Met-Ala-Lys-Asn-Pro
b: ------------------------------------------------------------)
```

Fig. 8.4. Complete amino acid sequence of the γ-subunit (a) and a partial amino acid sequence of the α-subunit (b) of murine 7S nerve growth factor. In the b sequence only the differing residues are indicated. Deletions (empty spaces) at positions 84 through 87 in the γ-subunit is introduced for a better match. Dashed lines indicate unidentified regions and x indicates unidentified derivatives blocking α-amino groups. Dashed lines in parentheses in the b sequence indicate compositional data only. Underlined residues represent a possible glycosylation site. (Partly from Isackson and Bradshaw 1984)

ministration (Aloe and Levi-Montalcini 1977, Bruni et al. 1982).

NGF exerts a general growth-promoting effect on sympathetic neurons in neonatal animals, an effect regularly preceded by a marked induction of ornithine decarboxylase (ODC). It promotes the differentiation of sympathetic neuroblasts to mature neurons, has a chemotactic effect on outgrowing neurons and acts as a survival factor for sympathetic neurons both in vivo and in vitro. Due to this latter effect, NGF prevents dying of "excess" neurons in the sympathetic ganglia, a process occurring physiologically in late embryonic and early postnatal de-

velopment of mammals, thereby causing an apparent neuronal hyperplasia. The adverse consequences of immuno-, surgical- or chemical-sympathetectomy can be counteracted by concomitant exogenous administration of NGF. NGF-responsive neural cells display a differential sensitivity towards NGF (Hamburger and Yip 1984), however, NGF-unresponsive sympathetic cells may be affected by other factors instead of NGF (Richardson and Ebendal 1982, Ebendal et al. 1983, Demeneix and Grant 1988). Ionic events seem to play an important role in the neurotropic action of NGF (Varon and Skaper 1985).

NGF is also involved in the survival, general growth and differentiation of sensory neurons and it guides growing axons or collaterals along its own concentration gradient, a phenomenon called neurotropism (Pfenninger and Johnson 1981, Seeley and Greene 1983).

Instead of being directly mitogenic as formerly suggested (Levi-Montalcini and Angeletti 1968), NGF merely supports the survival of "excess" neurons that normally degenerate and its mitogenic effect is restricted to the nonneural cells that are in contact with neurons.

An increasing number of observations suggests that NGF exerts a variety of actions also in CNS, such as accelerating axosomal outgrowth from, and enhancing regeneration of, transected neurons in the brain. The presence of NGF receptors (Szutowitz et al. 1976a,b) and expression of the gene coding for NGF in the brain (Shelton and Reichard 1986) also suggest a physiological role for this peptide in CNS.

Two distinct categories of NGF receptors have been assumed to exist in responsive cells: the high affinity class of receptors is thought to be associated with the plasma membrane, whereas low affinity receptors with the nucleus suggesting a bimodal mechanism of action for NGF. The molecular weight of hitherto isolated NGF receptors range between 98,000 and 190,000 depending on their cell source, but the major form seems to be a single chain polypeptide of M_r 130,000. NGF is internalized in sympathetic and sensory neurons, partly through the membrane of the cell body partly through retrograde axonal transport when NGF taken up and internalized at

the nerve terminals (synaptosomes) is transported in a retrograde fashion up the axon within membrane-bound vesicles into the nerve cell bodies where it exerts its characteristic effect.

At the present state of affairs one can only say with confidence that NGF apparently is finding its proper place in developmental neurobiology. In a rather wide spectrum of the phylogenetic scale, the action of NGF, though restricted to selected neuronal populations, seems to have a crucial role in activating differentiative programs in target cells as its main function. However, much remains to be done before all presently available information on NGF can be transformed into a comprehensive picture clearly delineating the physiological roles that NGF may play in living organisms. (For more information on EGF see Milbrandt 1986, Ricter-Landsberg and Jastroff 1986, Rush 1986, Levi-Montalcini 1987).

8.1.1.1.3. NEUROLEUKIN

The name neuroleukin (NL) refers to a M_r 56,000 protein of dual origin and function: it was detected both in mouse salivary glands and lectin-induced T cells as an agent exhibiting both neurotrophic/growth factor and B cell regulatory/lymphokine activities (Gurney et al. 1986a,b). Analysis of a NL-encoding cDNA revealed a 558 amino acid precursor (prepro-NL) (Fig.8.5). The difference found between the calculated molecular weight of the precursor (62,800) and that determined for the purified protein (56,000) suggests that mature NL may be a proteolytic cleavage product of the precursor. The partial sequence homology established between NL and a specific region of the external envelope protein of HTLV-III/LAV, the retrovirus that causes acquired immune deficiency syndrome (AIDS), represents the only homology found between NL and other known proteins.

NL promotes the survival of a selected group of embryonic spinal neurons and NGF-insensitive sensory neurons in culture without affecting sympathetic and parasympathetic neurons. On

```
                                                  10                  20
Met-Ala-Ala-Leu-Thr-Arg-Asn-Pro-Gln-Phe-Gln-Lys-Leu-Leu-Glu-Trp-His-Arg-Ala-Asn-
                                                  30                  40
Ser-Ala-Asn-Leu-Lys-Leu-Arg-Glu-Leu-Phe-Glu-Ala-Asp-Pro-Glu-Arg-Phe-Asn-Asn-Phe-
                                                  50                  60
Ser-Leu-Asn-Leu-Asn-Thr-Asn-His-Gly-His-Ile-Leu-Val-Asp-Tyr-Ser-Lys-Asn-Leu-Val-
                                                  70                  80
Asn-Lys-Glu-Val-Met-Gln-Met-Leu-Val-Glu-Leu-Ala-Lys-Ser-Arg-Gly-Val-Glu-Ala-Ala-
                                                  90                  100
Arg-Asp-Asn-His-Phe-Ser-Gly-Ser-Lys-Ile-Asn-Tyr-Thr-Glu-Asp-Arg-Ala-Val-Ile-His-
                                                  110                 120
Val-Ala-Leu-Arg-Asn-Arg-Ser-Asn-Thr-Pro-Ile-Lys-Val-Asp-Gly-Lys-Asp-Val-Met-Pro-
                                                  130                 140
Glu-Val-Asn-Arg-Val-Leu-Asp-Lys-Met-Lys-Ser-Phe-Cys-Gln-Arg-Val-Arg-Ser-Gly-Asp-
                                                  150                 160
Trp-Lys-Gly-Tyr-Thr-Gly-Lys-Ser-Ile-Thr-Asp-Ile-Ile-Asn-Ile-Gly-Ile-Gly-Gly-Ser-
                                                  170                 180
Asp-Leu-Gly-Pro-Leu-Met-Val-Thr-Glu-Ala-Leu-Lys-Pro-Tyr-Ser-Lys-Gly-Gly-Pro-Arg-
                                                  190                 200
Val-Trp-Phe-Val-Ser-Asn-Ile-Asp-Gly-Thr-His-Ile-Ala-Lys-Thr-Leu-Ala-Ser-Leu-Ser-
                                                  210                 220
Pro-Glu-Thr-Ser-Leu-Phe-Ile-Ile-Ala-Ser-Lys-Thr-Phe-Thr-Thr-Gln-Arg-Thr-Ile-Thr-
                                                  230                 240
Asn-Ala-Glu-Thr-Ala-Lys-Glu-Trp-Phe-Leu-Glu-Ala-Ala-Lys-Asp-Pro-Ser-Ala-Val-Ala-
                                                  250                 260
Lys-His-Phe-Val-Ala-Leu-Ser-Thr-Asn-Thr-Ala-Lys-Val-Lys-Glu-Phe-Gly-Ile-Asp-Pro-
                                                  270                 280
Glu-Asn-Met-Phe-Glu-Phe-Trp-Asp-Trp-Val-Gly-Gly-Arg-Tyr-Ser-Leu-Trp-Ser-Ala-Ile-
                                                  290                 300
Gly-Leu-Ser-Ile-Ala-Leu-His-Val-Gly-Phe-Asp-His-Phe-Glu-Gln-Leu-Leu-Ser-Gly-Ala-
                                                  310                 320
His-Trp-Met-Asp-Gln-His-Phe-Leu-Lys-Thr-Pro-Leu-Glu-Lys-Asn-Ala-Pro-Val-Leu-Leu-
                                                  330                 340
Ala-Leu-Leu-Gly-Ile-Trp-Tyr-Ile-Asn-Cys-Tyr-Gly-Cys-Glu-Thr-His-Ala-Leu-Leu-Pro-
                                                  350                 360
Tyr-Asp-Gln-Tyr-Met-His-Arg-Phe-Ala-Ala-Tyr-Phe-Gln-Gln-Gly-Asp-Met-Glu-Ser-Asn-
                                                  370                 380
Gly-Lys-Tyr-Ile-Thr-Lys-Ser-Gly-Ala-Arg-Val-Asp-His-Gln-Thr-Gly-Pro-Ile-Val-Trp-
                                                  390                 400
Gly-Glu-Pro-Gly-Thr-Asn-Gly-Gln-His-Ala-Phe-Tyr-Gln-Leu-Ile-His-Gln-Gly-Thr-Lys-
                                                  410                 420
Met-Ile-Pro-Cys-Asp-Phe-Leu-Ile-Pro-Val-Gln-Thr-Gln-His-Pro-Ile-Arg-Lys-Gly-Leu-
                                                  430                 440
His-His-Lys-Ile-Leu-Leu-Ala-Asn-Phe-Leu-Ala-Gln-Thr-Glu-Ala-Leu-Met-Lys-Gly-Lys-
                                                  450                 460
Leu-Pro-Glu-Glu-Ala-Arg-Lys-Glu-Leu-Gln-Ala-Ala-Gly-Lys-Ser-Pro-Glu-Asp-Leu-Glu-
                                                  470                 480
Lys-Leu-Leu-Pro-His-Lys-Val-Phe-Glu-Gly-Asn-Arg-Pro-Thr-Asn-Ser-Ile-Val-Phe-Thr-
                                                  490                 500
Lys-Leu-Thr-Pro-Phe-Ile-Leu-Gly-Ala-Leu-Ile-Ala-Met-Tyr-Glu-His-Lys-Ile-Phe-Val-
                                                  510                 520
Gln-Gly-Ile-Met-Trp-Asp-Ile-Asn-Ser-Phe-Asp-Asn-Thr-Gly-Val-Glu-Leu-Gly-Lys-Gln-
                                                  530                 540
Leu-Ala-Lys-Lys-Ile-Glu-Pro-Glu-Leu-Glu-Gly-Ser-Ser-Ala-Val-Thr-Ser-His-Asp-Ser-
                                                  550             558
Ser-Thr-Asn-Gly-Leu-Ile-Ser-Phe-Ile-Lys-Gln-Gln-Arg-Asp-Thr-Lys-Leu-Glu
```

Fig. 8.5. cDNA-derived amino acid sequence of mouse preproneuroleukin

the other hand, this peptid functionally resembles other lymphokines (see Chapter 7) in its capability of inducing immunoglobulin synthesis in a selected group of B cells (Gurney et al. 1986a). However, the exact physiological role of this neural growth factor/lymphokine remains to be established in the future (for a recent review see Gurney 1987).

8.1.1.1.4. MISCELLANEOUS GROWTH FACTORS IN THE SALIVARY GLAND

(1) Mesodermal growth factor (MGF). In the 1960s mouse salivary gland extracts were shown to contain a growth- and differentiation-promoting activity for cells of mesenchymal/mesodermal origin and it was named muscle diferentiation factor (MDF) by Attardi and associates (1965) referring to the responsive cells. Years later, partial purification of a male mouse salivary gland-derived growth factor with properties reminiscent of those of MDF but distinct from other known growth factors has been reported and the product termed MGF by Haraguchi et al. (1982). Recycling chromatography of salivary gland extract yielded a heterogenous pool of polypeptides of M_r 26,000 that was referred to as recycled MGF or R-MGF. The latter could be resolved into four active fractions (MGF I-IV) with MGF I as a major component carrying most of the biological activity.

Dissimilarly to EGF, cells of mesodermal origin appear to be the main target cells for MGF. As its most characteristic effect, MGF/R-MGF stimulates the DNA synthesis, division and general growth of fibroblasts both in vivo and in vitro, effects on which the in vivo observed wound-healing capability of this growth factor are based. The structural relatedness of MGF and R-MGF components to fibroblast growth factor, astroglial growth factor and other related growth factors remains to be established.

(2) Epithelial growth factor. The claim that male mouse submaxillary gland contains a growth factor acting specifically on epithelial cells of various origin (Jones 1966, Jones and Ashwood-Smith 1970) was seriously questioned by the observation that effects of this putative growth factor could be entirely mimicked by trypsin and pronase and could be markedly reduced by trypsin inhibitors, implicating a proteolytic enzyme as the acting agent in the extract (Banks and Walter 1972).

(3) Thymocte transforming factor (TTF). Since this structurally still uncharacterized factor that was recognized in mouse salivary gland extract by Naughton et al. (1966) was claimed to transform small lymphocytes into cells of the plasma series as its main action, it can be regarded as a differentiation factor rather than a true growth factor.

8.1.1.2. GROWTH FACTORS DERIVED FROM THE ENDOCRINE SYSTEM

8.1.1.2.1. PITUITARY-DERIVED GROWTH FACTORS

The failure of highly purified pituitary hormones to elicit growth factor activities similar to those displayed by their crude preparations suggested that growth stimulation by impure pituitary hormones might be ascribed to some extrahormonal factors lost during purification.

8.1.1.2.1.1. PITUITARY FIBROBLAST GROWTH FACTOR

While attempting to purify ovarian growth factor (OGF; see below) from bovine pituitary extracts, Gospodarowicz (1975) detected an additional mitogenic activity that he named fibroblast growth factor (FGF). The name refers to the cell on which FGF was first tested, though subsequent investigations revealed a much wider spectrum of FGF-responsive cells. Besides pituitary, FGF-like activity can be extracted from various brain areas and numerous other tissues and cell sources that all are characterized by their capability of preferentially stimulating the proliferation of endothelial cells suggesting a close relationship, perhaps identity, between FGFs and endothelial cells growth factors (ECGFs) discussed elsewhere in this chapter (for details and references see Klagsbrun and Smith 1980, Bohlen et al. 1984, 1985, 1986, Baird et al. 1985a,b,c, Esch et al. 1985a,b, Klagsbrun and Shing 1985, Gospodarowicz et al. 1985a,b, 1986a,b,c, Klagsbrun et al. 1986). Recent discovery of the unusual heparin-binding ability

```
                                                  10                  20
Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-His-Phe-Lys-Asp-Pro-
                                                  30                  40
Lys-Arg-Leu-Tyr-Cys-Lys-Asn-Gly-Gly-Phe-Phe-Leu-Arg-Ile-His-Pro-Asp-Gly-Arg-Val-
                                                  50                  60
Asp-Gly-Val-Arg-Glu-Lys-Ser-Asp-Pro-His-Ile-Lys-Leu-Gln-Leu-Asn-Ala-Glu-Glu-Arg-
                                                  70                  80
Gly-Val-Val-Ser-Ile-Lys-Gly-Val-Cys-Ala-Asn-Arg-Tyr-Leu-Ala-Met-Lys-Glu-Asn-Gly-
                                                  90                  100
Arg-Leu-Leu-Ala-Ser-Lys-Cys-Val-Thr-Asp-Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-
                                                 110                  120
Asn-Asn-Tyr-Asn-Thr-Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr-Val-Ala-Leu-Lys-Arg-
                                                 130                  140
Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Pro-Lys-Thr-Gly-Pro-Gly-Gln-Lys-Ala-Ile-Leu-Phe-Leu-
                                                146
Pro-Met-Ser-Ala-Lys-Ser
```

Fig. 8.6. Primary structure of bovine pituitary basic fibroblast growth factor

of these mitogens provided a powerful tool for their isolation by affinity chromatography and the occasion to alternatively designate them heparin-binding growth factors (HBGFs). Preliminary chemical data suggested that these mitogens may occur in two general structural forms referred to as acidic (aFGF) and basic FGF (bFGF), respectively. The structures of aFGF and bFGF are closely related irrespective of their tissue sources and both forms display structural homology to other distinctly named growth factors (for further information see brain-derived growth factors discussed elsewhere in this chapter). The primary structure of the 146 amino acid bovine pituitary bFGF as determined by Esch and associates (1985a) is shown in Fig.8.6. A cDNA-derived sequence (Abraham et al. 1986a) was shown to be nearly identical to that shwon in Fig.8.6 with only three amino acid substitutions: Gln(56), Asp(79), and Asp(101).

An N-terminal 8 residue-extended form of bFGF, termed Y-bFGF, has also been prepared from normal bovine pituitary (Ueno et al. 1986a). The extension has a blocked N-terminus and the likely sequence: AcAla-Ala-Gly-Ser-Ile-Thr-Thr-Leu, where AcAla indicates an alanine residue blocked by an acetyl group. Sequence analysis of a human hepatoma-derived growth factor (Klagsbrun et al. 1986) suggested its identity with the peptide reported by Ueno et al. (1986a).

Up to now, bFGFs with 130, 146 and 154 residues with full biological activity have been isolated from various sources

(Baird et al. 1985a,b, Gospodarowicz et al. 1985, 1986a, Esch et al. 1985a). Careful considerations suggest that natural bFGF corresponds to the 154 residue forms with blocked N-termini and that all other truncated forms represent artifacts generated during the extraction procedures (for supporting evidence see Ueno et al. 1986a; for structural data on ovine pituitary bFGF see Simpson et al. 1987).

In vitro, both aFGF and bFGF stimulate DNA synthesis and proliferation of multiple endothelial cell types such as capillary endothelial, adrenocortical, granulosa and even vascular smooth muscle cells, whereas bFGF is a potent angiogenic agent in vivo. The exact physiological role of pituitary bFGF is far from clear at present, though its involvement in regenerative processes have been implicated (reviewed by Mescher and Gospodarowicz 1979).

In the light of recent findings, the polypeptide growth factors distinctly named as FGFs, ECGFs, HBGFs, angiogenesis factor, etc., may represent a newly recognised family of peptides sharing important structural and biological properties. If their common belonging to a new category of growth factors can be firmly established, it may hopefully unnecessitate their separate naming, a prospect that probably would greatly clarify the present confusion experienced in the nomenclature of these growth factors.

8.1.1.2.1.2. MISCELLANEOUS PITUITARY-DERIVED GROWTH FACTORS

(1) Ovarian growth factor (OGF). Also called ovarian cell factor (OCF) is a carbohydrate-free polypeptide of basic character with a M_r between 10,000 and 13,000 that was isolated from bovine pituitary extract as a contaminant of partially purified LH preparations (Gospodarowicz et al. 1974). The qualitative amino acid composition of OGF has been established but its primary structure has not. OGF preferentially stimulates proliferation of the cells of ovarian origin (Jones and Gospodarowicz 1974). The physiological role of OGF remains unclear, but it has been implicated in the genesis of some ovarian tumors (Furth and Sobel 1974).

(2) Pituitary myoblast growth factor (MGF). Addition of a crude pituitary extract to low-density myoblast cultures promoted the proliferation and differentiation of these cells, an effect that was attributed to a specific proliferogenic agent termed MGF (Gospodarowicz et al. 1975a,b). The effect showed a pH dependency with a pronounced FGF and negligible MGF activity at low pH, and with a reversed activity ratio at slightly higher than neutral pH. Both the structure and the relation of MGF to other pituitary mitogens remain undetermined (brain-derived MGF is discussed elsewhere in this Chapter).

(3) Pituitary factor (PF). In a series of papers, Saxena (1978) Saxena and Saxena (1978a,b, 1979a,b,c) reported of a partially purified growth factor of peptide character (M_r 500), termed PF, from extracts of the anterior pituitary and from those of other organs of various mammals, that species nonspecifically stimulated DNA synthesis mainly in thymocytes but also in other lymphoid and in bone marrow cells in vitro. PF, of which the authors claimed to be a specific factor for a major subpopulation of thymocytes, has not been structurally defined.

(4) Pituitary endothelial cell growth factor (ECGF). It is highly probable that the ECGF activity described by Maciag et al. (1979) in pituitary extracts can be ascribed to pituitary FGF (similar but brain-derived activities are discussed elsewhere in this Chapter).

(5) Chondrocyte growth factor (CGF). The name refers to a component of human pituitary extracts that is cell-specifically mitogenic in rabbit chondrocyte cultures even if serum is present in low concentrations in the media (Kasper et al. 1982). Its estimated molecular weight (40,000) and physicochemical characteristics make its identity with other characterized pituitary growth factors very unlikely, except bFGF (for recent information see Too et al. 1987).

(6) Mammary growth factor. Although the late mammary growth factor of Kano-Sueoka et al. (1979a,b) turned out to be identical with ethanolamine, the recently described sheep pituitary-derived growth factor with a preferential activity on human and rat mammary tumor cells appears to be a true peptide (Ikeda et al. 1984).

(7) Glial growth factor. Purification of extracts from bovine pituitary and from different brain areas yielded a peptide product (M_r 30,000) having the capability of stimulating the proliferation of Schwann cells and astrocytes without any effects on oligodendrocytes and macrophage-like glial cells (Lemke and Brockes 1983). Specific activity of the product varies depending on the site of its origin. This factor was tentatively named glial growth factor referring to the importance of Schwann cells in its identification (astroglial growth factor is discussed elsewhere in this Chapter).

8.1.1.2.1.3. KNOWN PITUITARY PEPTIDE HORMONES

It is generally accepted that some peptide hormones of the adenohypophysis regulate not only the endocrine function of their target organs but additionally exert a trophic action on them, thereby displaying a growth factor-like activity in this restricted sense. Such functions have been attributed especially to growth hormone, prolactin (Liggins 1974, Bradshaw and Rubin 1980, Russel et al. 1987), gonadotrop hormones (Inoue et al. 1985), thyrotropin (Lamy et al. 1986, Roger and Dumont 1987) and also to α-melanocyte-stimulating hormone (Honnebier and Swaab 1974, Silman et al. 1977).

8.1.1.2.2. THYMUS-DERIVED GROWTH FACTORS

Several thymus peptides function not only as differentiation/maturation factors but also as growth factors, by promoting differentiation-connected proliferation of various immunogenic cells (for thymus peptides see Chapter 7).

8.1.1.2.3. GASTROENTEROPANCREATIC PEPTIDE HORMONES

Although the gastroenteropancreatic (GEP) peptides (reviewed in Chapter 6) have rather well defined functions as hormones or paracrine agents, simultaneously they also fulfill growth factor-like tasks by acting as mitogenic or trophic agents in different regions of the GEP system.

(1) Gastrin and cholecystokinin. Besides its overall trophic action on gut mucosa, gastrin is selectively mitogenic for the progenitor cells in oxynthic mucosa, for glandular stem cells and parietal cells in the stomach and for the cells in the duodenal crypts and colonic glands. Fatemi and associates (1984) reported of a differential mitogenic effect of gastrin, pentagastrin, cholecystokinin (CCK) and glucagon on mucosal cells along the alimentary canal. Cholecystokinin (CCK) was reported to exert a proliferogenic action mainly on exocrine pancreas, but also on gallbladder mucosal cells (Arnold 1985), while caerulein, a CCK analog was found to be active on cells in the antrum and the pancreas (Caes and Willems 1984). The caerulein-induced pancreatic growth may be mediated by regulating tissue concentration of somatostatin, a putative "anti-growth factor" (Sarfati et al. 1985). Gastrin, CCK and glucagon may also interfere with the proliferation of malignant cells (Arnold 1985).

(2) Gastrin-releasing peptide (GRP). It is the mammalian equivalent of bombesin both structurally and functionally. By all indications, GRP stimulates the clonal growth and colony-forming efficiency of normal human bronchial epithelial cells (Willey et al. 1984).

(3) Pancreatic polypeptide. The growth factor-like character of pancreatic polypeptide was suggested by its DNA synthesis stimulatory activity (Greenberg et al. 1977) and its trophic action (Laurentz and Hazelwood 1979).

(4) Insulin. Although the principal role of insulin is to regulate both distribution and utilization of metabolic fuels within the animal organisms, initial observations already indicated its growth-enhancing capacity (see e.g., Gey and Thalhimmer 1924). Early hopes, however, that insulin would prove to be a powerful mitogen of physiological importance did not fully come through: it is a rather poor mitogen for mammalian cells in general. However, the mitogenicity of insulin, though seemingly unimpressive, is relevant from a physiological point of view (reviewed by Straus 1984) and is an intrinsic property of the molecule (Petrides and Bohlen 1980) probably rooted in its crossreactivity with the receptors of structurally related serum peptides, the insulin-like growth factors. It also plays a permissive role by keeping cells metabolically in a good shape, thereby enabling them to respond to stimuli mediated by other growth factors whose effects are potentiated by insulin in a number of cell systems (Griffiths 1972, Gospodarowicz and Moran 1975). The tyrosine phosphorylation stimulatory and ODC-inducing effects of insulin may also be involved in its proliferogenic capability (Rinehart and Canellakis 1985, White et al. 1985). Insulin is also regarded as an important fetal growth factor (reviewed by Liggins 1974, Milner and Hill 1984).

(5) Glucagon. Glucagon may function as a "class II signal" potentiating cellular responses previously promoted by "class I signals" e.g., EGF, mainly in liver cells (Leffert et al. 1979). Glucagon was shown to stimulate the proliferation of mucosal cells in the small intestine (Fatemi et al. 1984).

8.1.1.3. GROWTH FACTORS DERIVED FROM THE NERVOUS SYSTEM

Surprisingly the nervous system proved to be an especially rich source of growth-regulating substances of various character. Up to now, most of these growth factors remained structurally poorly characterized and this has significantly contributed to the confusion experienced in their nomencla-

ture. This holds especially true for brain- and pituitary-derived growth factors. Species differences may have also contributed to the confusion by giving separate names to growth factors that were, in fact, functionally identical. Fortunately, this field of research now undergoes advantageous changes due to a steadily increasing number of structurally characterized growth factors of neural tissue origin.

8.1.1.3.1. CNS-DERIVED GROWTH FACTORS

8.1.1.3.1.1. BRAIN-DERIVED FIBROBLAST GROWTH FACTOR

Besides the pituitary, fibroblast growth factor (FGF) activity was also found in, and purified from, brain tissue extracts of various species (for FGF in nonneural tissue see Gautschi-Sova et al. 1987). Previous research on FGF has culminated in characterizing two forms (FGF-1 and FGF-2) of bovine brain-derived FGF (Gospodarowicz et al. 1978a) and in identifying this growth factor as a fragment of myelin basic protein (MBP), (Fig.8.7) within which an additional form of FGF (FGF-3) was also suggested to exist (Westall et al. 1978). Based on these results MBP was regarded by contemporary scientists as a precursor molecule from which CNS-derived FGFs are generated by proteolytic cleavage (for supporting evidence see Neville 1960, Whittaker 1977). In vivo proteolysis of MBP may have both physiological and pathological significance by producing MBP fragments promoting wound healing and formation of new capillaries on the one hand, and inducing encephalomyelitis on the other (Westall et al. 1971, Westall and Thompson 1977). The MBP-derived FGFs are unrelated to pituitary FGFs, while the latters and other brain-derived FGFs share many similarities indicating that closely related substances exist both in the brain and the pituitary, and that FGF-like activities may be bound to multiple molecules in various tissues (for current information on MBP see Chan et al. 1987, Kamholz et al. 1987).

```
1(44)                                10(53)                                20(63)
Phe-Gly-Ser-Asp-Arg-Gly-Ala-Pro-Lys-Arg-Gly-Ser-Gly-Lys-Asp-Gly-His-His-Ala-Ala-
                                     30(73)                                40(83)
Arg-Thr-Thr-His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys-Ala-Gln-Gly-His-Arg-Pro-Gln-Asp-Glu-
                                     50(93)                                60(103)
Asn-Pro-Val-Val-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro-Arg-Thr-Pro-Pro-Pro-Ser-Gln-
                                     70(113)                               80(123)
Gly-Lys-Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys-Pro-
                                     90(133)                               100(143)
Gly-Phe-Gly-Trp-Gly-Gly-Arg-Ala-Ser-Asp-Tyr-Lys-Ser-Ala-His-Lys-Gly-Leu-Lys-Gly-
                                     110(153)                              120(163)
His-Asp-Ala-Gln-Gly-Thr-Leu-Ser-Lys-Ile-Phe-Lys-Leu-Gly-Gly-Arg-Asp-Ser-Arg-Ser-
      123(166)
Gly-Ser-Pro
```

Fig. 8.7. Residues 44 through 166 of myelin basic protein (MBP). The entire sequence shown in the figure corresponds to brain fibroblast growt factor-1 (FGF-1) whereas residues 44 through 153 to FGF2, and those 91 through 153 to FGF-3. Numbers in parentheses indicate the positions of residues within the MBP sequence

There have always been scientists who have questioned the exclusive MBP origin of brain FGFs (Thomas et al. 1980, Lemmon and Bradshaw 1983). Supporting the sceptics, Thomas and coworkers (1984) have isolated from bovine brain an acidic mitogenic activity for fibroblasts in two microheterogenous forms with a M_r 16,000 and 16,800, respectively, and with properties distinctly different from those of MBP-derived FGFs. However, due to the lack of exact sequence data, the relation of this FGF to other partially characterized acidic FGFs (aFGFs) purified from human brain (Kellett et al. 1981), bovine hypothalamus (Maciag et al. 1982), retina (Barritoult et al. 1982) and the pituitary (Gambarini and Armelin 1982) remained unclear. Subsequently, however, two unique FGFs, one with an acidic (Thomas et al. 1984) and another with a basic pI (Bohlen et al. 1985) have been purified from bovine and human brain, respectively. The acidic form (aFGF) was purified in two microheterogenous forms, aFGF-1 (M_r 16,000) and aFGF-2 (M_r 17,000) differing in the presence and in the absence of an N-terminal hexapeptide sequence (Thomas et al. 1985). The complete amino acid sequences of human and bovine brain-derived aFGFs have been determined by Gimenez-Gallego and coworkers (1985,1986a,b) and Esch and associates (1985b), respectively. The amino acid sequence of bovine brain-derived bFGF has also been determined, partly by direct amino acid analysis (Esch et al. 1985a), partly as a cDNA-derived sequence (Abraham et al. 1986a), while for the human brain-derived bFGF, first a par-

tial N-terminal sequence (Gimenez-Gallego et al. 1986a) and subsequently a cDNA-derived full sequence (Abraham et al. 1986b, Kurokawa et al. 1987) have been reported (Fig.8.8; see also Bertolini and Hearn 1987, Iwane et al. 1987).

Both human and bovine brain-derived aFGFs have 140 amino acid residues and a M_r of 15,800. Their sequences are more than 90% homologous. On the other hand, both human and bovine brain-derived bFGFs have N-terminally 9 residues that are missing from the acidic forms. Homology between human and bovine bFGFs reaches 97%. Disregarding the 9 amino acid

```
                                                                  10
Ha:                                   Phe-Asn-Leu-Pro-Pro-Gly-Asn-Tyr-Lys-Lys-Pro-
Ba:                                   - - - - - Leu - - - - - - - -
Hb: Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-His-Phe-Lys-Asp-Pro-
Bb: - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
                                      20                          30
Ha: Lys-Leu-Leu-Tyr-Cys-Ser-Asn-Gly-Gly-His-Phe-Leu-Arg-Ile-Asp-Pro-Asp-Gly-Thr-Val-
Ba: - - - - - - - - - - - Tyr - - - - Leu - - - - - -
Hb: Lys-Arg-Leu-Tyr-Cys-Lys-Asn-Gly-Gly-Phe-Phe-Leu-Arg-Ile-His-Pro-Asp-Gly-Arg-Val-
Bb: - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
                                      40                          50
Ha: Asp-Gly-Thr-Arg-Asp-Arg-Ser-Asp-Gln-His-Ile-Gln-Leu-Gln-Leu-Ser-Ala-Glu-Ser-Val-
Ba: - - - Lys - - - - - - - - - - - - - - Cys - - - Ile
Hb: Asp-Gly-Val-Arg-Glu-Lys-Ser-Asp-Pro-His-Ile-Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-
Bb: - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
                                      60                          70
Ha: Gly-Glu-Val-Tyr-Ile-Lys-Ser-Thr-Glu-Thr-Gly-Gln-Tyr-Leu-Ala-Met-Asp-Thr-Asp-Gly-
Ba: - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Hb: Gly-Val-Val-Ser-Ile-Lys-Gly-Val-Cys-Ala-Asn-Arg-Tyr-Leu-Ala-Met-Lys-Glu-Asp-Gly-
Bb: - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
                                      80                          90
Ha: Leu-Leu-Tyr-Gly-Ser-Gln-Thr-Pro-Asn-Glu-Glu-Cys-Leu-Phe-Leu-Glu-Arg-Leu-Glu-Glu-
Ba: - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Hb: Arg-Leu-Leu-Ala-Ser-Lys-Cys-Val-Thr-Asp-Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-
Bb: - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
                                     100                         110
Ha: Asn-His-Tyr-Asn-Thr-Tyr-Ile-Ser-Lys-Lys-His-Ala-Glu-Lys-Asn-Trp-Phe-Val-Gly-Leu-
Ba: - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Hb: Asn-Asn-Tyr-Asn-Thr-Tyr-Arg-Ser-Arg-Lys-Tyr-Thr-Ser-Trp-Tyr-Val-Ala-Leu-Lys-Arg-
Bb: - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
                                     120                         130
Ha: Lys-Lys-Asn-Gly-Ser-Cys-Lys-Arg-Gly-Pro-Arg-Thr-His-Tyr-Gly-Gln-Lys-Ala-Ile-Leu-
Ba: - - - - - Arg-Ser - Leu - - - - - - Phe - - - - - - - -
Hb: Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Ser-Lys-Thr-Gly-Pro-Gly-Gln-Lys-Ala-Ile-Leu-Phe-Leu-
Bb: - - - - - - - - - Pro - - - - - - - - - - - - - - - - - - - - - - - - - - - -
                                     140
Ha: Phe-Leu-Pro-Leu-Pro-Val-Ser-Ser-Asp
Ba: - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Hb: Pro-Met-Ser-Ala-Lys-Ser
Bb: - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
```

Fig. 8.8 Amino acid sequences of human (H) and bovine (B) brain-derived acidic (a) and basic (b) fibroblast growth factors. Full sequences are given for the human peptides, acidic and basic. In the corresponding bovine sequences only the amino acid substitutions are indicated. Dashes indicate residues identical to those in the human peptides. The Ha, Ba and Bb sequences were chemically determined. Of these, the Bb sequence is identical to the corresponding pituitary peptide shown in Fig.8.6. The Hb sequence was deduced from cloned cDNA. Alignment was made to show the structural homology between the human and bovine peptides

N-terminal extension of bFGFs, a rather significant homology exists between aFGFs and bFGFs (see Fig.8.8) indicating that these two types of FGFs descended from a common ancestral protein. It is also noteworthy, that pituitary- and brain-derived bFGFs are identical (Bohlen et al. 1985, Esch et al. 1985a, Gimenez-Gallego et al. 1985, 1986a,b) while about 55% sequence homology exists between pituitary-derived bFGF and brain-derived aFGF (Esch et al. 1985b; compare Figs 8.6 and 8.8). There are indications that bFGF exists in multimolecular forms (Klagsbrun et al. 1987, Sommer et al. 1987, Story et al. 1987), and that bFGF or closely-related proteins are produced not only in the pituitary and brain but also by retinal cells (Schweiger et al. 1987), endothelial cells (Vlodavsky et al. 1987) and human hepatoma cells (Klagsbrun et al. 1986) in vitro as well as by the liver (Ueno et al. 1986b) and benign prostatic hyperplastic tissues (Story et al. 1987) in vivo. Apart from the aforementioned similarities, brain-derived human aFGF is structurally and functionally also related to human interleukin-1β (IL-1β; Gimenez-Gallego et al. 1985), to an interferon-inducing factor (Cameron et al. 1985) and to a form of prostatropins (PTs), polypeptide mitogens for prostate epithelial cells concentrated in neural tissues. As to the latter, two forms of PT have been purified to homogeneity: one has a M_r of about 16,000 and an unblocked N-terminus, whereas another has a M_r of about 18,000 and a blocked (acetylated) N-terminus (Crabb et al. 1986a). Surprisingly, the larger form of PT (Crabb et al. 1986b) proved to be an N-terminal 14 amino acid extended form of bovine brain-derived aFGF described by Esch et al. (1985b), and of class 1 HBGF (HBGF-1), a 140 amino acid bovine brain-derived protein (M_r 15,877) reported by Strydom and coworkers (1986). The 14 amino acid extension is N-terminally blocked and has the following sequence:
AcAla-Gly-Glu-Glu-Thr-Thr-Ile-Phe-Thr-Ala-Leu-Thr-Glu-Lys.
This HBGF-1 is also homologous to bovine brain-derived bFGF with about 50% of their amino acids being identical. On the other hand, a cDNA-derived amino acid sequence of the recently described human brain-derived endothelial cell growth factor (ECGF; Jaye et al. 1986) proved to be a 15 amino acid extended

form of human brain-derived aFGF reported by Gimenez-Gallego et al. (1985,1986b). The amino acid sequence of the extension of human ECGF is as follows:
Met-Ala-Glu-Gly-Glu-Ile-Thr-Thr-Phe-Thr-Ala-Leu-Thr-Glu-Lys,
which is homologous to the aforementioned extension of PT to a significant extent.

A decapeptide sequence contained within aFGFs at residue 102 through 111 is flanked on each end by a Lys-Lys dipeptide sequence. Since basic dipeptide sequences are signals for proteolytic processing of many active peptides from longer precursors, the mentioned decapeptide sequence was compared to those of other known peptides. It was established that, especially the C-terminal half of the decapeptide aligned well with various active neuropeptides such as neuropeptide-C and -K, substance P and substance K, bombesin, physalemin and eledoisin (Gimenez-Gallego et al. 1985). Thus, theoretically at least, the mentioned decapeptide sequence within the aFGF molecule may represent a unique neuropeptide.

The genes for human aFGF and bFGF were assigned to different chromosomes, chromosome 4 and 5, respectively, indicating that the two genes are not linked inspite of their presumably common evolutionary ancestor (Mergia et al. 1986).

Initially, the purification of FGF was monitored by its DNA synthesis-stimulating effect on a fibroblastic cell line. However, subsequent studies revealed that FGFs are very potent mitogens for vascular endothelial cells in vitro, and are potent angiogenic agents in vivo (for details and references see Thomas et al. 1985, Montesano et al. 1986). In the presence of heparin aFGF and bFGF have virtually equipotent specific mitogenic activity for human umbilical endothelial cells. However, in the absence of heparin bFGF is only slightly less active, but bFGF is about 1% or less as active as it is in the presence of heparin (Gimenez-Gallego et al. 1986a). Corresponding to their sequence homology, human and bovine aFGFs are equally active on human endothelial cells in culture (Schreiber et al. 1985, Thomas et al. 1985, Gimenez-Gallego 1986a) allowing to use them interchangeably in various in vivo and in vitro assay systems used to assess angiogenic activity. bFGF may be a na-

tural growth factor also for human melanocytes (Halaban et al. 1987) and FGFs have neurotrophic functions as well (Unsicker et al. 1987). In short, FGFs appear to be broad spectrum mitogens with potent angiogenic activities, properties that they share with brain-derived ECGFs discussed in the subsequent section. However, the physiological significance of the existence in the brain of several unique growth factors with similar spectra of biological activities remains to be clarified (for further information and references on FGFs see Gensburger et al. 1986, Gospodarowicz et al. 1986b,c,d, 1987, Juang et al. 1986, Morrison et al. 1986, Pelech et al. 1986, Thomas and Gimenez-Gallego 1986, Conolly et al. 1987, Mascarelli et al. 1987, Moscatelli 1987, Odedra and Weiss 1987, Thomas 1987).

8.1.1.3.1.2. ENDOTHELIAL CELL GROWTH FACTOR

The name endothelial cell growth factor (ECGF) refers to a whole array of polypeptide mitogens most abundantly present in neural tissues and tissues related to them (D'Amore and Klagsbrun 1984, Klein-Soyer et al. 1984, Lobb et al. 1985, Schreiber et al. 1985) that share many biological and chemical properties. They characteristically stimulate the proliferation of fibroblastic and endothelial cells (ECs) in vitro, induce angiogenesis in vivo and bind vividly to immobilized heparin, a property that greatly facilitated their purification (Conn and Hatcher 1984, D'Amore and Klagsbrun 1984, Maciag et al. 1984, Klagsbrun and Shing 1985, Terranova et al. 1985, Lobb et al. 1986). Thus FGF, ECGF, HBGF and angiogenesis factor would all be proper names for these peptides.

Applying a series of criteria, HBGFs can be classified into two broad categories. Class 1 HBGFs or HBGFs-1, formerly termed α-HBGFs, are acidic (anionic) mitogens with M_rs ranging between 15,000 and 17,000 that are almost exclusively found in neural tissues. They include the following distinctly named growth factors: brain- and pituitary-derived aFGFs (Gambarini and Armelin 1982, Lobb and Fett 1984, Thomas et al. 1984, 1985, Gimenez-Gallego et al. 1985, 1986a,b, Esch et al.

1985b), hypothalamus-derived acidic ECGF (aECGF) presumably having multiple molecular forms (Maciag et al. 1979, 1982, D'Amore and Klagsbrun 1984, Klagsbrun and Shing 1985); eye-derived growth factor-II (EDGF-II) demonstrated in the retina (hence its other name, retina-derived growth factor or RDGF) and in other ocular tissues (D'Amore et al. 1981, Barritault et al. 1981, 1982, D'Amore 1982, D'Amore and Klagsbrun 1984) of which two other forms, EDGF-I, and III are also known (Courty et al. 1985), and astroglial growth factor-1 (AGF-1), discussed elsewhere in this Chapter.

On the other hand, class 2 HBGFs or HBGF-2, formerly named β-HBGFs, are basic (cationic) mitogens with M_rs ranging between 18,000 and 20,000 that have a much wider tissue distribution than their acidic counterparts. They include pituitary- and brain-derived bFGFs (Gospodarowicz et al. 1982, Bohlen et al. 1985, Gimenez-Gallego et al. 1985a); hypothalamus-derived basic ECGF (bECGF; Klagsbrun and Smith 1980); cartilage-derived growth factor (CDGF; Klagsbrun and Smith 1980, Bekoff and Klagsbrun 1982, Sullivan and Klagsbrun 1985); kidney-derived ECGF (Baird et al. 1985a); EDGF/RDGF-I (Baird et al. 1985b); liver cell-derived factor (Hoshi and McKeehen 1984); platelet-derived ECGF (Clemmons et al. 1983); tumor- or chondrosarcoma-derived ECGF (Shing et al. 1984) and AGF-2 (see elsewhere in this Chapter).

Although a majority of these distinctly named growth factors has not yet been structurally characterized, sequence comparison of recently characterized human and bovine pituitary- and brain-derived aFGFs and bFGFs, human ECGF, bovine HBGF-1 and bovine prostatropin lent a strong support to the close chemical relatedness, occasionally identity, of these polypeptide growth factors. It should also be mentioned that, while there is a wide variety of cells and tissues endowed with the capability of producing EC mitogens (see e.g., Miyazono et al. 1987), studies on cultured bovine aortic and human umbilical vein endothelial cells have convincingly demonstrated the secretion by these cells of at least two distinct polypeptides, the M_r 30,000 and M_r 50,000 endothelium-derived growth factors having the capability of stimulating many con-

nective tissue cells in vitro (DiCorleto 1984, Gajdusek 1984, Scarra et al. 1985). Receptor-binding studies revealed platelet-derived growth factor (PDGF)-like characteristics of one of these growth factors. Both PDGF-like and PDGF-distinct mitogenic activities were found to be mediated by highly cationic substances. Although biochemical and immunological studies have indicated the structural unrelatedness of these mitogens to a number of known growth factors (DiCorleto 1984) their exact structures remain unknown for the time present.

Interest in ECGFs has been stimulated by the ubiquitous presence of ECs and their potential role in maintaining homeostasis. There are both physiological (e.g., wound healing, morphogenetic tissue rearrengement, embryogenesis, formation of corpus luteum, etc.) and pathological conditions(e.g., psoriasis, rheumatoid arthritis, diabetic retinopathies, arteriosclerosis, the genesis and metastases of tumors, etc.) that are characterized by a significant increase in EC proliferation that is frequently accompanied by angiogenesis or neovascularization. EC proliferation and angiogenesis, although are closely related, represent two distinct processes: angiogenesis always involves EC proliferation but the converse is not true. Angiogenesis often plays important etiological roles in the aforementioned pathological conditions. The idea that endogenous factors should participate in the induction of angiogenesis goes back many years, and the postulated molecular messenger(s) involved in the process has long been sought (for reviewing past and more recent literature see Gullino 1981, Vallee et al. 1985). These studies revealed a number of angiogenetic activities, nonpeptides and peptides. Of the nonpeptide angiogenesis factors, recent observations attribute an especially important role to heparin (see e.g., Folkman 1985a,b). Among the partially characterized angiogenic activities apparently carried by peptides/proteins, the "epidermal angiogenic factor" (Wolf and Harrison 1973), the "endothelium-stimulating factor" (Hoffman et al. 1976) and the unnamed polypeptides described by Tuan and coworkers (1973) and by Lundberg (1984) deserve mentioning. Testes, corpora lutea, kidneys and retinas are amongst the normal tissues

whose extract exert angiogenic activity. On the other hand, immunocompetent lymphocytes, leukocytes and macrophages are among the cells producing angiogenic factors (for angiotropin, a monocyte-derived angiogenic substance see Wissler and Renner 1981, Hockei et al. 1987). Of the fully known growth factors EGF, FGFs (Ben Ezra 1978, Gospodarowicz et al. 1978b, Petroutsos et al. 1984, Gospodarowicz et al. 1986b,c, McAuslan et al. 1985) and HBGFs (Lobb et al. 1985) were shown to elicit angiogenesis.

```
                                                  10                  20
pGlu-Asp-Asn-Ser-Arg-Tyr-Thr-His-Phe-Leu-Thr-Gln-His-Tyr-Asp-Ala-Lys-Pro-Gln-Gly-
                                                  30                  40
Arg-Asp-Asp-Arg-Tyr-Cys-Glu-Ser-Ile-Met-Arg-Arg-Arg-Gly-Leu-Thr-Ser-Pro-Cys-Lys-
                                                  50                  60
Asp-Ile-Asn-Thr-Phe-Ile-His-Gly-Asn-Lys-Arg-Ser-Ile-Lys-Ala-Ile-Cys-Glu-Asn-Lys-
                                                  70                  80
Asn-Gly-Asn-Pro-His-Arg-Glu-Asn-Leu-Arg-Ile-Ser-Lys-Ser-Ser-Phe-Gln-Val-Thr-Thr-
                                                  90                  100
Cys-Lys-Leu-His-Gly-Gly-Ser-Pro-Trp-Pro-Pro-Cys-Asn-Tyr-Arg-Ala-Thr-Ala-Gly-Phe-
                                                  110                 120
Arg-Asn-Val-Val-Val-Ala-Cys-Glu-Asn-Gly-Leu-Pro-Val-His-Leu-Asp-Gln-Ser-Ile-Phe-
      123
Arg-Arg-Pro
```

Fig. 8.9. Primary structure of human tumor angiogenin. pGlu indicates pyroglutamic acid

Solid tumors have been known for over 100 years to elicit angiogenesis and to be exceptionally reach sources of angiogenetic activities (see Vallee et al. 1985 and references cited therein). The idea that tumors themselves produce a potent angiogenic substance(s), termed tumor angiogenesis factor (TAF) has long been put forward and several attempts were made to identify TAF (Greenblatt and Shubik 1968, Folkman 1974, Nicolson 1977, Brem et al. 1978, Maiorana and Gullino 1978, Olander et al. 1982, Dodd and Kumar 1984, Shing et al. 1984). The major breakthrough came when Fett and coworkers (1985) reported of the successful isolation and extensive characterization of an angiogenic protein from media conditioned by HT-29 cells, a human adenocarcinoma cell line. This factor was designated as human tumor angiogenin by the authors to distinguish it from other similar factors encountered in normal and pathological sources. Soon thereafter the chemically determined and cDNA-derived amino acid sequences of angiogenin (which proved to be identical) have been reported from the

same laboratory (Kurachi et al. 1985, Strydom et al. 1985). Human angiogenin, whose amino acid sequence is shown in Fig.8.9 is a 123 amino acid, single-chain polypeptide of M_r 14,400 with a proline residue as its C-terminus. Three disulfide bonds link the half-cystinyl residues at positions 21 and 81, 39 and 92, 57 and 107, respectively.

A striking finding was that the sequence of human angiogenin proved to be homologous to those of human and horse pancreatic ribonuclease-As, with about 35% identity and many conservatively replaced residues in the rest of the molecule. The provocative finding that amino acid residues essential for the activity of ribonuclease are also conserved might have important physiological implications as to the mechanism of action of angiogenesis-inducing molecules, although to date, angiogenin has not been found to exhibit ribonuclease-A activity so far.

Since most neoplastic cells exert angiogenic activity, it was a plausible assumption that inhibiting angiogenesis might be an effective means for controlling tumor growth (Brem et al. 1976). It was also a plausible idea that antiangiogenic activities should be sought in avascular tissues such as cartilage, whose implants were shown never to be invaded by capillaries in appropriate assay systems. Antiangiogenic and EC growth-inhibiting activities could indeed be extracted from the cartilage (Eisenstein et al. 1975, Sorgente et al. 1975, Kaminski et al. 1978). The M_r 11,000 active factor possessed a collagenase inhibitory capacity (Kuettner et al. 1977) and was thought to be secreted by chondrocytes. A more recently described cartilage-derived antitumor factor (CATF) was shown to exert its antineoplastic activity through its antiangiogenic capacity (Takigawa et al. 1985). Apart from the cartilage and chondrocytes, corneal cells and extracts from the vitreous body also possess antiangiogenic activity (for recent information on angiogenic factors see Burgos 1986, Kull et al. 1987, Libermann et al. 1987, Rybak et al. 1987).

8.1.1.3.1.3. ASTROGLIAL GROWTH FACTOR

Partially purified brain-derived factors having the capability of affecting both the morphology and the proliferation of astroglial cells have been named differently in the past. Glial maturation factor (GMF), astroglial growth factor (AGF), brain-derived growth factor (BDGF), and glial growth factor (GGF) are the names most often encountered in the literature.

AGF, BDGF and GMF are acidic proteins with similar M_rs and they may be identical, whereas GGF appears to be a basic protein with a distinct M_r (for details see Pettman et al. 1985). The GGF present in both the pituitary and the brain (Lemke and Brockes 1983) has been mentioned earlier amongst the pituitary-derived growth factors. GMF is a differentiation factor for glioblasts rather than a growth factor. Following initial attempts, (Pettman et al. 1980, 1982) two forms of AGF, AGF-1 and AGF-2 have been extensively purified and characterized in chemical terms (Pettman et al. 1985). They differently modify the morphology of astroglial cells, but their effects on the proliferation of these cells are similar with only minor differences. Analytical data suggest that these AGFs are similar, or possibly identical, to brain-derived aFGFs.

A family of related peptides with a potential of stimulating the growth of specific macroglia populations has been identified in brain of rats and goldfishes and named glia-promoting factors (GPFs; Giulian and Young 1986, Giulian et al. 1986). Based on their effects GPF could be subdivided into four classes: GPF-1 (M_r 15,000) and GPF-3 (M_r 6,000) are secreted by microglial cells and preferentially act on oligodendroglial cells, whereas GPF-2 (M_r 9,000) and GPF-4 (M_r 3,000) are most abundantly present in growing neurons and preferentially act on astroglial cells. Available evidence suggests that specific GPFs are involved in regulating gliogenesis both in the developing and injured brain (for further information see Lim et al. 1985, Brockes and Kintner 1986, Giulian and Young 1986, Giulian et al. 1986 and references cited therein).

8.1.1.3.1.4. MISCELLANEOUS CNS-DERIVED GROWTH FACTORS

(1) Hippocampal growth factor. In rat hippocampal extracts two growth factors selectively acting on chick sympathetic or parasympathetic neurons have been identified and both named hippocampal growth factor (HGF; Crutcher and Collins 1982). Both HGFs accelerate neurite outgrowth from responsive neurons. The sympathetic neuron-specific HGF appears to be related to NGF. Their chemical structures are unknown at present. More recent data revealed that hippocampal extracts also exert a neurotrophic effect on cells in the medial septal nucleus (Ojika and Appel 1984).

(2) Keratinocyte growth factor. A mitogenic activity for epidermal keratinocytes, termed keratinocyte growth factor (KGF) has been partially purified from human and bovine hypothalami (Gilchrest et al. 1984) and also from human placenta (O'Keefe et al. 1985). The M_r 1,700 mitogen displays an anionic character. Its structure and even its peptide character awaits verification. It is clear, however, that the availability of KGF in a chemically pure form will clearly facilitate the serum-free cultivation of keratinocytes, and possibly that of other epithelial cells, a useful prospect also from a practical point of view.

(3) Brain myoblast growth factor. Apart from pituitary extracts, brain extracts were also found to stimulate the proliferation of myoblasts in sparse culture, an effect that was attributed to a specific factor, termed brain myoblast growth factor (Gospodarowicz et al. 1975b). Its chemical structure has not been identified thus far.

(4) Neuropeptides as growth factors. Vasopressin (VP), which is a neuropeptide only in the sense that it is produced in the hypothalamus, is a potent mitogen for quiescent fibroblasts and epithelial cells in culture (Rozengurt et al. 1979, Reznik et al. 1985). Arginine and lysine VP are equally potent and their effects are potentiated by several known growth fac-

tors. VP-occupied receptors interact with EGF receptors, thereby reducing activity of the latter (Rozengurt et al. 1981). Oxytocin is a 1000 fold less potent mitogen than VP.

Bombesin (see Cahpters 3,5 and 13) is pronouncedly mitogenic for fibroblasts (Rozengurt and Sinneth-Smith 1983) and epithelial cells (Willey et al. 1984). Its effect is potentiated by several identified growth factors and similarly to VP and the tachykinins, it stimulates a number of growth-related metabolic events.

Two related neuropeptides, substance P and substance K act as mitogens for connective tissue cells, notably for fibroblasts and vascular smooth muscle cells (Nilsson et al. 1985, Payan 1985). Their actions are mediated through specific techykinin receptors (for details see Hanley 1985). Somatostatin was also reported to exert a mitogenic action on mouse spleen lymphocytes in vitro (Pawlikowski et al. 1985).

The name gut-brain peptides refer to peptides in the brain that are identical or closely related to their counterparts in the gastroenteropancreatic (GEP) system. Whether these brain peptides are indeed identical to those in the GEP system and whether they also exert growth-related trophic actions like some GEP peptides do, remain to be determined.

(5) Chicken brain growth factor (CBGF). This is a brain-derived basic protein of M_r less than 6,000 with a potent mitogenicity for blastema cells and fibroblasts (Carlone and Rathbone 1985). CBGF may be functionally similar to the factors postulated to mediate growth stimuli from peripheral nerves to the blastema in regenerating amphibians limb. Its structure remains presently unknown.

(6) Spinal cord growth factor (SCGF). It was first isolated from the spinal cord of newborn calves, and subsequently also from other species as a M_r 1,100 basic protein with a mitogenic activity for fibroblasts (Jennings et al. 1979). Its physiological role and structure remain undetermined for the time being, as remain those of a more recently described, spinal cord-derived, NGF-unrelated neurotrophic activity (Lindsay and Peters 1984).

8.1.1.3.2. GROWTH FACTORS FROM THE PERIPHERAL NERVOUS SYSTEM

Limb regeneration, a rather common phenomenon in lower vertebrates, was suggested to be a nerve-dependent event as early as 1823 (for references see Gospodarowicz and Moran 1976). This was subsequently confirmed by Singer (1952, 1964, 1974) who has identified in brain extracts a FGF-like polypeptide with properties corresponding to those of the postulated neurotrophic substance(s) that is thought to mediate regeneration-promoting stimuli from the peripheral nerves to the blastema (Singer et al. 1976; reviewed by Brockes 1984).

The term growth associated proteins (GAPs) refers to a relatively small number of related proteins of acidic character having molecular weights ranging between 23,000 and 26,000 as well as between 43,000 and 49,000, respectively. For convenient references, they are distinguished by their M_rs (i.e., GAP-43, GAP-23, etc.). Induced synthesis of GAPs is regularly seen during axonal development and regeneration and it may be causally related to these processes. GAPs were shown to be associated with axonal membranes and their induced synthesis in neurons and glial cells as well as their rapid transport into axonal membranes seem to be a common feature of a wide range of neurons (for further information and references see Bijlsma et al. 1984, Skene 1984, Varon et al. 1984, Thoenen and Edgar 1985, Wood and Bunge 1986, Dekker et al. 1987).

8.1.1.4. GROWTH FACTORS DERIVED FROM THE REPRODUCTIVE ORGANS

8.1.1.4.1. PLACENTA-DERIVED GROWTH FACTORS

8.1.1.4.1.1. PROLIFERIN

A cloned cDNA prepared by Linzer and Nathans (1983, 1984) from murine 3T3 fibroblasts was shown to hybridize to mRNA species exclusively present in growing cells. Subsequent studies revealed that this cDNA encoded a 224 amino acid protein (M_r 24,000) showing significant sequence homology to human (pre)-prolactin (Linzer et al. 1985). Therefore, and because

of the exclusive detectability of this particular mRNA/cDNA in proliferating cells, the name proliferin (PLF) was coined to designate the encoded protein. Placenta was found to be the only organ containing PLF-related mRNAs in vivo. The sequence of the placental gene product slightly differed from that prepared from cultured 3T3 cell-lines. To distinguish between 3T3 cell-derived and placental PLFs they were named PLF-1 and PLF-2, respectively (Fig.8.10). The PLF gene is present in several copies in the mouse genome, and cultured 3T3 and placental cells express both forms of PLF (Wilder and Linzer 1986), of which placental PLF-2 was shown to be extensively glycosylated.

```
                                                  10                                  20
Met-Leu-Pro-Ser-Leu-Ile-Gln-Pro-Cys-Ser-Trp-Ile-Leu-Leu-Leu-Leu-Leu-Val-Asn-Ser-
                                                  30                                  40
Ser-Leu-Leu-Trp-Lys-Asn-Val-Ala-Ser-Phe-Pro-Met-Cys-Ala-Met-Arg-Asn-Gly-Arg-Cys-
                                                  50                                  60
Phe-Met-Ser-Phe-Glu-Asp-Thr-Phe-Glu-Leu-Ala-Gly-Ser-Leu-Ser-His-Asn-Ile-Ser-Ile-
                                                  70                                  80
Glu-Val-Ser-Glu-Leu-Phe-Thr-Glu-Phe-Glu-Lys-His-Tyr-Ser-Asn-Val-Ser-Gly-Leu-Arg-
                                                  90                                 100
Asp-Lys-Ser-Pro-Met-Arg-Cys-Asn-Thr-Ser-Phe-Leu-Pro-Thr-Pro-Glu-Asn-Lys-Glu-Gln-
                                                 110                                 120
Ala-Arg-Leu-Thr-His-Tyr-Ser-Ala-Leu-Leu-Lys-Ser-Gly-Ala-Met-Ile-Leu-Asp-Ala-Trp-
                                                 130                                 140
Glu-Ser-Pro-Leu-Asp-Asp-Leu-Val-Ser-Glu-Leu-Ser-Thr-Ile-Lys-Asn-Val-Pro-Asp-Ile-
                                                 150                                 160
Ile-Ile-Ser-Lys-Ala-Thr-Asp-Ile-Lys-Lys-Lys-Ile-Asn-Ala-Val-Arg-Asn-Gly-Val-Asn-
                                                 170                                 180
Ala-Leu-Met-Ser-Thr-Met-Leu-Gln-Asn-Gly-Asp-Glu-Glu-Lys-Lys-Asn-Pro-Ala-Trp-Phe-
                                                 190                                 200
Leu-Gln-Ser-Asp-Asn-Glu-Asp-Ala-Arg-Ile-His-Ser-Leu-Tyr-Gly-Met-Ile-Ser-Cys-Leu-
                                                 210                                 220
Asp-Asn-Asp-Phe-Lys-Lys-Val-Asp-Ile-Tyr-Leu-Asn-Val-Leu-Lys-Cys-Tyr-Met-Leu-Lys-
                                                224
Ile-Asp-Asp-Cys
```

Fig. 8.10. cDNA-derived amino acid sequence of proliferin-1 (PLF-1). PLF-2 differs from PLF-1 in residues at positions 5 (Ser), 67 (Asn), 107 (Ala) and 117 (Ser), respectively

A significant number of mammalian cells respond to growth factors by a selective increase in the synthesis of secreted proteins/glycoproteins. In Swiss 3T3 cells, for instance, EGF and FGF induce the synthesis of a glycoprotein termed mitogen-regulated protein (MRP; Nilsen-Hamilton et al. 1980), that shows a sequence homology to prolactin and has the same sequence near its C terminus as PLF (Parfett et al. 1985). MRP was shown to be a glycosylated protein whose carbohydrate-free molecular weight is identical

to that of PLF. This and other analytical and immunological data suggest that MRP and PLF are identical molecules. This prompted Nilsen-Hamilton and coworkers (1987) to propose that the molecule be referred to as MRP/PLF until a function suggesting a more proper name is identified.

8.1.1.4.1.2. KNOWN PLACENTAL HORMONES AS GROWTH FACTORS

Placental tissue produces a large number of endogenous substances including known poly- and glycopeptide hormones aiding the adaptation of maternal and fetal organisms to the altered metabolic needs characteristic of the pregnant state. A few of these placental products exert growth factor-like activities in both maternal and fetal tissues, (see, e.g., Moscatelli et al. 1986, Sen Majumdar et al. 1986a,b). Such secondary growth factor-like activities were attributed to human (MacMillan et al. 1973, Hurley et al. 1977) and rat placental lactogens (Astwood and Greep 1938, Averill et al. 1950, Robertson and Friesen 1975) and to human chorionic gonadotropin (Mukherjee and Das 1984). FGF and IGF-II have been reported to occur in the human (Gospodarowicz et al. 1985b) and mouse placenta (Stempien et al. 1986), respectively).

8.1.1.4.2. OVARY-DERIVED GROWTH FACTORS

Ovaries are rich storehouses of biologically active peptides including known hormones, especially during pregnancy, some of which also exhibit growth-promoting activity. Of these peptides, relaxin discovered in extracts of pregnant sow ovaries deserves a special attention (for structural data and physiological function see Chapter 4). This peptide belongs to the family of insulin-like growth factors sharing many structural and functional properties with other members of this peptide family. As a growth-promoting substance, relaxin increases mammary growth (Wada and Turner 1959a,b) by stimulating cell proliferation mainly periparturiently (Anderson et al. 1982). It is also mitogenic for uterus-derived fibroblastic cell types (Sheffield and Anderson 1983, Vasilenko and

Mead 1987) and affects DNA and RNA synthesis in mammary fibroblasts (Sheffield and Anderson 1984). By all indications, relaxin efficiently controls the stromal growth in general, and mammary growth in particular, especially during gestation.

8.1.1.4.3. UTERINE-DERIVE GROWTH FACTOR

Ikeda and Sirbasku (1984) have partially purified a peptide of M_r between 4,200 and 6,200 from pregnant sheep uteri with a mitogenic activity on several estrogen-responsive tumor cell lines, but not on normal fibroblasts. Referring to its organ source, this protein was termed uterine-derived growth factor (UDGF) whose primary structure, however, remains undetermined for the time being.

8.1.1.4.4. PROSTATE-DERIVED GROWTH FACTORS

Although both EGF and NGF have been demonstrated in rodent prostates, their roles in this organ remain unclear. More recently heparin affinity chromatography has been successfully applied to partial purification from human prostatic tissue of a protein having a M_r between 11,000 and 13,000 and a potent serum-dependent DNA synthesis-stimulating effect on murine 3T3 cell line (Nishi et al. 1985). This protein, termed human prostatic growth factor (hPGF) was found to be similar to bovine FGF in some of its properties but dissimilar in others. Its structure and physiological role remain to be established (see also Mbikay et al. 1987).

8.1.1.4.5. TESTIS-DERIVED GROWTH FACTORS

Homogenates and partially purified fractions prepared from the seminiferous tubes of adult mice were shown to stimulate the DNA synthesis in cultured Balb/c 3T3 cells, a fibroblastic cell line (Feig et al. 1980). This effect was attributed to an

approximately M_r 15,500 protein with a preferential localization to the Sertoli cells. The author of this chapter is unaware of any data that would indicate a more detailed characterization of this putative growth factor.

8.1.1.5. BONE- AND CARTILAGE-DERIVED GROWTH FACTORS

8.1.1.5.1. SKELETAL GROWTH FACTOR

Under normal conditions, vertebrate bone tissue is in a state of a dynamic equilibrium maintained between bone resorption and replacement processes. It has long been suspected that these two processes might be coupled, probably by chemicals, called coupling agents that are liberated during resorptive events and stimulate subsequent bone replacement. A number of coupling agent-like peptides has been described so far in animals such as chicken skeletal coupling factor (M_r 75,000) and similar proteins from cows and rats. Of the two related proteins isolated from embryonic rat bone cells one is a PDGF-like substance (M_r 25,000) and the other a somatostatin-like factor (M_r 10,000) both with a DNA synthesis stimulating potential on bone cells. They may be specialized fetal proteins related to the adult skeletal growth factor (SGF). Although similar proteins detected in rabbit, cow and human bones displayed a moderate mitogenic effect, thereby deserving the name bone mitogenic protein (BMP), their main function apparently is associated with promoting the differentiation of unspecialized bone cells. However, it was not until recently that a similar factor could unequivocally be demonstrated in humans. In the early 1980s, Farley and Baylink (1982) succeeded in isolating human SGF (hSGF) from the matrix of demineralized human femoral bone heads obtained at hip replacement surgery, as a homogenous protein of M_r 83,000 consisting of one major (M_r 65,000) and two smaller subunits with presently untested biological activities and undetermined primary structures. The M_r 83,000 hSGF cell-specifically stimulates both DNA synthesis and proliferation of chick and human embryonic

bone cells in vitro, and enhances the growth rate of chick tibiae and femurs in vivo (Farley et al. 1982). Apart from functioning as a putative physiological coupling agent, hSGF may also be involved in a number of bone diseases such as osteoporosis, periodontal disease, Paget's disease, etc. (for further information see Jaworski 1984, Linkhart et al. 1986, Wergedal et al. 1986 and references cited therein).

Besides the M_r 83,000 hSGF, a smaller form (M_r 11,000) has been isolated from human bones under dissociative conditions (Mohan et al. 1986a) that may be a product from the larger form. The M_r 83,000 hSGF may represent a complex between M_r 11,000 hSGF and a binding/carrier protein. Apart from human sources, both forms of hSGF could also be extracted from bovine bone matrix and from human bone cell-conditioned media. The smaller form is biologically more active than the larger one (Mohan et al. 1985b).

In the light of recent findings, bone tissue exhibits one of the most complex spectrum of growth factor activities of any tissue yet described. Besides hSGFs, bone-derived growth factors (BDGFs) include a number of presently insufficiently characterized mitogenic activities and growth factors that are closely related or are identical to fully characterized mitogenic factors such as PDGF, acidic FGF and cartilage-derived growth factor (Hauschka et al. 1986). More recently, an extracellular matrix-associated bone-inductive protein, called o s t e o g e n i n, has been isloted and partially characterized by Shampath and associates (1987).

8.1.1.5.2. CARTILAGE-INDUCING FACTOR

Cartilage-inducing factor (CIF), a M_r 26,000 dimeric peptide has been isolated in two related but distinct forms, CIF-A and CIF-B, from demineralized bovine long bones (Seyedin et al. 1985). Although this peptide is suggested to be involved in chondrogenesis, it plays a role in this process as a differentiation factor rather than a true growth factor by inducing rat embryonic mesenchymal cells to assume a cartilage

morphology. Recently collected data indicates a strcutural and functional relatedness between CIF-B and β -type transforming growth factor (Seyedin et al. 1987). A cartilage-derived growth factor with a capability of inducing cell proliferation, wound repair and collagen accumulation has been reported by Davidson et al. (1985; see also Golbus et al. 1980, Klagsbrun and Smith 1980, as well as sections 8.1.1.3.1.1. and 8.1.1.3.1.2. in this Chapter).

8.1.1.6. MESENCHYMAL FACTOR

Mesenchymal factor (MF) was first extracted from embryos and cultured fibroblats by Levine et al. (1973) and was suggested to be a M_r 60,000 glycoprotein (Ronzio and Rutter 1973). Its relation to "serum factor", also a M_r 60,000 glycoprotein with a growth-promoting potential on fibroblasts in vitro (Houck and Cheng 1973, Houck et al. 1973) is presently unclear. From experiences collected on the proliferation of epithelial cells it was concluded that nontissue-specific MFs may be widespread in mammals that promote mitosis in epithelial cells adjacent to connective tissues (or capillaries) through adenylate cyclase-dependent membrane-bound events (for further details see Ronzio and Rutter 1973).

8.1.1.7. KIDNEY-DERIVED GROWTH FACTOR

Burgess (1984) reporting on the presentations delivered at the M.D. Anderson Hospital's 37th Symposium on Fundamental Cancer Research, Houston, 1984, mentions the isolation by Sirbasku of a polypeptide named kidney-derived growth factor (KDGF) having the capability of specifically stimulating the proliferation of mesenchymal cells in the kidney. The author of this chapter is unaware of any further characterization of this peptide.

8.1.1.8. HEPATOTROPHIC FACTORS: HEPATOTROPHINS

Over the past twenty years, careful studies have clearly indicated the involvement of humoral factors in the control of liver regeneration. However, no such humoral factors have been fully characterized so far, nor has it been clearly established, whether the induction of the regenerative response in the liver can be attributed to the release of a stimulator(s) or to the decrease of a circulating inhibitor(s). However, several successful experiments have been performed that led to a partial characterization of a number of hepatotrophic factors also termed hepatotrophins. Two of these, fetal factor 1 and 2 (FF1 and FF2) were found to be peptides present in fetal and in lesser amounts in newborn calf sera, but absent from adult sera, that initiated DNA synthesis (FF1) and stimulated ODC activity (FF2) in mouse liver cells (Morley 1974). Rat factor 1 (RF1), is another peptide of M_r 17,000 exclusively found in sera of partially hepatectomized rats. It has a potent DNA synthesis stimulatory potential on liver parenchymal cells and bears some similarities to FF1. The regenerative stimulator substance (SS) described by LaBrecque et al. (1975) and LaBrecque (1979) stimulates not only the DNA synthesis in already regenerating liver but also the proliferation in resting liver in vivo. SS could be isolated from weanling and regenerating rat livers, but not from quiescent adult liver. Hepatic SS (HSS), a peptide similar or identical to SS (La Brecque et al. 1984) stimulates HTC hepatoma cell growth in vitro in the absence of serum. On the other hand, Liebermann (1984) has reported on liver membrane components with both stimulatory and inhibitory activities, with the stimulatory factor being an extrinsic membrane protein. Kadofuku and coworkers (1984) have reported on three serum proteins (with a M_r 800,000 - 900,000 each) that were exclusively present or greatly increased in posthepatectomy rat sera. Additional serum-born (Michalopoulos et al. 1984, Thaler and Michalopoulos 1985, Gohda et al. 1986, Diaz-Gill et al. 1987, Francavilla et al. 1987), platelet-derived (Russel et al. 1984a,b, Nakamura et al. 1986b,c, 1987) and liver-born factors (Suemori

et al. 1988) have been described in recent years with a capability of stimulating hepatocyte proliferation whose serum concentrations change characteristically following partial hepatectomy. Of the serum-born factors, hepatopoietin A (HPTA; M_r 150,000-250,000) and hepatopoietin B (HPTB; M_r 5,000) have been best characterized and shwon to be mitogenic on hepatocytes (Michalopoulos et al. 1984). Various cathepsins as well as calciferin were also shown to be mitogenic in intact livers in vivo (Terayama et al. 1985) as was a high molecular weight serum-born hepatocyte growth factor in humans (Selden et al. 1986). Although the authors assume a liver growth regulatory role for all these peptides, presently available data do not allow us to draw such a conclusion with satisfactory certainty (for reviews see Starzl et al. 1978, Leffert et al. 1979, Michalopoulos et al. 1984).

8.1.1.9. UBIQUITOUS TISSUE GROWTH FACTORS

8.1.1.9.1. CALMODULIN

Similarly to cyclic nucleotides, calcium is a major "second messenger" for the regulation of numerous physiological processes and it has also been implicated as an important regulatory component for cell proliferation (Boynton et al. 1977, Hazelton et al. 1979, Whitfield et al. 1979, Cheung 1984). Ca^{2+} exerts its "second messenger" function via interaction with calcium-binding proteins, a family of small structurally similar acidic proteins of cytosolic localization which bind Ca^{2+} through a helix-loop-helix structure (Kretsinger 1980). They include calmodulin (CaM), troponin C, parvalbumin, intestinal Ca-binding protein (IB), oncomodulin that has initially been detected exclusively in tumor tissues (MacManus et al. 1982) and only recently as a normal constituent of human and rodent placentas (MacManus et al. 1985), the somewhat larger two subunit-containing calcineurin (Klee et al. 1979) and the multimolecular forms of S-100 protein (Isobe et al. 1981, 1983). The most multifaceted member of this fami-

ly is CaM which occurs in different molecular forms and in relatively high concentrations in a rather wide spectrum of the phylogenetic scale, including plants, animals and protozoa. Although CaM is considered ubiquitous in mammals, its relative concentration varies considerably from one cell type to the other. CaM has been purified and sequenced from a large variety of tissues of many eukaryotes extending from mammals to unicellular organisms (for details and references see Klee and Vanaman 1982, Dedman and Kaetzel 1983, Vanaman 1983, Nairn et al. 1984, Lukas et al. 1985). CaM contains four internally homologous domains containing a high affinity Ca^{2+}-binding site each, and requiring at least 3 bound Ca^{2+} for activity (Cheung 1984). This protein is phylogenetically highly conserved both structurally and functionally commensurate with its essential role as a mediator of the "second messenger" function of Ca^{2+}. CaM has been demonstrated to be involved in regulating activity or the rate of several key enzymes including a variety of protein kinase/phosphatase systems (Cheung 1984), and it also regulates nucleotide metabolism and interacts with several cytoskeleton components. Much of the structural properties of CaM (Babu et al. 1985, O'Neil and DeGrado 1985), the structural organization of the chicken CaM gene (Simmen et al. 1985) and the nucleotide sequence of a human CaM cDNA (Wawrzynczak and Perham 1984) have recently been reported.

The consensus sequence of vertebrate CaM is shown in Fig.8.11 (Vanaman 1983). Vertebrate CaM undergoes a fairly extensive posttranslational modification (Murtaugh et al. 1983). This results, for instance, in trimethylation of the lysine residue at position 115 which distinguishes CaM from other calcium-binding proteins. CaM is acetylated at its N-terminus. It is unique among calcium-binding proteins inasmuch, besides binding Ca^{2+}, it also binds peptides, hydrophobic drugs and target enzymes (see e.g., O'Neil and DeGrado 1985).

It is now well established that CaM mediates most if not all of the Ca^{2+}-regulated events in eukaryotic cells (reviewed by Means et al. 1982) and a significant body of circumstantial evidence suggests that CaM is an important mediator of cell growth and malignant transformation (reviewed by Veigl et al. 1984; see also Rasmussen and Means 1987).

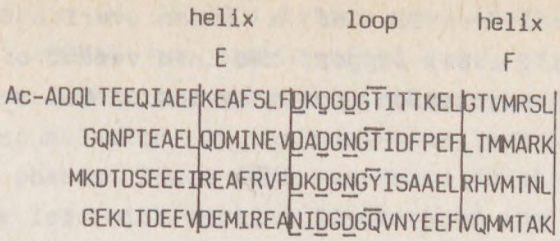


Fig. 8.11. Consensus amino acid sequence of vertebrate calmodulin. Regions thought to form the four Ca-binding sites (helix-loop-helix) are indicated by vertical bars. The lysine residue at position 115 is fully trimethylated. Underlined residues are ligands for the Ca^{2+} ions, whereas overlined residues contribute backbone carbonyls for complexation with calcium. The one letter amino acid symbols are used

Although, a transient increase in CaM content has been observed in several systems, cells can obviously proliferate under conditions where intracellular CaM content does not exhibit unique and specific synthesis-associated changes relative to the bulk of soluble cellular proteins. However, several studies utilizing CaM antagonists have provided circumstantial evidence indicating that CaM should have a specific function in initiating DNA synthesis and cell division, inasmuch treatment of various cells with CaM antagonists markedly inhibited cell proliferation. There also is evidence indicating that CaM itself may act as a mitogen for rat liver cells. Involvement of CaM in normal cell cycle regulation is further supported by a recent observation suggesting that CaM and its mRNA change specifically and characteristically during reentry of quiescent (G_0) cells into the cell cycle (Chafouleas et al. 1984).

It has been indicated that extracellular Ca^{2+} is necessary for DNA synthesis and cell proliferation in normal cells but not in cancer cells (Whitfield et al. 1979). However, cancer cells have increased amounts of Ca-binding proteins through which initiation of DNA synthesis can be permanently activated without the extracellular Ca^{2+} surge serving as a trigger (Whitfield et al. 1980). The positive correlation established between the growth rate of Morris hepatomas and their CaM levels (Wei et al. 1982), and the finding that CaM antagonists

inhibited proliferation of a human ovarian tumor cell line (Kikuchi et al. 1984) support the involvement of CaM in tumor cell growth regulation (for a review on CaM see Veigl et al. 1984).

8.1.1.9.2. CYCLIN-PROLIFERATING CELL NUCLEAR ANTIGEN

An ubiquitous polypeptide whose relative proportion increases in S phase HeLa cells and in various established cell lines undergoing viral transformation has been identified by Bravo et al. (1981). The peptide, named cyclin, was shown to be ubiquitous and localized in the cell nucleus. Its relative proportion decreased dramatically with a decreasing rate of cell proliferation. Up to now, two forms of cyclin have been identified in various species: an acidic and a basic form, respectively, that differ by one charge unit but exhibit very similar molecular weight, approx. 36,000 (Celis et al. 1984). Cyclin is induced in quiescent mouse 3T3 fibroblasts upon stimulation by serum or growth factors, and the induction correlates well with DNA synthesis of the cells (Bravo and Mac-Donald-Bravo 1984). As a general rule (a) noncycling normal cells synthesize very little cyclin, or do not synthesize it at all; (b) cycling cultured normal cells synthesize this peptide at low passage, and the synthesis ceases as cell division and DNA synthesis decline; (c) transformed cells synthesize this peptide "constitutively" in varying amounts. Transformed cells seem to lose their capacity to regulate the synthesis of cyclin, thereby becoming committed to cycle permanently, a state preceeding tumorigenicity.

Apart from cyclin, another polypeptide with cytoplasmic localization resembling tropomyosin was also identified, whose relative proportion increased significantly with a decreasing rate of cell proliferation. Based on presently available data, no regulatory role can be attributed to cyclin in cell proliferation with certainty, but the peptide may turn out to be a useful marker for cycling cells (for a recent review see Celis et al. 1984). Cleveland and coworkers (1977) have re-

ported of a protein (M_r 35,000) termed proliferating cell nuclear antigen (PCNA) in synchronized cultures whose levels and distribution fluctuated throughout the cell cycle, with a conspicuous accumulation in the nucleolus late in the G_1 and early in the S phases. Since PCNA was shown to share many important properties with cyclin, identity of the two polypeptides has been suggested (Mathews et al. 1984).

More recently, molecular cloning of a cDNA encoding rat PCNA/cyclin and a cDNA-derived amino acid sequence of the encoded protein have been reported by Matsumoto and associates (1987). The rat PCNA gene encodes a 261 amino acid protein with a calculated M_r of 28,748 whose predicted amino acid sequence is shown in Fig.8.12.

```
                                   10                                  20
Met-Phe-Glu-Ala-Arg-Leu-Ile-Gln-Gly-Ser-Ile-Leu-Lys-Lys-Val-Leu-Glu-Ala-Leu-Lys-
                                   30                                  40
Asp-Leu-Ile-Asn-Glu-Ala-Cys-Trp-Asp-Ile-Ser-Ser-Gly-Gly-Val-Asn-Leu-Gln-Ser-Met-
                                   50                                  60
Asp-Ser-Ser-His-Val-Ser-Leu-Val-Gln-Leu-Thr-Leu-Arg-Ser-Glu-Gly-Phe-Asp-Thr-Tyr-
                                   70                                  80
Arg-Cys-Asp-Arg-Asn-Leu-Ala-Met-Gly-Val-Asn-Leu-Thr-Ser-Met-Ser-Lys-Ile-Leu-Lys-
                                   90                                 100
Cys-Ala-Gly-Asn-Glu-Asp-Ile-Ile-Thr-Leu-Arg-Ala-Glu-Asp-Asn-Ala-Asp-Thr-Leu-Ala-
                                  110                                 120
Leu-Val-Phe-Glu-Ala-Pro-Asn-Gly-Glu-Lys-Val-Ser-Asp-Tyr-Glu-Met-Lys-Leu-Met-Asp-
                                  130                                 140
Leu-Asp-Val-Glu-Gln-Leu-Gly-Ile-Pro-Glu-Gln-Glu-Tyr-Ser-Cys-Val-Val-Lys-Met-Pro-
                                  150                                 160
Ser-Gly-Glu-Phe-Ala-Arg-Ile-Cys-Arg-Asp-Leu-Ser-His-Ile-Gly-Asp-Ala-Val-Val-Ile-
                                  170                                 180
Ser-Cys-Ala-Lys-Asp-Gly-Val-Lys-Phe-Ser-Ala-Ser-Gly-Glu-Leu-Gly-Asn-Gly-Asn-Ile-
                                  190                                 200
Lys-Leu-Ser-Gln-Thr-Ser-Asn-Val-Asp-Lys-Glu-Glu-Glu-Ala-Val-Ser-Ile-Glu-Met-Asn-
                                  210                                 220
Glu-Pro-Val-Gln-Leu-Thr-Phe-Ala-Leu-Arg-Tyr-Leu-Asn-Phe-Phe-Thr-Lys-Ala-Thr-Pro-
                                  230                                 240
Leu-Ser-Pro-Thr-Val-Thr-Leu-Ser-Met-Ser-Ala-Asp-Val-Pro-Leu-Val-Val-Glu-Tyr-Lys-
                                  250                                 260 261
Ile-Ala-Asp-Met-Gly-His-Leu-Lys-Tyr-Tyr-Leu-Ala-Pro-Lys-Ile-Glu-Asp-Glu-Glu-Gly-Ser
```

Fig. 8.12. cDNA-derived amino acid sequence of rat proliferating cell nuclear antigen (PCNA)/cyclin

Certain portions of PCNA, a DNA-binding protein itself, shows a generally weak homology with parts of other DNA-binding proteins such as the E1A antigen of type 5 adenovirus, the human c-myc oncogene product and the so called infected cell polypeptide 8 (ICP8) of type 1 herpes simplex virus.

The level of PCNA falls in resting cells and, conversely, it becomes particularly prominent in transformed and tumor cells. Its precise function remains presently unclear. The maximal synthesis of PCNA coincides with its accumulation in the nucleus preceding the peak of DNA synthesis indicating a role for this protein in some specific aspect of DNA replication (for details see Cleveland et al. 1977, Mathews et al. 1984 and Celis et al. 1986). This role has been more clearly specified by recent findings suggesting a functional (Prelich et al. 1987) and structural identity (Bravo et al. 1987) between PCNA and the auxiliary protein of DNA polymerase-δ see also Celis et al. 1987a).

Celis and Nielsen (1986) have also described a M_r 54,000 nuclear phosphoprotein whose rate of synthesis was sensitive to changes in the rate of cell proliferation. This protein, called d i v i d i n, exclusively is present in cells committed to divide. Presently available data suggest a role for this protein in events leading to DNA replication and cell division. More recently, Celis and associates (1987b) have reported of a proliferation-sensitive and cell cycle-dependent protein, called p r o g r e s s i n, whose rate of synthesis increased at the G_1/S boundary of the cell cycle (for further readings see Bravo and Bravo 1985, Celis and Celis 1985, Madsen and Celis 1985, Bravo 1986, Celis et al. 1986, Kurki et al. 1986, Sadaie and Mathews 1986, Celis et al. 1987a, Murray 1987).

8.1.1.9.3. $_p$53 CELLULAR TUMOR ANTIGEN

The term p53 cellular tumor antigen, also called p53 protein or simply p53, refers to a family of cellular proteins predominantly of nuclear localization that vary in size from 48,00 to 55,000 daltons in different species. (p53 refers to the median M_r of these proteins). cDNAs encoding p53 have been recently cloned both from human (Matlashewski et al. 1984, Harlow et al. 1985) and murine sources (Zakut-Houri et al. 1983, Benchimol et al. 1984, Jenkins et al. 1984 and Pennica et al. 1984a). The human gene was assigned to chromosome 7

(Isobe et al. 1986, Miller et al. 1986) and was characterized (Lamb and Crawford 1986). Predicted amino acid sequences of the human and mouse proteins are shown in Fig.8.13. The human

```
                                                10                                                  20
h: Met-Glu-Glu-Pro-Gln-Ser-Asp-Pro-Ser-Val-Glu-Pro-Pro-Leu-Ser-Gln-Phe-Thr-Phe-Ser-
m:  -   -   -   Ser -   -   -   Ile - Leu - Leu -   -   -   -   Glu -   -   -
                                                30                                                  40
h: Asp-Leu-Trp-Lys-Leu-Leu-Pro-Glu-Asn-Asn-Val-Leu-Ser-Pro-Leu-Pro-Ser-Gln-Ala-Met-
m: Gly -   -   -   -   -   -   -   Pro-Glu-Asp-Ile -       -   Ser -       His-Cys -
                                                50                                                  60
h: Asp-Asp-Leu-Met-Leu-Ser-Pro-Asp-Asp-Ile-Glu-Gln-Trp-Phe-Thr-Glu-Asp-Pro-Gly-Pro-
m:  -   -   -   -   -   -       -   Gln - Val - Glu-Phe -       -       -   -
                                                70                                                  80
h: Asp-Glu-Ala-Pro-Arg-Met-Pro-Glu-Ala-Ala-Pro-Pro-Val-Ala-Pro-Ala-Pro-Ala-Ala-Pro-
m: Ser -   -   Leu -   Val-Ser-Gly -   Pro-Ala-Ala-Gln-Asp -   Val-Thr-Glu-Thr -
                                                90                                                  100
h: Thr-Pro-Ala-Ala-Pro-Ala-Pro-Ala-Pro-Ser-Trp-Pro-Leu-Ser-Ser-Ser-Val-Pro-Ser-Gln-
m: Trp -   Val -   -   -   -   -   Thr-Pro -   -   -   -   -   Phe -   -   -   -
                                                110                                                 120
h: Lys-Thr-Tyr-Gln-Gly-Ser-Tyr-Gly-Phe-Arg-Leu-Gly-Phe-Leu-His-Ser-Gly-Thr-Ala-Lys-
m:  -   -   -   -   -   -   Asn -   -   -   His -   -   -   -   Gln-      -   -   -   -
                                                130                                                 140
h: Ser-Val-Thr-Cys-Thr-Tyr-Ser-Pro-Ala-Leu-Asn-Lys-Met-Phe-Cys-Gln-Leu-Ala-Lys-Thr-
m:  -   -   Met -   -   -   -   -   Pro -   -   -   Leu -   -   -   -   -   -   -   -
                                                150                                                 160
h: Cys-Pro-Val-Gln-Leu-Trp-Val-Asp-Ser-Thr-Pro-Pro-Pro-Gly-Thr-Arg-Val-Arg-Ala-Met-
m:  -   -   -   -   -   -   -   -   Ser-Ala -   -   -   Ala -   Ser -   -   -   -   -
                                                170                                                 180
h: Ala-Ile-Tyr-Lys-Gln-Ser-Gln-His-Met-Thr-Glu-Val-Val-Arg-Arg-Cys-Pro-His-His-Glu-
m:  -   -   -   -   Lys -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
                                                190                                                 200
h: Arg-Cys-Ser-Asp-Ser-Asp-Gly-Leu-Ala-Pro-Pro-Gln-His-Leu-Ile-Arg-Val-Glu-Gly-Asn-
m:  -   -   -   -   Gly -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
                                                210                                                 220
h: Leu-Arg-Val-Glu-Tyr-Leu-Asp-Asp-Arg-Asn-Thr-Phe-Arg-His-Ser-Val-Val-Val-Pro-Tyr-
m:  -   Tyr-Pro -   -   -   Glu -   -   Gln -   -   -   -   -   -   -   -   -   -   -
                                                230                                                 240
h: Glu-Pro-Pro-Glu-Val-Gly-Ser-Asp-Cys-Thr-Thr-Ile-His-Tyr-Asn-Tyr-Met-Cys-Asn-Ser-
m:  -   -   -   -   -   Ala -   -   Glu-Tyr -   -   -   -   -   -   Lys -   -   -   -   -   -
                                                250                                                 260
h: Ser-Cys-Met-Gly-Gly-Met-Asn-Arg-Arg-Pro-Ile-Leu-Thr-Ile-Ile-Thr-Leu-Glu-Asp-Ser-
m:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
                                                270                                                 280
h: Ser-Gly-Asn-Leu-Leu-Gly-Arg-Asn-Ser-Phe-Glu-Val-His-Val-Cys-Ala-Cys-Pro-Gly-Arg-
m:  -   -   -   -   -   -   -   -   Asp -   -   -   -   Arg -   -   -   -   -   -   -   -
                                                290                                                 300
h: Asp-Arg-Arg-Thr-Glu-Glu-Glu-Asn-Leu-Arg-Lys-Lys-Gly-Glu-Pro-His-His-Glu-Leu-Pro-
m:  -   -   -   -   -   -   -   -   -   -   Phe -   -   -   Glu-Val-Leu-Cys-Pro -   -   -
                                                310                                                 320
h: Pro-Gly-Ser-Thr-Lys-Arg-Ala-Leu-Pro-Asn-Asn-Thr-Ser-Ser-Ser-Pro-Gln-Pro-Lys-Lys-
m:  -   -   -   Ala -   -   -   -   -   -   Thr-Cys -   -   Ala -   -   Pro-Gln -   -
                                                330                                                 340
h: Lys-Pro-Leu-Asp-Gly-Glu-Tyr-Phe-Thr-Leu-Gln-Ile-Arg-Gly-Arg-Glu-Arg-Phe-Glu-Met-
m:  -   -   -   -   -   -   -   -   -   -   -   Lys -   -   -   -   -   Lys -   -   -   -
                                                350                                                 360
h: Phe-Arg-Glu-Leu-Asn-Glu-Ala-Leu-Glu-Leu-Lys-Asp-Ala-Gln-Ala-Gly-Lys-Glu-Pro-Gly-
m:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   His -   Thr-Glu -   Ser -
                                                370                                                 380
h: Gly-Ser-Arg-Ala-His-Ser-Ser-His-Leu-Lys-Ser-Lys-Lys-Gly-Gln-Ser-Thr-Ser-Arg-His-
m: Asp -   -   -   -   -   -   -   Tyr -   -   Thr -   -   -   -   -   -   -   -   -
                                                390         393
h: Lys-Lys-Leu-Met-Phe-Lys-Thr-Glu-Gly-Pro-Asp-Ser-Asp
m:  -   -   Thr -   Val -   Lys-Val -   -   -   -   -
```

Fig. 8.13. cDNA-derived amino acid sequences of human (h) and murine (m) p53. Dashes indicate identical residues. Deletions (empty spaces) in the m sequence were introduced to achieve maximal homology

protein contains 393 residues whereas the hitherto published murine proteins contain 387 and 386 (or 390 and 389) residues, respectively (for reasons of the uncertainty see Harlow et al. 1985). The human and mouse polypeptides are about 80% homologous and only minor differences exist between sequences of the murine proteins derived from different sources. p53 has a distinctly domained structure characterized by a very acidic N-terminal portion, a neutral central part and a basic C-terminal domain.

The positively charged C-terminal region may be involved in DNA-binding, the only activity of p53 hitherto recognized with certainty (Oren 1985). The anomalous behavior of p53 on SDS-PAG resulting in false M_r values is shared with a number of oncogene products and may reflect common structural features related to their common functions.

Very little is known of the exact function(s) of p53. It is overproduced or is accumulated due to its substantial stability in various transformed and in embryonic cells (Schmieg and Simmons 1984). It may collaborate with the Ha-ras oncogene in the transformation of rodent fibroblasts suggesting a role for p53 in at least some types of malignant transformations (reviewed by Oren 1985). p53 is a cell cycle-dependent protein (see e.g., Mercer and Baserga 1985): p53 levels increase during cell proliferation and decrease markedly upon cessation of cell division (Dippold et al. 1981, Milner and Milner 1981); its production in normal cells appears to be cell cycle regulated (Milner and Milner 1981, Mercer et al. 1984). This protein may be required for the reentry of serum-starved mitotically resting cells (G_0 cells) into the G_1 or S phase of the cell cycle upon serum or mitogen stimulation. Thus, p53 may be regarded as a regulatory agent involved in the transition of quiescent cells into the state of proliferation (for details see Zakut-Houri et al. 1983, Mercer et al. 1984, Reich and Levine 1984, Harlow et al. 1985, Oren 1985, Wynford-Thomas et al. 1985, Lane 1987).

8.1.2. BLOOD-BORN GROWTH FACTORS

8.1.2.1. SERUM-DERIVED GROWTH FACTORS

A recognition of paramount importance was the strict serum dependence of cultured mammalian cells, now accounted for the known presence in serum of a number of growth factors required for optimal cell growth. Thus, blood serum has been recognized as a major source of growth factors whose isolation and characterization has remarkably advanced during the past few years.

8.1.2.1.1. SOMATOMEDINS: INSULIN-LIKE GROWTH FACTORS

By the 1950s, it became gradually clear that effects on cartilage metabolism formerly attributed to growth hormone (GH) were in fact mediated by a GH-dependent serum factor. Because this factor stimulated sulfate uptake of in vitro cultured cartilagenous tissue, it was initially termed the "sulfation factor" (Salmon and Daughaday 1957, Daughaday and Kipnis 1966). It was also recognized soon that only 10% of the insulin-like activity present in plasma could be neutralized by anti-insulin antibodies, and the remaining part was termed "non-suppressible insulin-like activity" (NSILA; Froesch et al. 1967). NSILA could be separated into two peptide-containing fractions NSILA-S and NSILA-P, where S stands for soluble and P for precipitable. The peptides contained within NSILA-S were found to be similar to those present in the "sulfation factor" (Van Wyk et al. 1969). This similarity was the decisive evidence leading to the recognition that NSILA-S and sulfation factor were in fact the same substance of which the common term somatomedin (SM) was proposed (Daughaday et al. 1972), referring to its capability of mediating anabolic effects of GH or somatotropin. Subsequent isolation of NSILA-I and NSILA-II from NSILA-S by Rinderknecht and Humbel (1976), and especially their recognized biological activities made it clear that the growth-promoting

effect of these two NSILAs was more pronounced than their insulin-like activity. Therefore, and because their structure was found to be similar to that of proinsulin, NSILA-I and NSILA-II were renamed insulin-like growth factor I (IGF-I) and insulin-like growth factor II (IGF-II), respectively. Also part of the terminology story is that initially SMs were named according to their net charges and distinction was made between neutral, acidic and basic SMs largely corresponding to the fractions termed SM A, SM B and SM C in the literature. The exact nature of SM A, which was subdivided into SM A1 and SM A2 components by Fryklund et al. (1974), is still in doubt, but it probably contains both IGF I and IGF II derivatives. There are more explicit opinions maintaining that both human SM A and rat multiplication-stimulating activity (MSA) correspond to IGF II (see Goustin et al. 1986). On the other hand SM B was specified as an acidic peptide fraction of the human plasma with mitogenic activity on glial cells. Subsequent investigations, however, revealed that EGF present as a minor component in AM B preparations was in fact responsible for the mitogenic activity, and this is why SM B is no longer considered to be an SM peptide fraction. Finally the determination of the primary structure of both IGF I, (Rinderknecht and Humbel 1978a) and SM C (Klapper et al. 1983) showed identity of these peptides.

Accordingly NSILA, SM and IGF are synonyms for one and the same group of substances. The term NSILA is not in use any more, and the name SM is retained as a generic term to designate closely related plasma peptides, and is used particularly when mediation of GH action is implied. On the other hand, the term IGF is employed when referring to a specific, structurally identified SM peptide.

Bioassays, as well as radioimmuno, radioreceptor and protein binding assays are now equally available for measuring SMs (for a review see Daughaday 1984).

(1) Insulin-like growth factors. Until recently, two forms of IGFs, IGF I and II, had been isolated from human serum. They are closely related peptides sharing many properties not

only with each other, but also with insulin. IGF I was recognized as a 70 amino acid peptide with a M_r of 7,649, whereas IGF II has 67 amino acid residues and a M_r of 7,471.

Human IGF I and IGF II have been sequenced by Rinderknecht and Humbel (1978a,b). The chemical synthesis of IGF II (Li et al. 1985, Yamashiro and Li 1985), the molecular cloning and expression of a biologically active IGF I analog have also been accomplished (Peters et al. 1985). The primary amino acid sequence of both human IGFs and that of human proinsulin are demonstrated in Fig.8.14.

```
B domain

HPI:          Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly
1-20
IGFI:            Gly-Pro-Glu-Thr - - - Ala-Glu - - Asp - - Gln-Phe - - -
1-19
IGFII: Ala-Tyr-Arg-Pro-Ser - - - - - Gly - - - - Thr - - - - - - -
1-22

HPI:    Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-
21-30
IGFI:   Asp - - - Tyr-Phe-Asn-Lys-Pro -
20-29
IGFII:   - - - - - - Ser-Arg - Ala
23-32

C domain

HPI:    Arg-Arg-Glu-Ala-Glu-Asp-Leu-Gln-Val-Gly-Gln-Val-Glu-Leu-Gly-Gly-Gly-Pro-Gly-Ala-
31-50
IGFI:   Gly-Tyr-Gly-Ser-Ser-Ser-Arg-Arg-Ala-Pro-Gln-Thr
30-41
IGFII:  Ser-Arg-Val - Arg-Arg-Ser -
33-40
HPI:    Gly-Ser-Leu-Gln-Pro-Leu-Ala-Leu-Glu-Gly-Ser-Leu-Gln-Lys-Arg
51-65

A domain

HPI:    Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn
66-86
IGFI:    - - - Asp-Glu - - Phe-Arg-Ser - Asp - Arg-Arg - - Met - - Ala-
42-62
IGFII:   - - - Glu - - - - - - - - - - Ala-Leu - - Thr - - -
41-61

D domain

IGFI:   Pro-Leu-Lys-Pro-Ala-Lys-Ser-Ala
63-70
IGFII:  Thr       - - - - Glu
62-67
```

Fig. 8.14. Amino acid sequences and domain structures of human proinsulin (HPI), insulin-like growth factor I (IGF I) and IGF II, respectively. Amino acids are aligned in such a way as to give maximal homology. Dashes indicate residues identical to those positioned above them

Both IGFs have a remarkable structural homology to proinsulin: 43 and 41% of the amino acids in IGFs and those in the A and B chains of insulin are in identical positions. IGFs and proinsulin have similar domain structures as well, as demonstrated in Fig.8.14. Recent analysis of the genes encoding human and mouse liver IGF Is revealed two distinct precursors, preproIGF IA and IB, that differ both in the size and the amino acid sequence of the C-terminal extension (domain-D), but not in those of the encoded mature proteins (Bell et al. 1984, 1986b, Rotwein 1986). The sequences of human and mouse preproIGF IAs are highly conserved (94% identity), whereas those of preproIGF IBs from the two species are significantly different. There are 3 intrachain disulfide bridges in both human IGFs. The two IGFs show extensive homology also to each other: nearly 70% of their residues are identical. The isolation and nucleotide sequence of the genes for human (Ullrich et al. 1984) and mouse IGF Is (Bell et al. 1986b) and rat IGF II (Rechler et al. 1985) have been reported. The mature proteins from these species are highly homologous: more than 90% of their amino acids are in identical position. Human IGF I and II genes were mapped to chromosome 12 and 11, respectively (Brissenden et al. 1984, Tricoli et al. 1984, Hoppener et al. 1985).

Human IGFs apparently have their counterparts in other mammals (Scanes et al. 1986). The basic SM described and partially sequenced by Rubin and associates (1982) seems to be the main SM in adult rat serum and, according to partial sequence data, it seems to be the rat equivalent of human IGF I. On the other hand, rat multiplication stimulating activity (MSA) is held to be the rat equivalent of human IGF II.

SM peptides are present in high molecular weight complex forms in the plasma, of which at least two types can be distinguished. Approximately 80% of SM in normal plasma is carried as a component of a M_r 150,000 complex, called the 150 K complex, whereas the remainder is in the form of a somewhat smaller complex, whose M_r was variously estimated to range between 30,000 and 50,000, hence the name 45K complex. The physiological significance of the binding of SMs to carrier

proteins in plasma is far from clear. It is clear, however, that SM peptides in complex form behave a great deal differently from those in free form, a point which should be kept in mind when physiological function of SM peptides are assessed. (for a review see Hintz 1984).

Similarly to other growth factors, SMs/IGFs interact with specific membrane proteins to accomplish their cellular functions. According to the present state of our knowledge, two distinct forms of SM/IGF receptors exist. Type 1 receptors show homology to the insulin receptor, which is a M_r 450,000 heterotetrameric complex consisting of two M_r 98,000 transmembrane β subunits each of which is disulfide-bonded to one α subunit (M_r 130,000). The α subunit represents the insulin (or IGF)-binding domain whereas the β subunit displays ATPase and tyrosine kinase activities. Recent studies on a cloned cDNA encoding the insulin receptor revealed a surprising homology of the α region to the extracellular domain of human EGF receptor. On the other hand the β domain of the insulin receptor shows a moderate homology to members of the src oncogene family, and a more extensive homology to the ros oncogene. These homologies are consistent with the suggestion that some of these oncogenes may encode growth factor receptors (for references see Goustin et al. 1986). Type 1 receptor displays a substantially higher affinity toward IGF I than IGF II, and only a weak interaction with insulin. On the other hand, type 2 receptor prefers IGF II to IGF I and does not interact with insulin at all. In general, it is a more simple molecule than its IGF I counterpart, probably consiting of a single polypeptide chain. In the light of recent findings, the old idea that IGF receptors mediate growth-stimulating action both for insulin and IGFs while insulin receptors are mediating acute metabolic effects for both types of molecules, has proved to be untenable. The demonstration of SM/IGF receptors on human fibroblasts and circulating mononuclear cells will probably help to establish biological systems that are suited for investigating patients with putative end-organ resistance to SM/IGF (for further details on SM/IGF receptors see Nissley and Rechler 1984).

(2) Somatomedin-A. In the early 1970s SM-A was regarded as a separate entity existing in two forms, SM-A1 (60 residues) and SM-A2 (63 residues; Fryklund et al. 1974). However, this view has changed considerably for today: serum SM-A fraction now is held to be either a mixture of IGF-I and IGF-II or the human equivalent of rat MSA corresponding to IGF II (for further information see Hall and Sara 1984).

(3) Multiplication stimulating activity. In the 1970s, the term MSA was used to designate more or less characterized peptide fractions prepared from sera or conditioned media that stimulated fibroblast proliferation. The peptides contained within these fractions were shown to have similar molecular weights and to be chemically related. Moreover purified MSA peptides showed a remarkable similarity to IGF-II. Of the several MSA peptides reported previously, those termed MSA II-1 and MSA III-2 by Moses et al. (1980a,b) have been most extensively characterized (for past results see Pierson and Temin 1972, Dulak and Temin 1973a,b, Nissley et al. 1976, Nissley and Rechler 1978).

It was Marquardt and his associates (1981) who have first achieved full purification and determined the amino acid sequence of MSA derived from media conditioned with rat liver cells. This particular MSA, a 67 amino acid peptide of Mr 7,484 whose primary structure is demonstrated in Fig.8.15 showed 93% homology to human IGF-II, a reason why the name rat IGF-II was proposed for this peptide. Because the latter represents the main form of IGFs in fetal rat serum (Moses et al. 1980c) the name fetal MSA is used alternatively to desig-

```
                                        10                  20
Ala-Tyr-Arg-Pro-Ser-Glu-Thr-Leu-Cys-Gly-Gly-Glu-Leu-Val-Asp-Thr-Leu-Glu-Phe-Val-
                                        30                  40
Cys-Ser-Asp-Arg-Gly-Thr-Tyr-Phe-Ser-Arg-Pro(Ser)Gly-Arg-Ala-Asn-Arg-Arg(Ser)Arg-
                                        50                  60
Gly-Ile-Val-Glu-Glu-Cys-Cys-Phe-Arg-Ser-Cys-Asp-Leu-Ala-Leu-Leu-Glu-Thr-Tyr-Cys-
                                    67
Ala(Thr)Pro-Ala-Lys-Ser-Glu
```

Fig. 8.15. Primary structure of rat multiplication stimulating activity Tentative assignments are in parentheses

nate rat IGF-II. The structure and characteristics of rat IGF-II precursor protein has also been determined from a cloned cDNA (Whitfield et al. 1984).

Biological properties of SMs/IGFs

SMs/IGFs mediate important metabolic effects independent of growth regulation (reviewed by Clemmons and Van Wyk 1981a, Daughaday 1984). However, here only their growth-related effects will be mentioned. SMs/IGFs stimulate proliferation-related metabolic events in, and are mitogenic for a broad range of cells. SM-C/IGF-I is one of the important growth factors in the serum and plasma (Svoboda et al. 1980). Rothstein and associates (1980) provided the first evidence for the effectiveness of a growth factor in vivo by showing that the complete cessation of DNA synthesis as well as mitosis in bullfrog lens epithelia caused by previous hypophysectomy, an intervention resulting in lowered plasma levels of SM-C/IGF-I, could be reinitiated by exogenous administration of SM-C/IGF-I. IGF-I responsive cells include embryonic and adult fibroblasts, corneal epithelium, chondrocytes and bone cells from various species such as human, rat and chicken and the responses elicited on these cells include the stimulation of DNA synthesis, ODC activity, mitosis as well as differentiation (see e.g., Rechler et al. 1975, Haselbacher and Humbel 1976, Canalis 1980, Schmid et al. 1983 and the references cited therein). SM-C/IGF-I may also be causally involved in regulating compensatory growth of the kidney (Polychronakos et al. 1985). SM-C/IGF-I probably exhibits a progression factor-like activity requiring the cooperation of initiating factor-type growth factors for a full scale mitotic activity (Stiles et al. 1979) The findings that serum levels of IGF-I, but not IGF-II, are elevated in acromegaly (Zapf et al. 1978), with low serum concentrations in pituitary dwarfs (Daughaday and Parker 1963, Hall 1971 may help to evaluate the roles that SMs play within the organism. As a gross approximation, both SM-A/IGF-II and MSA seem to act on a similar spectrum of cells and elicit similar responses as SM-C/IGF-I does, with only minor differen-

ces (see e.g., Wasteson et al. 1975, Nissley et al. 1977). In addition, however, SM-A/MSA/IGF-II may be a major fetal growth factor (reviewed by Underwood and D'Ercole 1984). SM production is GH-dependent in some cells, but it is dependent on other growth factors, e.g., PDGF in others. These two mechanisms may represent two distinct model systems by which GH can stimulate generalized cell growth, whereas PDGF would be involved in local growth stimulation as it is required during wound healing (Clemmons and Van Wyk 1981a,b.)

Available data suggest the liver as the major site of SM synthesis, though several other organs also contain SMs in varying concentrations. However, no organs could be identified which contained SMs in concentrations greater than in serum (for nonserum sources of SMs/IGFs see Haselbacher et al. 1985, Bar et al. 1986, Carlsson-Skwirut et al. 1986, Le Bouc et al. 1986, Lund et al. 1986, Minuto et al. 1986, Sara et al. 1986, Casella et al. 1987, Hoppener et al. 1987).

8.1.2.1.2. MAMMARY-STIMULATING FACTOR

Mammary-stimulating factor (MSF) was first identified as a serum protein fraction with a M_r of 100,000 at neutral pH that preferentially stimulated the growth of cultured mouse mammary epithelial cells (Hsueh and Stockdale 1973, 1974, 1975). However, when purification was carried out at low pH, the highest MSF activity could be found in a fraction with a M_r ranging between 10,100 and 10,400 (Ptashne et al. 1979). A highly purified form of this low molecular weight fraction displayed several properties distinct from those of other identified growth factors, but it also had SM-like activity. Thus, the possibility that MSF belongs to the SM family of polypeptides cannot be excluded.

8.1.2.1.3. GLYCYL-HISTIDYL-LYSINE

Glycyl-histidyl-lysine (GHL) is present in human plasma in association with the albumin and α-globulin fractions. In low concentrations, GHL is mitogenic for a broad spectrum of cells, preferentially for liver-related cells (Pickart and Thaler 1973, Pickart et al. 1973), but it inhibits the proliferation of glial cells (Sensenbrenner et al. 1975). GHL reduces the serum requirement of several cell types. In higher concentrations GHL mediates inhibitory signals rather than stimulatory ones. This tripeptide can be isolated from plasma as an iron and copper complex and its involvement in Cu^{2+} transport processes was seriously considered. An HGL analog affecting neuronal electrical activity was isolated from the spinal cord (Lote et al. 1976; for further information see Pickart and Thaler 1979).

8.1.2.1.4. PROTEASES

Although proteases may not unconditionally be of serum origin, thrombin, the most thoroughly studied protease with a proliferogenic activity is most abundantly present in the serum, that is why proteses are discussed as blood-born growth factors in this Chapter.

It is now well established that certain proteases have a pronounced activating effect on a large number of important biological processes, including the proliferation of cultured animal cells. For instance, trypsin, pronase, ficin (Sefton and Rubin 1970), DNAse and hyaluronidase were demonstrated to be mitogenic for a variety of cultured cells (Vasiliev et al. 1970). Thrombin was found to be a potent mitogen for avian (Buchanan et al. 1976) but a less potent mitogen for human and mouse embryo fibroblasts (Pohjanpelto 1977, Cunningham et al. 1979).

Up to now, thrombin is the most extensively studied protease with respect to its mechanism of action. It was repeatedly demonstrated that proteolytic activity directed on mem-

brane components is essential for cell activation by thrombin (Carney et al. 1984). Specific binding sites (receptors) were demonstrated on the membrane surface of responsive cells (Cunningham et al. 1979), that must be cleaved by thrombin before stimulation of proliferation occurs (Carney et al. 1978, Glenn and Cunningham 1979). Although thrombin does not need to be internalized for inducing cell division, the putative peptide fragments generated by the proteolytic activity of thrombin might be internalized and might bring about secondary thrombin actions. Recently, a partial thrombin sequence with growth factor activity on macrophage has been identified (Bar-Shavit et al. 1986).

According to recent observations, human foreskin fibroblasts release into the medium a cellular component termed protease nexin (PN), which has the capability of forming heat stable complexes with exogenous ^{125}I-urokinase, and ^{125}I α-thrombin. The contribution of the formation of protease-PN complex to the protease-induced mitogenesis is unclear, and might have nothing to do with the latter process (for more information on PN see Baker et al. 1980).

Although presently available data do not allow us to attribute a physiological significance to protease-stimulated mitogenesis, thrombin may be among the candidates involved in tissue repair processes following tissue damage in general, and wound healing in particular (for a recent review see Scott 1987).

8.1.2.1.5. TRANSFERRIN

Transferrin (Tf) is the major iron-binding protein in vertebrate serum, although it occurs in distinct molecular forms also in species at a lower grade of phylogenetic development. Tf is a member of a family of Tf-like proteins including hen ovotransferrin, lactotransferrin or lactoferrin, melanoma antigen p97 and ChBlym-1, a transforming protein from chicken lymphoma, that all share strong amino acid homologies. The chemically determined amino acid sequence of human Tf (M_r

```
                                                                10
Val-Pro-Asp-Lys-Thr-Val-Arg-Trp-Cys-Ala-Val-Ser-Glu-His-Glu-Ala-Thr-Lys-Cys-Gln-
                                                                30
Ser-Phe-Arg-Asp-His-Met-Lys-Ser-Val-Ile-Pro-Ser-Asp-Gly-Pro-Ser-Val-Ala-Cys-Val-
                                                                50
Lys-Lys-Ala-Ser-Tyr-Leu-Asp-Cys-Ile-Arg-Ala-Ile-Ala-Ala-Asn-Glu-Ala-Asp-Ala-Val-
                                                                70
Thr-Leu-Asp-Ala-Gly-Leu-Val-Tyr-Asp-Ala-Tyr-Leu-Ala-Pro-Asn-Asn-Leu-Lys-Pro-Val-
                                                                90
Val-Ala-Glu-Phe-Tyr-Gly-Ser-Lys-Glu-Asp-Pro-Gln-Thr-Phe-Tyr-Tyr-Ala-Val-Ala-Val-
                                                               110
Val-Lys-Lys-Asp-Ser-Gly-Phe-Gln-Met-Asn-Gln-Leu-Arg-Gly-Lys-Lys-Ser-Cys-His-Thr-
                                                               130
Gly-Leu-Gly-Arg-Ser-Ala-Gly-Trp-Asn-Ile-Pro-Ile-Gly-Leu-Leu-Tyr-Cys-Asp-Leu-Pro-
                                                               150
Glu-Pro-Arg-Lys-Pro-Leu-Glu-Lys-Ala-Val-Ala-Asn-Phe-Phe-Ser-Gly-Ser-Cys-Ala-Pro-
                                                               170
Cys-Ala-Asp-Gly-Thr-Asp-Phe-Pro-Gln-Leu-Cys-Gln-Leu-Cys-Pro-Gly-Cys-Gly-Cys-Ser-
                                                               190
Thr-Leu-Asn-Gly-Tyr-Phe-Gly-Tyr-Ser-Gly-Ala-Phe-Lys-Cys-Leu-Lys-Asp-Gly-Ala-Gly-
                                                               210
Asp-Val-Ala-Phe-Val-Lys-His-Ser-Thr-Ile-Phe-Glu-Asn-Leu-Ala-Asn-Lys-Ala-Asp-Arg-
                                                               230
Asp-Gln-Tyr-Glu-Leu-Leu-Cys-Leu-Asp-Asn-Thr-Arg-Lys-Pro-Val-Asp-Glu-Tyr-Lys-Asp-
                  Glu                                          250
Cys-His-Leu-Ala-Gln-Val-Pro-Ser-His-Thr-Val-Val-Ala-Arg-Ser-Met-Gly-Gly-Lys-Glu-
                                                               270
Asp-Leu-Ile-Thr-Glu-Leu-Leu-Asn-Gln-Ala-Gln-Glu-His-Phe-Gly-Lys-Asp-Lys-Ser-Lys-
                                                               290
Glu-Phe-Gln-Leu-Phe-Ser-Ser-Pro-His-Gly-Lys-Asp-Leu-Leu-Phe-Lys-Asp-Ser-Ala-His-
                                                               Asn                320
Gly-Phe-Leu-Lys-Val-Pro-Pro-Arg-Met-Asp-Ala-Lys-Met-Tyr-Leu-Gly-Tyr-Glu-Tyr-Val-
                                                               330
Thr-Ala-Ile-Arg-Asn-Leu-Arg-Glu-Gly-Thr-Cys-Pro-Glu-Ala-Pro-Thr-Asp-Glu-Cys-Lys-
                                                               350
Pro-Val-Lys-Trp-Cys-Ala-Leu-Ser-His-His-Glu-Arg-Leu-Lys-Cys-Asp-Glu-Trp-Ser-Val-
Ser Asp                                                        370
Asn-Ser-Val-Gly-Lys-Ile-Glu-Cys-Val-Ser-Ala-Glu-Thr-Thr-Glu-Asp-Cys-Ile-Ala-Lys-
                                                               390
Ile-Met-Asn-Gly-Glu-Ala-Asp-Ala-Met-Ser-Leu-Asp-Gly-Gly-Phe-Val-Tyr-Ile-Ala-Gly-
                                                               410                Asp 420
Lys-Cys-Gly-Leu-Val-Pro-Val-Leu-Ala-Glu-Asn-Tyr-Asn-Lys-Ser-Asp-Asn-Cys-Glu-Asp-
                                                               430
Thr-Pro-Glu-Ala-Gly-Tyr-Phe-Ala-Val-Ala-Val-Val-Lys-Lys-Ser-Ala-Ser-Asp-Leu-Thr-
                                                               450
Trp-Asp-Asn-Leu-Lys-Gly-Lys-Lys-Ser-Cys-His-Thr-Ala-Val-Gly-Arg-Thr-Ala-Glu-Trp-
                                                               470
Asn-Ile-Pro-Met-Gly-Leu-Leu-Tyr-Asn-Lys-Ile-Asn-His-Cys-Arg-Phe-Asp-Glu-Phe-Phe-
                                                               490
Ser-Glu-Gly-Cys-Ala-Pro-Gly-Ser-Lys-Lys-Asp-Ser-Ser-Leu-Cys-Lys-Leu-Cys-Met-Gly-
                                                               510
Ala-Lys-Asn-Leu-Asn-Gly-Lys-Asp-Tyr-Glu-Lys-Glu-Gly-Tyr-Tyr-Gly-Tyr-Thr-Gly-Ala-
                                                               530                Thr 540
Phe-Arg-Cys-Leu-Val-Glu-Lys-Gly-Asp-Val-Ala-Phe-Val-Lys-His-Gln-Thr-Val-Pro-Gln-
   Pro                                                         550                    560
Asn-Thr-Gly-Gly-Lys-Asn-Pro-Asp-Pro-Trp-Ala-Lys-Asn-Leu-Asn-Glu-Lys-Asp-Tyr-Glu-
                                                               570          Gln       580
Leu-Leu-Cys-Leu-Asp-Gly-Thr-Arg-Lys-Pro-Val-Gln-Glu-Tyr-Ala-Asn-Cys-His-Leu-Ala-
                                                               590                    600
Arg-Ala-Pro-Asn-His-Ala-Val-Val-Thr-Arg-Lys-Asp-Lys-Glu-Ala-Cys-Val-His-Lys-Ile-
                                                               610                    620
Leu-Arg-Gln-Gln-Gln-His-Leu-Phe-Gly-Ser-Asn-Val-Thr-Asp-Cys-Ser-Gly-Asn-Phe-Cys-
                                                               630                    640
Leu-Phe-Arg-Ser-Glu-Thr-Lys-Asp-Leu-Leu-Phe-Arg-Asp-Asp-Thr-Val-Cys-Leu-Ala-Lys-
                                                               650          Gln       660
Leu-His-Asp-Arg-Asn-Thr-Tyr-Glu-Lys-Tyr-Leu-Gly-Glu-Glu-Tyr-Val-Lys-Ala-Val-Gly-
                                                               670                679
Asn-Leu-Arg-Lys-Cys-Ser-Thr-Ser-Ser-Leu-Leu-Glu-Ala-Cys-Thr-Phe-Arg-Arg-Pro
```

Fig. 8.16. Amino acid sequence of human transferrin. The residues indicated by two amino acid symbols represent differences between chemically determined residues (upper symbols) and those predicted from cDNA (lower symbols)

79,500) has been reported by MacGillivray and associates (1983) and is shown in Fig.8.16. Shortly thereafter cDNAs for human Tf have been cloned and sequenced (Uzan et al. 1984, Yang et al. 1984), and the structure of the human gene determined (Adrian et al. 1986, Schaeffer et al. 1987).

Human Tf is a glycoprotein whose carbohydrate moiety amounts to 6% of its total molecular weight. The Tf gene and its receptor has been mapped to chromosome 3 in the human genome. The specific receptors binding Tf-iron complexes occur ubiquitously (for further information on Tf receptor and its gene see Sutherland et al. 1981, Newman et al. 1982, McLelland and associates 1984, Schneiderm et al. 1984).It is interesting to note that Tf receptors are present both on the cell surface and in endocytic vesicles, and there is a continuous exchange between the two locations (Dautry-Varsat et al. 1983). The cell surface receptors show a cell cycle-dependent fluctuation in number (Chitamber et al. 1983, Musgrove et al. 1984, Pauza et al. 1984, Sager et al. 1984). In the mouse, Tf is a major milk protein which is synthesized by mammary epithelial cells (Lee et al. 1987). It has been known for years that Tf is an obligatory growth factor for cultured cells in general (Barnes and Sato 1980, Breitman et al. 1980). More recent data indicate that Tf is a major pituitary-derived mitogen for some mammary tumor cells (Riss and Sirbasku 1987) while lactoferin acts as a myeolopoietic factor (Poppas et al. 1986). It has also become apparent that cell surface receptors for Tf are expressed on proliferating cells in general, both in vitro and in vivo (Galbraith et al. 1980, Shindelman et al. 1981, Hirose-Kumagi et al. 1984; for further information on Tf see Amouric et al. 1984, Bomford and Munro 1985, Baker et al. 1987, Huebers and Finch 1987, Kamboh and Ferrel 1987, Lee et al. 1987).

8.1.2.1.6. FIBRONECTIN

Fibronectin (FN) is a high molecular weight glycoprotein present on the external surface of a variety of normal cells, and is conspicuously absent, or is greatly reduced after malignant transformation (Ali and Hunter 1981). FN is also secreted into the culture medium of cells in vitro, and a similar but not identical protein is present in blood plasma in vivo (reviewed by Grinnel 1981).

Although the major role of FN is in the cell-cell and cell-substratum adhesion as well as in cellular migration, enzyme-digested FN was demonstrated to stimulate DNA synthesis of serum-deprived quiescent fibroblasts (Humpries and Ayad 1983), but is inhibiting the growth of cultured endothelial cells (Homandberg et al. 1985). Fragmented FN was isolated from several sources, including human plasma, supporting the assumption that cleavage of FN can generate "activation peptides" with various regulatory functions. It is also worth remembering that mitogenic action of some proteases is preceded by a cleavage of cell-surface proteins including FN, which is fully compatible with the generation of mitogenic FN fragments upon protease stimulation. As to the general characteristics and primary structure of human cellular FN, see Bernard et al. (1985) Kornblihtt et al. (1985), Proctor (1987).

8.1.2.1.7. FETUIN AND EMBRYONIN

Fetuin first was partially purified from fetal calf serum (FCS) by Pedersen (1947) as a heterogenous fraction containing an about M_r 48,000 glycoprotein that, among others, displayed growth factor activity. However, subsequent purification attempts failed to support Pedersen's claim concerning fetuin (Deutsch 1954, Spiro 1960, Salomon et al. 1981). Using a purification scheme different from that previously used for purification of Pedersen's fetuin preparation, Salomon and coworkers (1982) identified a M_r 270,000 protein, termed embryonin that showed growth factor activity. In more recent inves-

tigations, identity of embryonin with bovine α_2-macroglobulin ($\alpha_2 M_b$) was discovered (Feldman et al. 1984). Recently, Libby and associates (1985) expressed caution in regard to the mitogenic effect of fetuin preparations showing that such preparations vividly bind PDGF and the latter may be responsible for the mitogenic effect attributed to fetuin preparations and to components therefrom (for recent information see Dziegielewska et al. 1987).

8.1.2.1.8. MISCELLANEOUS SERUM-BORN GROWTH FACTORS

α_1-acid glycoprotein (AG), also termed orosomucoid, is a normal serum constituent having the capability of stimulating the growth of a variety of cells in culture. Asialo-AG, (AG lacking the sialyl residue) exhibits similar activity indicating that Ca^{2+} is not responsible for the observed effect (Maeda et al. 1980).

Slotta and associates (1975) reported of the growth-promoting activities of salt-free human serum fractions. Proliferogenic activities were also found in the proteolytic digest of serum proteins and of the liver. Also the erythrocytes proved to be an exceptionally rich source of cell growth-promoting factors (Slotta 1980), however, none of these activities have been structurally identified.

Rossow et al. (1979) have described a serum-dependent "initiator" (R) protein, whose amount should rise to a critical value before the cells reach the S phase. The authors proposed that serum growth factors may act by inducing this protein.

8.1.2.2. PLATELET-BORN GROWTH FACTORS

The platelet proved to be an extraordinarily rich source of various biologically active substances including those with growth-promoting activity. Of these, platelet-derived growth factor is best characterized, but important data were presented from numerous other platelet-derived factors.

(1) Platelet-derived growth factor. Earlier attempts to isolate human platelet-derived growth factor (PDGF) from variously prepared platelet preparations have unexceptionally yielded protein products whose M_rs ranged between 28,000 and 35,000 under unreducing conditions that were replaced by proteins with M_r of about 13,000 and 14,000 upon reduction (Antoniades et al. 1979, Heldin et al. 1979, Ross et al. 1979). Results of subsequent structural studies can be summarized as follows (Deuel et al. 1981, Johnsson et al. 1982, Raines and Ross 1982).

Purified PDGF can be separated into two distinct glycoproteins designated PDGF-I. (M_r 31,000) and PDGF-II (M_r 28,000) that share many properties in common and have similar amino acid composition, but their exact relationship presently is unclear. The main difference between the two appears to be in their carbohydrate content amounting to 7% in PDGF-I and 4% in PDGF-II. Both have 9 disulfide bonds required for biological activity. Under reducing conditions both molecules are cleaved into two subunits of uneven size: the smaller A-chain, also referred to as PDGF-1 and the larger B-chain also termed PDGF-2 both of which proved to be extraordinarily stable molecules. Sequences of the N-terminal 109 and 75 amino acids of the A- and B-chain, respectively, have been determined by Waterfield and associates (1983). A striking result of a computer-assisted comparison of these sequences with those stored in the protein data base NEWAT (Doolittle 1981) was the extensive homology found between the sequenced stretch of the B-chain of human PDGF and the corresponding region of the predicted sequence of $p28^{v\text{-}sis}$, the putative transforming protein of simian sarcoma virus (SSV; Devare et al. 1983) and the 100% homology found between PDGF B-chain and $p28^{c\text{-}sis}$, the cellular counterpart of $p28^{v\text{-}sis}$. The homology is less extensive between PDGF A-chain and the two types of p28. By these data, the product of an oncogene was, for the first time, identified as a protein with known physiological effects on normal cells that can be of paramount importance in our future understanding of the mechanism of action of physiological growth factors, and ultimately the transformation process itself. It is

```
                                                                10
A:                                  Ser-Ile-Glu-Glu-Ala-Val-Pro-Ala-Val-Cys-Lys-Thr-Arg-Thr-
                                                  10                                        20
B: Ser-Leu-Gly-Ser-Leu-Thr-Ile-Ala  -  Pro  -  Met-Ile  -  Glu  -     -     -     -

                                20                                                  30
A: Val-Ile-Tyr-Gln-Ile-Pro-Arg-Ser-Gln-Val-Asp-Pro-Thr-Ser-Ala-Asn-Phe-Leu-Ile-Trp-
                                                  30                                        40
B: Glu-Val-Phe-Glu  -  Ser  -  Arg-Leu-Ile  -  Arg  -  Asn  -     -     -     -  Val  -

                                40                                                  50
A: Pro-Pro-Cys-Val-Glu-Val-Lys-Arg-Cys-Thr-Gly-Cys-Cys-Asn-Thr-Ser-Ser-Val-Lys-Cys-
                                                  50                                        60
B:  -     -     -     -     -     -  Cys  -     -  Ser  -     -     -     -  Asn-Arg-Asn  -  Gln  -

                                60                                                  70
A: Gln-Pro-Ser-Arg-Val-His-His-Arg-Ser-Val-Lys-Val-Ala-Lys-Val-Gln-Tyr-Val-Arg-Lys-
                                                  70                                        80
B: Arg  -  Thr-Gln  -  Gln-Leu  -  Pro  -  Gln  -  Arg  -  Ile-Glu-Ile  -     -     -

                                90                                                  90
A: Lys-Pro-Lys-Leu-Lys-Glu-Val-Gln-Val-Arg-Leu-Glu-Glu-His-Leu-Glu-Cys-Ala-Cys-Ala-
                                                  90                                       100
B:  -     -  Ile-Phe  -  Lys-Ala-Thr  -  Thr  -     -  Asp  -     -  Ala  -  Lys  -  Glu-

                               100                                                 110
A: Thr-Thr-Ser-Leu-Asn-Pro-Asp-Tyr-Arg-Glu-Glu-Asp-Thr-Gly-Arg-Pro-Arg-Glu-Ser-Gly-
                                                 110                                       120
B:  -  Val-Ala-Ala-Ala-Arg-Pro-Val-Thr-Arg-Ser-Pro-Gly  -  Ser-Gln-Glu-Gln-Arg-Ala-

                               120                 125
A: Lys-Lys-Arg-Lys-Arg-Lys-Arg-Leu-Lys-Pro-Thr
                                                 130                                       140
B:  -  Thr-Pro-Gln-Thr  -  Val-Thr-Ile-Arg  -  Val-Arg-Val-Arg-Arg-Pro-Pro-Lys-Gly-

                                                 150                                       160
B: Lys-His-Arg-Lys-Phe-Lys-His-Thr-His-Asp-Lys-Thr-Ala-Leu-Lys-Glu-Thr-Leu-Gly-Ala
```

Fig. 8.17. cDNA-derived amino acid sequence of the mature forms of the A- and B-chains of human PDGF. Dashes indicate identities. Alignment was made for maximizing homology

also intriguing that PDGF induces a rapid but transient expression of the c-fos gene (Kruijer et al. 1984) and a more protracted expression of the c-myc gene (Armelin et al. 1984), both belonging to the family of cellular (proto-) oncogenes.

Subsequent efforts led not only to the identification of further partial sequences of both chains of human PDGF from various sources (Antoniades and Hunkapiller 1983, Devare et al. 1983, Robbins et al. 1983, Johnsson et al. 1984, Josephs et al. 1984, Deuel et al. 1985b), but also to the isolation of cDNA clones encoding A- and B-chain precursors, from which amino acid sequences of the mature proteins could be deduced (Fig.8.17; Bestholtz et al. 1986).

There is an internal structural homology within the PDGF molecule: amino acids 3-95 of the mature A-chain is 56% homo-

logous to the corresponding stretch of the B-chain. Although subunit composition of the PDGF molecule has not yet been fully elucidated, available evidence suggests that PDGF is a disulfide-bonded heterodimer with an A-B construction. (Heldin et al. 1979, Deuel et al. 1981, Raines and Ross 1982), though the possibility that PDGF is a mixture of homodimers with an A-A and B-B structure, respectively, has not been ruled out either. It should be stressed, however, that PDGF from nonhuman species and PDGFc (where c stands for cellular), the non-platelet-derived PDGF-like growth factors secreted by various cells, normal or transformed, are homodimers of either A-A or B-B construction (for further information see Bowen-Pope and Seifert 1985, Collins et al. 1985, Heldin et al. 1985, 1986, Betsholtz et al. 1986, Wich et al. 1986, Tong et al. 1987). The PDGF A-chain gene has been mapped to chromosome 7, (Swann et al. 1982) whereas the PDGF B-chain gene (c-sis) to chromosome 22 (Betsholtz et al. 1986) within the human genome.

It is interesting to note that the predicted secondary structures and hydrophobic cores of PDGF, interleukin-2 and the sis oncogene protein are similar to those of interferons (Zavyalov and Denesyuk 1985).

Concentrations of PDGF in human serum measured by RIA were reported to be around 50 ng/ml, but PDGF cannot be detected in the plasma. A binding protein covalently and specifically bound to PDGF was described in the plasma, that was later identified as α_2-microglobulin, whose physiological significance has not yet been clearly defined. Protein phosphorylation is an immediate consequence of PDGF binding to cell surface receptors. PDGF stimulates tyrosine-specific kinase activity in membranes of responsive fibroblasts, the target probably being the PDGF receptor, a M_r 180,000 glycoprotein with both intrinsic and PDGF-inducible tyrosine kinase activities. Autophosphorylation of the receptor was also described, when the receptor serves as a substrate for its own ligand. Receptors for PDGF were found on a variety of mesenchymal cells but not on most epithelial cells with the exception of placental cytotrophoblasts (for more information on PDGF receptor see Ek and Heldin 1982, Williams et al. 1984, Heldin et al. 1985).

When serum is formed from plasma, platelets (thrombocytes) are exposed to thrombin. As a result of this exposure, but also seen with ADP or collagen, the platelets aggregate and undergo a series of events alternatively designated as "platelet degranulation", "platelet release reaction", or simply "platelet activation" resulting in the liberation of several platelet products and constituents including PDGF (Kohler and Liptin 1974, Ross et al. 1974). PDGF is biosynthesized in the megakariocytes and subsequently stored in platelets' α-granules, from where it is released locally during platelet activation when blood vessels are injured. Beside PDGF, the α-granules also contain platelet factor 4 (PF4), β-thromboglobulin as well as fibrinogen.

PDGF synthesis has also been demonstrated in endothelial cells (DiCorleto and Bowen-Pope 1983), smooth muscle cells (Seifert et al. 1984) cytotrophoblasts, activated macrophages and in various transformed cells (for details see Deuel and Huang 1984, Deuel et al. 1985b, Heldin et al. 1985, Betsholtz et al. 1986, Graves et al. 1986, Westermark et al. 1986). It is especially intriguing that the c-sis transcripts, which encode the B-chain of PDGF, are present in the cytotrophoblastic shell of human placenta, and that placental explants synthesize a PDGF-like molecule (Goustin et al. 1985). Cultured cells from early placentas express cell surface receptors for PDGF and respond to exogenous PDGF with activation of the c-myc gene and DNA synthesis (Goustin et al. 1985). Since the cells of the cytotrophoblastic shell are the most invasive and proliferative normal cells known, the expression of PDGF receptors on these cells may account for their "pseudomalignant phenotype" (Beaconsfield et al. 1980).

The precise physiological role of PDGF remains elusive. PDGF is a potent chemoattractant, first of all for inflammatory cells, suggesting that one of its major physiological roles may be to act as a mediator of inflammation and repair. There are suggestions that the mitogenic and chemotactic ability of PDGF might be bound to different structural segments of the molecule. PDGF may play a pivotal role in wound healing and

this may be the primary function of PDGF in vertebrates. Available evidence suggests that PDGFc may be developmentally regulated (Seifert et al. 1984).

Although vasoconstriction was recently described as a new activity for PDGF (Berk et al. 1986), the principal biological effect of PDGF remains the initiation of DNA synthesis in responsive cell populations, mainly fibroblasts, followed by cell division in an appropriate environment. A loss of the requirement for PDGF appears to be characteristic of most transformed cell lines (Vogel and Pollack 1974), and may also be a marker of tumorigenicity (Scher et al. 1979). PDGF is believed to be a competence factor rather than a directly acting mitogen, rendering responsive cells competent to enter the cycle and to respond to progression factors in the plasma which eventually enable the cells to undergo progression from the G_0/G_1 boundary to the S phase. Convincing evidence suggests that SM-C/IGF-I is, at least one of the progression factors present in plasma. PDGF-induced competence can be transferred to incompetent cells by cell fusion.

Exposure of the responsive cells to PDGF prior to their exposure to EGF results in a transient loss in EGF binding activity, and vice versa probably by down regulation (Wrann et al. 1980) suggesting that receptors for PDGF and EGF may be clustered in similar loci on the cell surface. The observation that PDGF and EGF exert a similar action in the prereplicative phase of human fibroblasts suggests that these two growth factors use a common intracellular pathway (Westermark and Heldin 1985).

The metabolism of responsive cells undergoes rapid and dramatic changes following their exposure to PDGF. Of these, changes in pinocytic activity, lipid metabolism, prostaglandin synthesis, actin and membrane organization, EGF binding and the expression of specific genes including proto-oncogenes should especially be stressed. The recent findings that cycloheximide or puromycin can substitute for PDGF in inducing DNA synthesis in quiescent 3T3 cells (Kaczmarek et al. 1986), and that PDGF and double-stranded ribonucleic acids stimulate the expression of identical genes in 3T3 cells (Zullo et al.

1986) should also be considered when the mechanism of action of PDGF as a mitogenic agent is interpreted.

PDGF appears to be one of the principal mitogens in whole blood serum that initiates DNA synthesis and proliferation in cultured cells. However, no firm evidence is presently available which would indicate that PDGF is capable of reaching the sites of cell proliferation in vivo, and that this factor is in fact responsible for the fibroproliferative events seen during wound healing and atherosclerosis. Recall that serum, not plasma, is the fluid which contains the factors derived from platelets and from the coagulation cascade of enzymes. In adult organisms, tissue cells are exposed to the equivalent of plasma, not serum, or a filtrate of plasma, and would only be exposed to the equivalent of serum during tissue injury eliciting platelet activation. This, on the other hand, implies that to expose cells to whole blood serum in culture is a situation analogous to when cells become exposed to the milieu created during response to injury in vivo. Such a response usually results in connective tissue cell proliferation and scar formation.

Previous data suggested that platelets might play a critical role in initiating the lesions characteristically seen in experimentally induced atherosclerosis (Harker et al. 1976, Moore et al. 1976, Friedman et al. 1977, Fuster et al. 1978). However, presently available data do not allow us to deduce the exact role what platelets play in the release of PDGF in vivo, allowing the latter to act at sites of endothelial injury.

There are a number of hereditary diseases in which abnormalities in platelet granules or in the platelets themselves play a critical role (Weiss et al. 1977, White 1978 and Gerrard et al. 1980). An increasing number of evidence also indicates that PDGF or similar proteins might play a critical role in cellular events ultimately leading to malignant transformation of the target cells (for details see Antoniades 1984, Antoniades et al. 1985, Deuel et al. 1985a,b,c, Pantazis et al. 1985, Rozengurt et al. 1985, Anzano et al. 1986, Bernabe et al. 1986). PDGF and platelet functions are extensively dis-

cussed in reviews by Deuel and Huang (1984), Deuel et al. (1985b), Heldin et al. (1985), Ross et al. (1986), Heldin and Westermark (1987), Williams et al. (1987), Williams (1988).

(2) Additional platelet-born growth factors. Besides PDGF, platelets also contain several other mitogenic substances. A mitogenic protein with an estimated M_r of 70,000, and a thermostable polypeptide with a M_r of 30,000-50,000 were shown to stimulate DNA synthesis in rat mammary endothelial cells. Other platelet factors displaying mitogenic activity include the connective tissue activating peptide III (CTAP III; Castor et al. 1977, Castor and Whitney 1978), low affinity platelet factor IV (LAPF IV) and platelet basic protein (PBP; Rucinski et al. 1979, Paul et al. 1980, Dresow and Delbrieck 1984).

More recently, platelets have been shown to contain an acidic protein of M_r around 20,000 endowed with the capability of stimulating both DNA synthesis and proliferation of porcine aorta-derived endothelial cells (Miyazono et al. 1985). Chemical, physicochemical and biological properties of this partially purified growth factor indicate that it is distinct from other platelet-derived factors hitherto described.

8.1.3. GROWTH FACTORS DERIVED FROM CONDITIONED MEDIA

The term conditioned or used media is applied to designate culture media in which certain cells are grown for a period of time and which, as a consequence, contain a number of diffusible factors with various biological activities released by the cells. Experience collected over the past decade or so provides ample evidence indicating that media conditioned by certain cells or tissues may occasionally be strikingly rich sources of substances promoting the growth and/or differentiation of various cells. Although some of these growth-promoting substances have also been detected in other biological sources (e.g., cultured cells, serum, urine, tissues), conditioned media are frequently used even today for studying, isolating and characterizing these factors.

8.1.3.1.1. COLONY-STIMULATING FACTORS

Hematopoiesis, a life-long process through which an unknown, but certainly a restricted number of self-renewing stem cells gives rise to lineage-specific progenitor cells that, by subsequent proliferation and differentiation, produce the mature blood cells found in the circulation, requires the continuous availability and cooperation of a group of glycoprotein factors, collectively termed colony-stimulating factors (CSFs). This term refers to the observation that CSFs stimulate progenitor cells of different lineages to form discrete colonies consisting of a single type of maturing cells. The different CSFs are operationally defined by prefixes denoting the major type of colony produced, e.g., granulocyte macrophage CSF (GM-CSF). CSFs are necessary, not only for the proliferation of progenitor cells, but for the function of mature cells as well, and they are also the factors that regulate increased cell production during states of increased demand. Their pathogenic role in some forms of leukemia remains to be elucidated.

By cloning the genes for a number of murine and human CSFs, recombinant forms with similar activity to their natural counterparts have been made available, allowing a large scale production and intensive investigation into the actions of various CSFs.

Recent studies on the biological activities of highly purified recombinant CSFs have revealed the inadequacy of existing CSF nomenclature. For example, GM-CSF was initially thought to stimulate the proliferation of granulocytes and macrophages. However, it is now clear that, with erythropoietin, it also induces the formation of colonies that comprise granulocytes, erythroid cells, macrophages and megakaryocytes indicating that GM-CSF is in fact a multipoietin as are other CSFs with pleiotropic activities. The confusion arisen by introducing new terms to describe the expanded target cell range of this and other CSFs and from many investigators' intention to for-

mulate their own pet names for the factors they have isolated, and also because many differently named factors proved to be a single factor upon more precise studies while their different names persisted in the literature, has been considerable. It should also be noted that CSFs and their role in hematopoiesis have been more clearly defined and more thoroughly studied in murine species, and the murine factors have often been named differently from their human counterparts. The information presented in Table 8.1 is to promote inquiry into variously named hematopoietic growth factors. It is apparent from Table 8.1 that CSFs are produced by various cell types and they have overlapping activities, stressing the importance of the purity both of the producer and the target cell populations while studying biological action of CSFs. However, the purification of normal hematopoietic cells in quantities sufficient for obtaining intricate information on the biochemical pathways involved and the point of attack of various CSFs within the cell cycle has not yet been accomplished. When comparing the actions of purified factors it is indispensable to differentiate between the effects on self-renewal or survival, proliferation and on differentiation, respectively. Having this in mind, it now seems very probable that at least two or perhaps three classes of factors may be necessary for completing cell development in a number of lineage: class I factors such as multi-CSF and GM-CSF act on pluripotent cells (stem cells) and immature progenitor cells in a not lineage-specific manner. They are required throughout differentiation, are important for cell renewal and proliferation of their target cells and contribute to their differentiation as well. On the other hand, class II factors such as G-CSF, M-CSF and erythropoietin (EP) act on more mature progenitor cells, and are only required during a later phase of the development of specific cell lineages, affecting both proliferation and differentiation of the progenitor cells, and the survival of mature end cells. Available evidence suggests a third class represented by a factor termed hematopoietin 1, that acts in synergism with multi-CSF or M-CSF (Bartelemez and Stanley 1985, Jubinsky and Stanley 1985, Stanley et al. 1986), but has no

Table 8.1. Human and murine myeloid hematopoietic cell growth factors

Human factors			Murine factors		
Name	Alternative name	Main target cells	Name	Alternative name	Main target cells
Multipotential CSF (Multi-CSF)	IL-3	CFU-MIX, CFU-GM/G/M, CFU-EO, CFU-MEG, BFU-E	Multi-CSF	IL-3, BFA, BPA, EEA, HCGF, MCGF, PCSF	CFU-MIX, CFU-GM/G/M CFU-S, CFU-EO, CFU-MEG, BFU-E, mast cells
Granulocyte-Macrophage-CSF, (GM-CSF)	CSF-α Pluripoietin	CFU-MIX, CFU-GM, CFU-EO, CFU-MEG, BFU-E, granulocytes, monocytes, eosinophils, KGI and HL60 cell lines	GM-CSF	MGI-1GM, CSF-2	CFU-MIX, CFU-GM, CFU-EO, CFU-MEG, BFU-E
Granulocyte-CSF (G-CSF)	CSF-β Pluripoietin	CFU-G, HL-60 cell line	G-CSF	MGI-2, GM-DF, D-factor	CFU-MIX, CFU-G, BFU-E, WEHI-3B cell line
Macrophage-CSF (M-CSF)	CSF-1	CFU-M, monocytes, myelomonocytic cell lines	M-CSF	CSF-1, MGI-1M, L-cell-CSF, CSA	CFU-M
Erythropoietin (Epo)	---	BFU-E, CFU-E	Epo	---	BFU-E, CFU-E

Abbreviations: BFA= burst-forming activity; BFU= burst-forming unit; BPA= burst-promoting activity; CSA= colony-stimulating activity; CSF= colony-stimulating factor; CFU= colony-forming unit; DF= differentiation factor; D-factor-differentiation factor; E= erythroid; EEA= erythropoiesis enhancing activity; EO= eosinophil; G= granulocyte; GM= granulocyte-macrophage; HCGF= hematopoietic cell growth factor; HL-60, KGI and WEHI-3B cell lines are myelomonocytic leukemic cell lines; IL-3= interleukin-3; M= macrophage; MCGF= mast cell growth factor; MEG= megakaryocyte; MGI= macrophage-granulocyte inducer; PCSF= permanent cell stimulatory factor.

colony-stimulating activity (CSA) on its own. The cooperation between this factor and M-CSF results in the formation of giant murine macrophage colonies in vitro (for recent reviews on CSFs see Arai et al. 1986, Dexter and Moore 1986, Metcalf 1986, Quesenberry 1986, Schrader et al. 1986, Whetton and Dexter 1986, Clark and Kamen 1987, Dexter 1987, Dexter and Spooncer 1987, Groopman 1987, Kaushansky 1987, Stadler et al. 1987, Sieff 1987, Vadas et al. 1987).

Responsive cells possess specific high affinity membrane receptors for CSFs (for a review see Nicola 1987). Each type of the receptors appears to bind only a single species of CSF. Since most target cells respond to more than one CSF, these cells simultaneously exhibit more than one type of receptors. It is unclear at present how many hemopoietic cells exhibit only a single species of CSF receptors. Receptors for different CSFs differ in molecular weight: GM-CSF: 50,000, G-CSF: 150,000, multi-CSF: 50,000, M-CSF: 16,500 (for references see Metcalf 1985). The number of receptors per cell for GM-CSF, G-CSF and multi-CSF ranges between 100 and 500, whereas that for M-CSF ranges between 3,000-16,000. Despite these relatively low receptor numbers, half maximal proliferative effects are achieved by G-CSF and GM-CSF with a receptor occupancy of only 5 to 10%. There are substantial differences between the speed of receptor degradation; M-CSF receptors being degraded very rapidly and G-CSF receptors more slowly with a half-life of about six hours. In general, murine CSFs do not stimulate human target cells, despite the often considerable sequence homology observed between the human and murine molecules. However, murine G-CSF and human CSF- are exceptions, showing considerable interspecies reactivity. It may be of interest to note that incubation of bone marrow adhesive cells with either CSF-1 or multi-CSF will induce up-regulation of the number of CSF-1 receptors (Bartelemez and Stanley 1985). Autophosphorylation of receptors and changes in the rate of synthesis of a number of cytoplasmic and nuclear proteins following binding of CSFs to the receptors have been reported (for references see Metcalf 1985a). It may be of interest to mention that GM-CSF has been shown to increase the phosphorylation of some

proto-oncogen products, e.g., p21, the cellular ras gene product, and p53, a tumor associated protein. It is also an intriguing observation that the M_r 165,000 product of c-fms, a proto-oncogene is closely related to the CSF-1 receptor (Sherr et al. 1985): the c-fms gene product displays tyrosine kinase activity and appears to be a truncated version of CSF-1 receptor, a situation reminiscent of the v-erb B gene and the EGF receptor. Although it has been established with certainty that the proliferative action of CSFs is direct on responsive cells, the complexity of events has so far prevented the exact identification of changes crucial for cell division. Although CSF-induced proliferation is regularly followed by maturation of affected cells, it is still uncertain whether CSFs have a direct effect on the maturation process.

All mouse tissues hitherto tested were shown to synthesize one or another form of CSFs in vitro. The tissue concentrations of extractable CSFs surpass those in the serum. As to the CSF-producing cell types, macrophages, T-lymphocytes, endothelial cells, fibroblasts and skin epithelial cells have all been shown to synthesize one or more types of CSFs. Several tumors can also synthesize CSF and this is the reason why granulocyte levels in tumor bearing animals and patients are often grossly elevated.

(1) Multipotential colony-stimulating factor. As it can be inferred from its name, multipotential colony-stimulating factor or simply multi-CSF is a multifunctional molecule acting on a large number of target cells. The acronyms registered in Table 8.1 stem from previous periods when the activities referred to by the acronyms were attributed to different biochemical entities. Among others, multi-CSF now is known to be identical to interleukin-3 (IL-3), a member of the interleukin family of polypeptides, whose other members are discussed in Chapter 7 as immunopeptides. Multi-CSF/IL-3 was first assumed by Ihle and associates (1982) to be a glycoprotein. In subsequent studies, murine IL-3 derived from WEHI-3 cells, a myelomonocytic leukemia cell-line, was purified to apparent homogeneity and the sequence of its 15

N-terminal amino acids, as well as some of its biochemical and biological properties have been established (Bazill et al. 1983, Ihle et al. 1983). Similarly to GM-CSF and G-CSF, the protein portion of multi-CSF is a monomer. More recently, two cDNA clones for multi-CSF have been isolated from a mouse T-cell cDNA library under the alternative names mast cell growth factor (MCGF; Yokota et al. 1984) and IL-3 (Fung et al. 1984). The cDNA-derived amino acid sequence of the 140 amino acid mature murine IL-3 (mIL-3) reported by Fung et al. (1984) is shown in Fig.8.18. The formation of mIL-3 from its precursor probably involves a proteolytic removal not only of the signal peptide, but also six additional N-terminal residues (see Fig.8.18) Whether this step occurs in vivo, or simply represents an adventitious proteolysis during purification remains to be determined. If the additional proteolysis occurs physiologically, then mIL-3 will be a 134 amino acid polypeptide having a calculated molecular weight of about 15,000, The four possible glycosylation sites may account for the differences found between the M_rs of purified IL-3s and that calculated from the predicted sequence.

On the other hand, the cloned cDNA reported by Yokota and associates (1984) was shown to encode a murine polypeptide with mast cell growth factor activity whose predicted amino acid sequence proved to be practically identical to that of IL-3 shown in Fig.8.18. The differences are confined to four residues: in the predicted sequence reported by Yokota and associates (1984) threonine is substituted for alanine at position 119 and the tripeptide sequence Pro-Arg-Arg for the sequence Arg-Pro-Pro at positions 121 through 123 (see Fig.8.18) indicating identity or a very close relatedness between these two molecules. More recently, the nucleotide sequence of a BALB/c mouse cell-derived cDNA encoding IL-3 was determined by Campbell and associates (1985) and found to be fully homologous to that derived from the murine leukemia cell line WEHI-3 indicating that the unprocessed primary structure of IL-3 is identical in both strains (for recent structural data on murine multi-CSF see Clark-Lewis et al. 1986, Todokoro et al. 1986). cDNA-derived amino acid sequences of the human and gib-

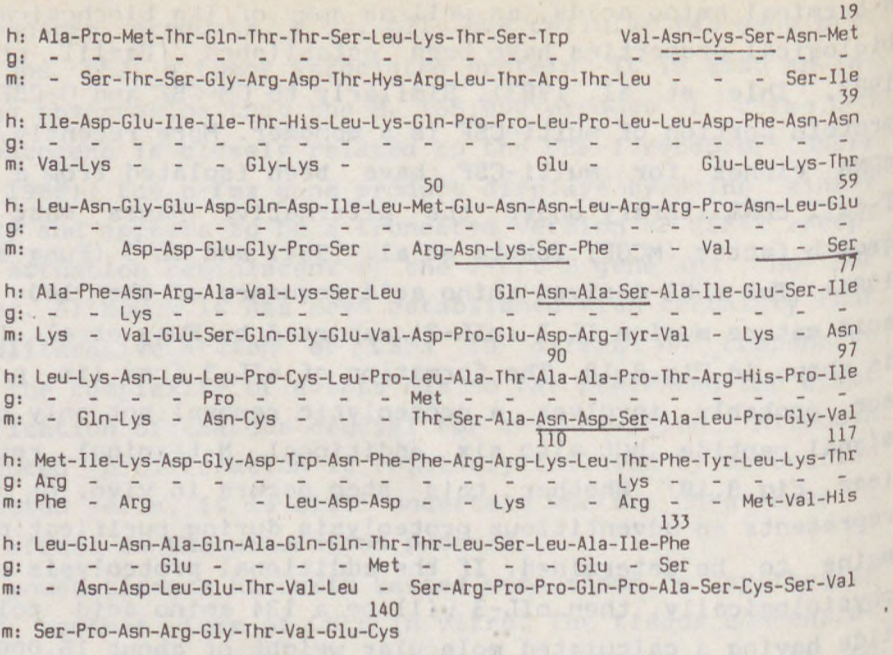


Fig. 8.18. cDNA-derived amino acid sequences of the putative mature forms of human (h), gibbon (g) and murine (m) multi-CSFs/IL-3s. The N-terminal 15 amino acids of the m sequence has also been chemically determined. The N-terminal seventh residue (Asp) in mIL-3 is the probable N-terminus of the mature protein (see text). Underlined residues represent possible glycosylation sites. Deletions (empty spaces) are introduced for a better match

bon IL-3s are also shown in Fig.8.18 (Yang et al. 1986). While there is a high degree of sequence homology between the human and the gibbon proteins both at the amino acid and the nucleic acid levels, the homology is much less marked betwen the former two and the murine proteins. The same is true for rat IL-3 not shown in the figure (Cohen et al. 1986) whose sequence differs rather significantly from those of IL-3s of the higher vertebrate species that are only 53% homologous to their murine counterpart. Similarly to GM-CSF, the gene for multi-CSF exists in a single copy form on chromosomes 11 and 5 in the mouse and human genomes, respectively (Fung et al. 1984, Yokota et al. 1984). A detailed structural analysis of the murine chromosomal gene for IL-3 has been accomplished by Miyatake and associates (1985b).

Biologically, IL-3 somewhat resembles IL-2, promoting differentiation and being required for the growth and activity of

various lymphocyte lines. However, dissimilarly to IL-2, IL-3 primarily influences specific stages of immune lymphocyte differentiation. Purified IL-3 is devoid of activity in several IL-2-specific assays and lacks many other activities which IL-2 has. IL-3 stimulates the self-renewal of multipotent and some of the committed myeloid precursor cells, and has an especially marked growth-promoting activity practically on all cells of the myeloid lineage including both multipotent and committed progenitor cells (see Table 8.1). Some cloned bone marrow-derived nonleukemic cell lines exhibit an absolute dependence on the continuous presence of hemopoietic cell growth factor (HCGF), another name for IL-3, for their survival and proliferation; in the absence of HCGF the cells die within hours. It is also worth mentioning that, while erythropoietin plays a role only in a relatively late phase of erythropoiesis and erythroid differentiation, the early proliferative events of the process are under the control of another regulator molecule with CSF-like characteristics. The latter, variously named erythrocyte CSF (E-CSF), burst-promoting activity (BPA), burst-feeder activity (BFA) and erythroid-enhancing activity (EEA), now is held to be identical with IL-3. IL-3 also enhances the proliferation of T-cell clones and of cloned mast cells. It also has a B-cell stimulatory activity inducing immunoglobulin secretion. According to recent data, distinct mechanisms may account for the growth-promoting activity of IL-3 on the cells of lymphoid and myeloid origin (Palacios and Garland 1984). A recombinant IL-3 was reported to exhibit synergistic factor activities (McNiece et al. 1984).

More recently, Greenberger and associates (1985) reported of a molecularly cloned and expressed murine T-cell gene product displaying biological activities similar to those of IL-3, while Welte and coworkers (1985) succeeded in isolating and characterizating a protein from low serum-containing medium conditioned by a human bladder carcinoma cell line (5637), which constitutively produces the isolated protein, termed pluripotent hematopoietic CSF (pluripotent CSF). The approximately M_r 18,000 protein supports the growth of human mixed colonies, granulocyte-macrophage colonies and early erythroid

colonies. It also induces the differentitation of both human and murine leukemia cell lines (for more information on IL-3 regulation of the growth of normal and transformed hemopoietic cells and on other IL-3 activities, see Ihle et al. 1985, Burstein 1986, Chen and Clark 1986, Kindler et al. 1986, Schrader 1986).

(2) Granulocyte-macrophage colony-stimulating factor. Although it has been possible to purify various CSFs to a significant degree (for recent results see Burgess et al. 1986, Gaffney et al. 1986) a detailed analysis of their biology and biochemistry has been hampered by the limited quantity available of these molecules. Resolution of this problem could be realistically expected only from introducing molecular cloning and recombinant DNA technology into this field. As to the GM-CSF, the first success on this line was achieved by Gough and associates (1984, 1985a), Lee et al. (1985b), Wong and coworkers (1985a,b) and by Miyatake et al. (1985a), who have cloned and sequenced both human and mouse GM-CSF cDNAs and determined the structure of the corresponding genes. Deduced amino acid sequences of human (Wong et al. 1985a,b) and mouse GM-CSFs (Gough et al. 1985a,b) are shown in Fig.8.19. Accordingly, the mature human protein has 127, whereas its murine counterpart has 124 amino acid residues, respectively. Both proteins possess two potential N-glycosylation sites. Position of the C-terminus within the precursor is identical in both proteins, as are the positions of the four cysteine residues, indicating the possible importance of disulfide bond formation in the structure of these molecules. The human cDNA sequence has an insertion of nine nucleotides relative to the mouse sequence, giving rise to three additional amino acids at positions 23-25. The nucleotide sequence of human GM-CSF cDNA (and the amino acid sequence predicted therefrom) exhibits a strong homology with its murine counterpart both in the coding and noncoding regions. The homology is about 70% at the nucleotide level and approximately 50% at the (deduced) amino acid level.

Two additional human GM-CSF cDNAs have been sequenced: one was prepared from T7 cells, a concanavalin A-activated human

```
                                    10                                  20
h: Ala-Pro-Ala-Arg-Ser-Pro-Ser-Pro-Ser-Thr-Gln-Pro-Trp-Glu-His-Val-Asn-Ala-Ile-Gln-
m:  -   -  Thr  -   -   -  Ile-Thr-Val   -  Arg  -   -  Lys  -   -  Glu  -   -  Lys-
                                    30                                  40
h: Glu-Ala-Arg-Arg-Leu-Leu-Asn-Leu-Ser-Arg-Asp-Thr-Ala-Ala-Glu-Met-Asn-Glu-Thr-Val-
m:  -   -   -   -   -   -  Leu-Asp  -  Met-Pro-Val-Thr-Leu  -   -  Glu  -
                                    50                                  60
h: Glu-Val-Ile-Ser-Glu-Met-Phe-Asp-Leu-Gln-Glu-Pro-Thr-Cys-Leu-Gln-Thr-Arg-Leu-Glu-
m:  -   -  Val  -  Asn-Glu  -  Ser-Phe-Lys-Lys-Leu  -   -  Val  -   -   -   -  Lys-
                                    70                                  80
h: Leu-Tyr-Lys-Gln-Gly-Leu-Arg-Gly-Ser-Leu-Thr-Lys-Leu-Lys-Gly-Pro-Leu-Thr-Met-Met-
m: Ile-Phe-Glu  -   -   -   -   -  Asn-Phe  -   -   -   -   -  Ala  -  Asn  -  Thr-
                                    90                                 100
h: Ala-Ser-His-Tyr-Lys-Gln-His-Cys-Pro-Pro-Thr-Pro-Glu-Thr-Ser-Cys-Ala-Thr-Gln-Thr-
m:  -   -  Tyr  -  Gln-Thr-Tyr  -   -   -   -   -   -   -  Asp  -  Glu  -   -  Val-
                                   110                                 120
h: Ile-Thr-Phe-Glu-Ser-Phe-Lys-Glu-Asn-Leu-Lys-Asp-Phe-Leu-Leu-Val-Ile-Pro-Phe-Asp-
m: Thr  -  Tyr-Ala-Asp  -  Ile-Asp-Ser  -   -  Thr  -   -  Thr-Asp  -   -   -  Glu-
                                   127
h: Cys-Trp-Glu-Pro-Val-Gln-Glu
m:  -  Lys-Lys  -  Ser  -  Lys(124)
```

Fig. 8.19. cDNA-derived amino acid sequences of the mature forms of human (h) T-cell-derived and murine (m) lung-derived granulocyte-macrophage colony stimulating factors (GM-CSF). Hyphens indicate identical residues. Deletions (empty spaces) are introduced to obtain maximum homology. Underlined residues indicate possible glycosylation sites. The number in parenthesis indicates the number of constituting amino acids in mGM-CSF

T-cell line (Lee et al. 1985b), and the other from HUT-102, a human T-cell line, as well as from mitogen-stimulated peripheral blood T lymphocytes (Cantrell et al. 1985). The cDNA derived amino acid sequence of the former differs in two residues [Thr(84) and Ile(100)], whereas that of the latter differs only in residue Ile(100) from the sequence published by Wong and coworkers (1985b). A genomic sequence has been reported for human GM-CSF by Kaushansky and associates (1986) that showed about a 98% homology to the aforementioned human sequences. On the other hand, the amino acid sequence of mature GM-CSF predicted from a gibbon ape continuous T-cell line-derived cDNA clone was found to differ in six residues [Ser(3), Arg(10), Ile(36), Val(60), Ile(100) and Gly(127)] from the human sequence shown in Fig.8.19. Some human GM-CSF cDNA clones were used to produce recombinant GM-CSF using both yeast (Cantrell et al. 1985) and mammalian (monkey and simian COS cells) expression systems (Dunn et al. 1985, Lee et al. 1985b, Wong et al. 1985a,b). In all instances, the products were similar to the natural protein purified to homogeneity from media conditioned by human T-cell lines. Six N-terminal

residues of the purified natural human GM-CSF and 16 N-terminal residues of a human recombinant GM-CSF were microsequenced and found to be identical to cDNA-derived sequence of the corresponding N-terminal stretch of the GM-CSF shown in Fig.8.19 (for references see Wong et al. 1985a,b).

The genes for both GM-CSF (Gough et al. 1984) and multi-CSF (Fung et al. 1984, Yokota et al. 1984, Miyatake et al. 1985a,b) exist in a single-copy form on chromosomes 11 and 5 in the mouse and human genome, respectively. This is consistent with the contention that size differences of GM-CSF molecules derived from different mouse tissues are the likely results of differences in glycosylation (Nicola et al. 1979). This makes it likely that the multiple forms represent post-translational modifications of a single product rather than products of multiple related genes.

GM-CSF is required continuously for in vitro proliferation of the progenitor cells of all lineage. Furthermore, this factor controls the irreversible commitment of these progenitors to the formation of mature end cells and it also regulates the functional activities of the latters (Metcalf 1981, Metcalf and Burgess 1982). The proliferative effect of GM-CSF seems to be directed on the G_1 phase of the cell cycle (Pluznik et al. 1984). Responsive cells that are out of cycle are forced by GM-CSF to enter the S phase and, in the absence of GM-CSF, they die or lose their capability to proliferate, a phenomen analogous to IL-3, EGF, NGF and FGF.

Various infections cause a gross elevation in serum and urine GM-CSF levels. In full harmony with this, GM-CSF levels in germ-free mice are extremely low. This sensitive reaction of serum GM-CSF levels to the exposure to infection by microbial agents may explain the fluctuation of GM-CSF levels in the serum observed in several species including man. Endotoxin also evokes a rapid and huge increase in serum and urine GM-CSF levels, but the effect decreases gradually upon repeated endotoxin injections. Some tumor populations including spontaneously developing ones, have the capacity to produce a large quantity of GM-CSF, and this may explain the elevated serum GM-CSF in many tumor patients. Although the effects of

GM-CSF were found to be relatively small and often inconsistent, a considerable body of evidence suggests that GM-CSF might be a physiologic agent, and its functions under in vivo conditions are identical to those experienced in vitro (see e.g., Welte et al. 1987). This is also supported by the observation that recombinant human GM-CSF induces granulocytosis in vivo (Fujisawa et al. 1986).

Despite a high degree of sequence homology between human and murine GM-CSFs, the expressed human gene product has no activity on murine bone marrow cells (Lee et al. 1985b). Although the main biological activity of GM-CSF encoded by the cDNA clone described by Lee and associates (1985b) was a stimulation of the development of neutrophil and macrophage colonies when incubated with human bone marrow cells or cord blood cells, the gene product expressed in COS-7 cells had a minor stimulatory effect also on eosinophil precursors. Because there may exist one or more classes of human GM-CSFs whose activity is specific only for cells of a given lineage as is the case with CSF-β (G-CSF; Nicola et al. 1979), the observed activity on eosinophil progenitors needs further confirmation before it can be accepted as an activity characteristic of all human GM-CSFs. Recently, the recombinant human GM-CSF described by Wong and coworkers (1985a,b) was shown to be identical with a lymphokine, termed T-cell-derived neutrophil migration inhibitory factor (NIF-T) purified to homogeneity by Gasson and associates (1984), which is a potent inhibitor of the migration of human neutrophils in vitro. Thus a single CSF cannot only stimulate the growth (and differentiation) of progenitor cells in the bone marrow but can also activate the biological functions of the circulating mature blood cells. This is consistent with the findings that purified murine GM-CSF (and G-CSF) can activate murine neutrophil cytotoxic functions, and that semipurified human CSF from placental-conditioned medium can enhance antibody dependent cell-mediated cytotoxicity of human neutrophils (for references see Wong et al. 1985a,b, and for a review Metcalf 1985).

(3) Granulocyte colony-stimulating factor. Two CSFs, namely GM-CSF and M-CSF, were purified to homogeneity years ago (Burgess et al. 1977, Stanley and Heard 1977) and tested for their biological activities. In these initial studies M-CSF showed no apparent capacity to induce differentiation, but GM-CSF was found to induce a limited differentiation (and stem cell suppression) in myeloid leukemic cells in vitro. Contradictory results obtained with these factors finally led to the purification of a third, chemically distinct type of regulator with an exceptional ability to induce differentiation in murine myelomonocytic leukemia cells (Nicola et al. 1983). This particular factor was variously termed type 2 macrophage-granulocyte inducer (MGI-2), differentiation factor (DF) and G-CSF, since its primary effect on normal cells was to stimulate the proliferation and differentiation of granulocyte precursors. This naturally occurring inducer of terminal differentiation in WEHI-3B cells, a murine myelomonocytic leukemia cell line, was purified to apparent homogeneity from culture media conditioned by lungs of mice previously injected with bacterial endotoxin. Since the differentiation-promoting activity could be completely separated from GM-CSF activity at an early stage of a multistep separation procedure, but it was copurified at each remaining step with the G-CSF activity, the two activities were suggested to be mediated by the same substance, namely G-CSF. This factor (M_r 24,000-25,000) was active not only on murine myelomonocytic leukemia cells, but also on normal granulocyte progenitor cells, inducing differentiation in these cells with a simultaneous suppression of stem cell replication in the leukemic cells. In contrast to earlier assumptions, this regulator molecule proved to be a typical CSF because, in addition to its striking differentiation-promoting activity, it also mediated a proliferative effect mainly on granulocytic precursor cells. This G-CSF was identified as a glycoprotein with considerable hydrophobicity containing one or more disulfide bonds essential for maintaining structural integrity and biological activity of the molecule (for previous results on assumedly pure macrophage-

granulocyte differentiation factors or inducers (MGIs-2) see Lotem et al. 1980, Nicola et al. 1983, Olsson et al. 1984).

While previous results have shed some light on many properties of granulocyte-active substances, 1986 has been a real milestone in chemical characterization of G-CSF molecules from various species, and also in providing decisive evidence on their distinct entity. In 1986, G-CSF cDNAs have been isolated and characterized from both murine and human sources. Murine G-CSF (mG-CSF) cDNA was isolated from NFS-60 cells, a murine fibrosarcoma cell line, that constitutively synthesizes G-CSF mRNAs. The latter were used for constructing a cDNA library (Tsuchiya et al. 1986). On the other hand, Nomura (cited by Nagata et al. 1986a,b) has demonstrated that CHU-2 cells, a squamous carcinoma cell-line derived from a human oral cavity tumor, produces a high quantity of G-CSF constitutively. The produced protein was purified to homogeneity, and was partially sequenced (Nagata et al. 1986a). Subsequently, an oligonucleotide probe corresponding to a fragment of the partially purified human protein, (hG-CSF) was constructed and used for screening and isolating hG-CSF mRNAs that allowed the construction of an appropriate cDNA library. From the nucleotide sequences of murine and human G-CSF cDNAs, amino acid sequences of the encoded proteins, precursor and mature, could be predicted (Fig.8.20; Nagata et al. 1986a, Tsuchiya et al. 1986). The murine protein consists of 178 residues, whereas there are two distinct mRNA species for hG-CSF directing the synthesis of two distinct mature proteins, termed G-CSFa and G-CSFb, respectively, both having authentic G-CSF activity (Nagata et al. 1986b).

The 174 amino acid human G-CSFb differs from G-CSFa in having three amino acids (Val-Ser-Glu) deleted at positions 36 through 38 or, conversely, hG-CSFa has three amino acids inserted at the mentioned positions in comparison to G-CSFb (see Fig.8.20). The protein purified from the conditioned medium of human CHU-2 cells proved to be a G-CSFa species. On the other hand, when a cloned chromosomal gene for hG-CSF was introduced into monkey COS cells, the latter produced mainly G-CSFb mRNAs. Analysis of the chromosomal gene structure of hG-CSF

```
h: Thr-Pro-Leu                                  Gly-Pro-Ala-Ser      10  Ser-Leu-Pro-Gln-Ser-Phe-Leu-
                                                                    10                              20
m: Val  -  -  Val-Thr-Val-Ser-Ala-Leu  -  Pro  -  Leu-Pro  -  -  Arg  -  -  -
                                                20                              30
h: Leu-Lys-Cys-Leu-Glu-Gln-Val-Arg-Lys-Ile-Gln-Gly-Asp-Gly-Ala-Ala-Leu-Gln-Glu-Lys-
                                                30                              40
m:  -  -  Ser  -  -  -  -  -  -  -  -  Ala-Ser  -  Ser-Val  -  Leu  -  Gln-
                                                40                              50
h: Leu-Val-Ser-Glu-Cys-Ala-Thr-Tyr-Lys-Leu-Cys-His-Pro-Glu-Glu-Leu-Val-Leu-Leu-Gly-
                                                50
m:  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
                                                60                              70
h: His-Ser-Leu-Gly-Ile-Pro-Thr-Ala-Pro-Leu-Ser-Ser-Cys-Pro-Ser-Gln-Ala-Leu-Gln-Glu-
                                        60                              70
m:  -  -  -  -  -  -  -  Lys  -  Ser  -  -  Gly  -  Ser  -  -  -  -  -  Gln-
                                                80                              90
h: Ala-Gly-Cys-Leu-Ser-Gln-Leu-His-Ser-Gly-Leu-Phe-Leu-Tyr-Gln-Gly-Leu-Leu-Gln-Ala-
                                        80                              90
m: Thr-Gly  -  -  -  -  -  -  -  -  -  -  -  Cys  -  -  -  -  -  -  -  -  -  -
                                                100                             110
h: Leu-Glu-Gly-Ile-Ser-Pro-Glu-Leu-Gly-Pro-Thr-Leu-Asp-Thr-Leu-Gln-Leu-Asp-Val-Ala-
                                100                             110
m:  -  Ser  -  -  -  -  -  -  -  Ala  -  -  -  -  Leu  -  -  -  -  -  -  -
                                                120                             130
h: Asp-Phe-Ala-Thr-Thr-Ile-Trp-Gln-Gln-Met-Glu-Glu-Leu-Gly-Met-Ala-Pro-Ala-Leu-Gln-
                                120                             130
m: Asn  -  -  -  -  -  -  -  -  -  -  -  -  Asn  -  -  Val  -  -  Thr-Val  -
                                                140                             150
h: Pro-Thr-Gln-Gly-Ala-Met-Pro-Ala-Phe-Ala-Ser-Ala-Phe-Gln-Arg-Arg-Ala-Gly-Gly-Val-
                                140                             150
m:  -  -  -  Ser  -  -  -  -  -  Thr  -  -  -  -  -  -  -  -  -  -  -  -  -
                                                160                             170
h: Leu-Val-Ala-Ser-His-Leu-Gln-Ser-Phe-Leu-Glu-Val-Ser-Tyr-Arg-Val-Leu-Arg-His-Leu-
                                160                             170
m:  -  Ala-Ile  -  Tyr  -  -  Gly  -  -  -  Thr-Ala-Arg-Leu-Ala  -  His  -  -
                                177
h: Ala-Gln-Pro
   178
m:  -
```

Fig.8.20. cDNA-derived amino acid sequences of the mature forms of human (h) and murine (m) granulocyte colony stimulating factor (G-CSF). The human sequence shown represents a G-CSFa species. In the G-CSFb species the residues at positions 43 through 45 are deleted. Deletions (empty spaces) are introduced for maximizing homology

revealed five exons (and four introns) within the gene (Nagata et al. 1986b). A comparison of the nucleotide sequences of hG-CSFa and hG-CSFb cDNAs has indicated that an alternative use of the 5'splice donor sequence in intron 2 is responsible for the production of two different mRNAs for hG-CSFs. G-CSFb appears to be responsible for in vivo activity (Komatsu et al. 1987). The human and the murine G-CSFs exhibit 69% homology at the amino acid level. There is evidence that both the murine and human G-CSFs contain internal disulfide bonds. The gene for hG-CSF has been assigned to chromosome 17 within the human genome (Le Beau et al. 1987).

Both the mouse and human G-CSF cDNAs were expressed in monkey COS cells and purified to homogeneity (Nagata et al. 1986a,b, Tsuchiya et al. 1986). The purified proteins, and also a recombinant species reported by Souza and associates (1986) were shown to have authentic G-CSF activity. G-CSF proteins produced in COS cells stimulate granulocyte colony formation of bone marrow cells and support the proliferation of murine NFS-60 myeloid leukemic cells in vitro, and it also has a capability to induce granulocytosis in vivo (Cohen et al. 1987, Tamura et al. 1987). On the other hand, G-CSF induces terminal differentiation of WEHI-3B D cells, a subtype of a murine myelomonocytic leukemia cell line, but not of NFS-60 cells.

Although progress has been made in acquiring important knowledge of G-CSF, much remains to be done until we have a clear picture of the molecular characteristics and biological properties of G-CSF. The results presented above are encouraging and certainly will aid in promoting further progress in this area of research (for further information on G-CSF see Metcalf 1985a, Nicola et al. 1985, Takahashi et al. 1985, Whetton and Dexter 1986).

(4) Macrophage colony-stimulating factor. The CSF derived from mouse L cell-conditioned media that stimulates colony formation predominantly from macrophages has been rather exhaustively studied by Stanley and Heard (1977). This macrophage colony-stimulating factor (M-CSF), formerly known as CSF-1, L-cell CSF, MGI-1M and colony stimulating activity (CSA), was demonstrated in various murine and human sources including serum, urine, various tissues, and in media conditioned by leukocytes and other cells (for references see Stanley and Heard 1977). These CSFs of widely different origin were demonstrated to be physicochemically and immunologically similar glycoproteins. The overall CSF activity detected in L cell-conditioned medium was evidently mediated by a heterogenous group of substances having both low (23,000) and high M_r (70,000) components, with the high M_r components predominating. The protein purified by Stanley and Heard (1977) to

apparent homogeneity belongs to the high molecular weight group and represents about 70% of total CSF activity present in L cell-conditioned medium. It proved to be a glycoprotein with a M_r of approximately 70,000 and was shown to be composed of two disulfide-bonded polypeptide subunits with a M_r of approximately 35,000 each. Most conspicuously, the factor stimulated the formation of macrophage colonies but also promoted the production of granulocytes in cultures of mouse bone marrow cells. On the other hand, separated subunits proved to be biologically inactive. Microheterogeneity of the native form of this CSF was demonstrated by isoelectrofocusing, a feature shared with many other glycoproteins. This CSF had many properties common with those of most reported GM-CSFs derived from various murine and human sources. In subsequent studies Lotem and associates (1980) isolated an M-CSF activity, termed MGI-1M from sera of endotoxin (ES)-treated mice that showed a M_r of about 30,000 on gel-filtration, but this figure changed to 24,000 upon gel-filtration under dissociating conditions, suggesting a considerable inclination of this molecule to aggregate, or the presence of some binding proteins. This particular M-CSF proved to be distinguishable from other CSF activities present in ES by several criteria, including its disappearance time course from the serum in vivo, and its immunological properties. Similar studies with media conditioned by mouse lung tissue and peritoneal macrophages resulted in the isolation of a M-CSF that, in its native form, had a M_r of about 41,000 but 24,000 under dissociating conditions, a figure identical to that found for the ES-induced M-CSF mentioned above. It is generally true that, irrespective of the molecular weight species, treatment of M-CSF/CSF-1 preparations with reducing agents halves their apparent sizes, indicating that each consists of two similar disulfide-linked subunits whose molecular weights are species- and tissue source-dependent.

In recent years, a significant progress has been made in characterizing the structure of M-CSF/CSF-1. The glycoprotein nature of human- and mouse-derived molecules and the dimeric character of their protein portion have been definitively established. Ben-Avram and associates (1985) have reported the

amino acid composition, and the sequence of a region as well as the triptic map of murine L929 cell-derived CSF-1, and soon thereafter a corrected sequence (Ben-Avram 1985). This CSF-1 contains several cysteine residues per subunit and approximately 50% carbohydrate by weight. Available data also suggest that sulfhydril bridging plays a major role in maintaining the conformation of the protein, and that CSF-1 probably contains two identical subunits, suggesting a homodimeric construction of the protein. Based on the comparison of the N-terminal amino acid sequence of this CSF-1 to that of other identified growth factors, only a limited homology to insulin and insulin-like growth factors and practically no homology to GM-CSF and IL-3 could be established. Also in 1985, cDNA clones encoding human M-CSF/CSF-1 have been isolated from a human pancreatic carcinoma cell-line (Kawasaki et al. 1985). The nucleotide sequence of this cDNA predicted a 224 amino acid subunit polypeptide with a molecular weight of approximately 26,000 which, however, may be further processed to a M_r 20,000 form by proteolytic processing after residue 188.

The human CSF-1 appears to be encoded by a single copy gene, whose expression, however, results in the synthesis of several mRNA species ranging in size from 1.5 to 4.5 kilobase (see also Csejtey and Boosman 1986). In subsequent experiments a cDNA encoding the human urinary protein has been cloned from a simian virus (SV40)-transformed human trophoblast cell line (Wong et al. 1987). This cDNA coding for a 522 amino acid protein (M_r 60,500) proved to be homologous to that isolated from the murine L929 cell line and could also be isolated from the aforementioned human pancreatic carcinoma cells (Ladner et al. 1987). This means that these cells produce both 224 and 522 amino acid CSF-1s. Analysis of the predicted amino acid sequence of the larger protein revealed that it consists of an N-terminal 149 amino acid portion of the 224 amino acid protein, followed by a 298 amino acid insertion that is flanked by the C-terminal 75 amino acids of the 224 residue protein (Fig. 8.21). It has been established that the diverse sequences of CSF-1 mRNAs arise from a differential splicing of a large primary transcript, the resulting transcript coding for either

```
                                                  10                  20
Glu-Glu-Val-Ser-Glu-Tyr-Cys-Ser-Hys-Met-Ile-Gly-Ser-Gly-His-Leu-Gln-Ser-Leu-Gln
                                                  30                  40
Arg-Leu-Ile-Asp-Ser-Gln-Met-Glu-Thr-Ser-Cys-Gln-Ile Thr-Phe-Glu-Phe-Val-Asp-Gln
                                                  50                  60
Glu-Gln-Leu-Lys-Asp-Pro-Val-Cys-Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-Val-Gln-Asp-Ile
                                                  70                  80
Met-Glu-Asp-Thr-Met-Arg-Phe-Arg-Asp-Asn-Thr-Pro-Asn-Ala-Ile-Ala-Ile-Val-Gln-Leu
                                                  90                  100
Gln-Glu-Leu-Ser-Leu-Arg-Leu-Lys-Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-Asp-Lys
                                                  110                 120
Ala Cys-Val-Arg-Thr-Phe-Thr-Glu-Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-Val-Lys-Asn-Val
                                                  130                 140
Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-Asp-Lys-Asp-Trp-Asn-Ile-Phe-Ser-Lys-Asn-Cys-Asn
                                                  150                 160
Asn-Ser-Phe-Ala-Glu-Cys-Ser-Ser-Gln-Asp-Val-Val-Thr-Lys-Pro-Asp-Cys-Asn-Cys-Leu
                                                  170                 180
Tyr-Pro-Lys-Ala-Ile-Pro-Ser-Ser-Asp-Pro-Ala-Ser-Val-Ser-Pro-His-Gln-Pro-Leu-Ala
                                                  190                 200
Pro-Ser-Met-Ala-Pro-Val-Ala-Gly-Leu-Thr-Trp-Glu-Asp-Ser-Glu-Gly-Thr-Glu-Gly-Ser
                                                  210                 220
Ser-Leu-Leu-Pro-Gly-Glu-Gln-Pro-Leu-His-Thr-Val-Asp-Pro-Gly-Ser-Ala-Lys-Gln-Arg
                                                  230                 240
Pro-Pro-Arg-Ser-Thr-Cys-Gln-Ser-Phe-Glu-Pro-Pro-Glu-Thr-Pro-Val-Val-Lys-Asp-Ser
                                                  250                 260
Thr-Ile-Gly-Gly-Ser-Pro-Gln-Pro-Arg-Pro-Ser-Val-Gly-Ala-Phe-Asn-Pro-Gly-Met-Glu
                                                  270                 280
Asp-Ile Leu-Asp-Ser-Ala-Met-Gly-Thr-Asn-Trp-Val-Pro-Glu-Glu-Ala-Ser-Gly-Glu-Ala
                                                  290                 300
Ser-Glu-Ile-Pro-Val Pro-Gln-Gly-Thr-Glu-Leu-Ser-Pro-Ser-Arg-Pro-Gly-Gly-Gly-Ser
                                                  310                 320
Met-Gln-Thr-Glu-Pro-Ala-Arg-Pro-Ser-Asn-Phe-Leu-Ser-Ala-Ser-Ser-Pro-Leu-Pro-Ala
                                                  330                 340
Ser-Ala-Thr-Gly-Gln-Gln-Pro-Ala-Asp-Val-Thr-Gly-Thr-Ala-Leu-Pro-Arg-Val-Gly-Pro
                                                  350                 360
Val-Arg-Pro-Thr-Gly-Gln-Asp-Trp-Asn-His-Thr-Pro-Gln-Lys-Thr-Asp-His-Pro-ser-Ala
                                                  370                 380
Leu-Leu-Arg-Asp-Pro-Pro-Glu-Pro-Gly-Ser-Pro-Arg-Ile-Ser-Ser-Leu-Arg-Pro-Gln-Gly
                                                  390                 400
Leu-Ser-Asn-Pro-Ser-Thr-Leu-Ser Ala-Gln-Pro-Gln-Leu-Ser-Arg-Ser-His-Ser-Ser-Gly
                                                  410                 420
Ser-Val-Leu-Pro-Leu-Gly-Glu-Leu-Glu-Gly-Arg-Arg-Ser-Thr-Arg-Asp-Arg-Arg-Ser-Pro
                                                  430                 440
Ala-Glu-Pro-Glu-Gly-Gly-Pro-Ala-Ser-Glu-Gly-Ala-Ala-Arg-pro-Leu-Pro-Arg-Phe-Asn
                                                  450                 460
Ser-Val-Pro-Leu-Thr-Asp-Thr-Gly-His-Glu-Arg-Gln-Ser-Glu-Gly-Ser-Ser-Ser-Pro-Gln
                                                  470                 480
Leu-Gln-Glu-Ser-Val-Phe-His-Leu-Leu-Val-Pro-Ser-Val-Ile-Leu-Val-Leu-Leu-Ala-Val
                                                  490                 500
Gly-Gly-Leu-Leu-Phe-Tyr-Arg-Trp-Arg-Arg-Arg-Ser-His-Gln-Glu-Pro-Gln-Arg-Ala-Asp
                                                  510                 520
Ser-Pro-Leu-Glu-Gln-Pro-Glu-Gly-Ser-Pro-leu-Thr-Gln-Asp-Asp-Arg-Gln-Val-Glu-Leu
    522
Pro-Val
```

Fig. 8.21. cDNA-derived amino acid sequence of the 522 amino acid human macrophage colony-stimulating factor (M-CSF or CSF-1). The N-terminal 13 residues are the chemically determined N-terminal fragment of human urinary CSF-1. Underlined residues indicate possible glycosylation sites. The 298 amino acid insertion between residues at positions 149 and 447 separates a 149 amino acid N-terminal and a 75 amino acid C-terminal portion of the 224 amino acid human CSF-1 species

a 224 or a 522 amino acid CSF-1 protein, respectively. By all indications, the N-terminal 149 amino acid fragment is responsible for the biological activity of all CSF-1s, whereas the remaining amino acids may impart a correct configuration for proteolytic processing and membrane binding (for more details see Ladner et al. 1987). A partial amino acid sequence for a murine CSF-1 has also been reported (Boosman et al. 1987) and established that the human and murine proteins show 74% amino acid identity over the 65 residues of the region that have been sequenced, and are thus highly homologous.

It has been fairly well documented that hemopoietic growth factors involved in the early steps of the maturation pathway stimulate both proliferation and differentiation of cells capable of forming more than one blood cell type (hence the name multi-lineage growth factors), whereas those controlling the later stages act on cells committed to forming one particular blood cell type (hence the name lineage-specific growth factors). Synergism between these two types of growth factors appears to be an important aspect of the regulation of hemopoiesis. Hemopoietin-1 (H-1) and hemopoietin-2 (H-2) are multilineage growth factors endowed with the ability to stimulate the proliferation of developmentally early CSF-1 receptor bearing cells (for references see Jubinsky and Stanley 1985). H-1 and H-2 are able to induce an increased expression of CSF-1 receptors, an event that is soon followed by differentiation of the target cells into adherent mononuclear phagocytes (macrophages). H-2 may be identical to IL-3, but it differs from H-1 in several respects including its physical properties, target cell specificity and its activity in generating CSF-1 receptor bearing cells which require the synergistic action of CSF-1. To date, H-1 has been isolated from serum-free media conditioned by a human urinary bladder carcinoma cell line (5637) and purified approximately 62,000 fold. It has a M_r of about 17,000 and has been recently characterized both chemically and biologically by Jubinsky and Stanley (1985). For the molecular and biological properties of human CSF-1 see Ralph and associates (1986), Shadduck et al. (1987).

(5) Eosinophil colony-stimulating-factor. Until recently, attempts to characterize eosinophil (EO)- and megakaryocyte (MEG)-CSF activities have been far less successful than those for other CSFs, mainly because these activities usually are associated with sub-sets of hemopoietic growth factors that nonlineage-specifically also promote the colony formation of eosinophil progenitors (e.g., GM-CSF, IL-3, etc.). Thus the question of whether EO-CSF is a separate entity remained unanswered for long. Data on eosinophilopoietin, a postulated agent acting on more mature precursors have also remained difficult to be reproduced.

More recently, however, convincing evidence has been presented on the existence of a human eosinophil differentiation factor (EDF) that, apart from promoting the differentiation of EO progenitor cells, has also been established as an EO-CSF that selectively promotes the clonal proliferation of EO progenitors. EDF cDNAs have been molecularly cloned and isolated both from human (Campbell et al. 1987) and murine sources (Lopez et al. 1986). Predicted amino acid sequences of the mature human and murine proteins show an about 70% sequence homology and the recombinant molecules are active on cells of the opposite species as well. The murine protein proved to be identical to the recently identified B-cell growth factor-2 (BCGF-2)/interleukin-5 (IL-5) (Campbell et al. 1988) that is discussed in more details in Chapter 7 (for a novel human lymphokine with an EO-CSF activity see Yokota et al. 1987).

(6) Megakaryocyte colony-stimulating factor. Platelet production is controlled on at least two levels. The transition of stem cells into megakaryocyte progenitors is initiated by megakaryocyte (MEG)-CSF, whereas the ploidization and cytoplasmic maturation of the committed precursor cells into fully differentiated megakaryocytes is modulated by a humoral factor(s) whose blood concentration is altered by changes in the numbers of circulating platelets. This latter component has been termed thrombopoietin (TP) or thrombopoietic stimulatory factor (TSF) depending on the site of isolation. Although

TP increases platelet number and megakaryocyte frequency, it does not stimulate megakaryocyte colony formation and it acts only at a relatively late stage of megakaryocyte maturation and platelet production, as zerythropoietin does during erythropoiesis (for recent data see Greenberg et al. 1987, Tayrien and Rosenberg 1987).

A MEG-CSF activity partially purified from spleen cell conditioned media indicated a M_r 24,000 glycoprotein as the active agent (Burgess et al. 1978, Metcalf et al. 1978). MEG-CSF activity has also been detected in media conditioned by WEHI-3B cells, a myelomonocytic tumor cell line, and L cells a murine fibroblastic cell line. There are known CSFs that also exhibit MEG-CSF activity (see Table 8.1). The apparent capacity of erythropoietin to induce megakaryocyte colony formation (McLeod et al. 1976) probably is due to contamination. It follows from the foregoing discussion that the exact chemical structure of MEG-CSF remains unknown for the time being (for a recent review on the regulation of megakaryocytopoiesis see Hoffman et al. 1987).

(7) Allogenic effect factor. Bone marrow cell cultures established in the absence of an adherent cell layer looses stem cell activity. A soluble factor derived from short-term secondary mixed lymphocyte cultures (MLC) of the in vivo allo-antigen activated murine T cells was described, which was capable of supporting the growth of bone marrow stem cells in vitro in the absence of a pre-established adherent cell layer (Armerding and Katz 1974, Altman et al. 1980). The factor, probably a protein, was designated allogenic effect factor (AEF), and was shown to be highly mitogenic for bone marrow cells in vitro, and to maintain cellular heterogeneity in such cultures. Since the addition of IL-2 to the culture failed to evoke a similar effect, and AEF displays properties dissimilar to those of colony-stimulating activity (CSA), AEF is regarded as a distinct molecular entity. The primary structure of AEF has not been reported yet.

8.1.3.1.2. ERYTHROPOIETIC FACTORS

(1) Erythropoietin and erythroid potentiating activity. Neither erythropoietin nor erythrotropin (see in the subsequent section) were discovered in conditioned media, and thus do not fit into section 8.1.3. The reason why they are included here is that both exhibit growth factor-like activities on erythroid precursor cells, which also belong to cells of myeloid lineage whose growth factors are dealt with in this paragraph.

Erythropoietin (Epo) is regarded as the main inducer of erythropoiesis which in cultures of adult bone marrow cells induces the formation of erythroid colonies and of bursts, as well as the synthesis of hemoglobin. Thus, like IL-3 and GM-CSF, Epo is a regulator of both the proliferation and differentiation of the eryhtroid family.

To date, Epo has been purified from plasma of anemic sheep, and from urine of anemic patients (Goldwasser and Kung 1971, Miyake et al. 1977). Both types of Epo proved to be an acidic glycoprotein with a molecular weight of 46,000 (sheep) and 34,000 (human) and with 24% (sheep) and about 59% (human) carbohydrate content, respectively. The human and sheep Epos differ immunologically. The human Epo was shown to exist in two forms, α and β Epo (Miyake et al. 1977) having identical potency and qualitative amino acid composition, but a slightly different number of amino acid residues and a slight difference in their electrophoretic mobilities.

Truly valuable information on the primary structure of human Epo was obtained when Lee-Huang (1984) succeeded in cloning and expressing human Epo cDNA in E. c o l i, and Lin and associates (1985) in establishing the nucleotide sequence of a gene encoding human Epo (see also Beru et al. 1987). The amino acid sequence of the mature Epo deduced from this cDNA is shown in Fig.8.22. The gene was isolated from a human fetal liver genomic library using mixed 20-mer and 17-mer oligonucleotide probes synthesized on the basis of partial amino acid sequences of tryptic fragments obtained from purified Epo isolated from the urine of patients with aplastic anemia.

The Epo gene encodes a 166 amino acid mature protein (see Fig.8.22) with a calculated molecular weight of 18,399. The Epo gene introduced into Chinese hamster ovary cells was shown to produce Epo that was biologically active both in vivo and in vitro. At a first approximation, Epo consists of three domains. The N-terminal and C-terminal portions are highly structured, and protease resistant, each having one disulfide bond. These two domains are joined by a rather unstructured, protease sensitive connecting region. This latter region appears to contain the active site of the molecule since limited proteolysis causes inactivation. The Epo gene has been mapped to chromosome 7 in the human genome (Powel et al. 1986). The murine (Shoemaker and Mitsock 1986), human (Lin et al. 1985) and the monkey Epo genes (Lin et al. 1986) have also been cloned and their strong homology to the human gene demonstrated (for differences between Epos from various species see Browne et al. 1986). Human urinary Epo has also been chemically sequenced and structurally characterized (Lai et al. 1986). The chemically determined sequence proved to be identical with the deduced sequence shown in Fig.8.22. The two disulfide bonds demonstrated in the molecule were shown to span between the paired cysteine residues at positions 7-161 and 29-33, respectively.

An impressive number of in vivo studies convincingly demonstrated that Epo has the ability to stimulate both hemoglobin synthesis and erythropoiesis (for reviews see Peschele et al. 1978, Goldwasser 1981, 1984). Epo binds to specific membrane receptors on Epo-responsive cells, each of which has about 600-700 receptors. Besides having a growth factor-like effect on erythroid precursor cells, Epo was also shown to interfere with both globin and hem synthesis (Goldwasser 1984). In contrast to previous beliefs suggesting the kidney as the sole organ source of Epo precursors, it now seems more than probable that other cell types may also be able to synthesize Epo or its precursor (Naughton et al. 1978, Zucali and Mirand 1978). The finding that Epo equally induces proliferation and hemoglobin synthesis in responsive cell populations indicates that Epo may have multiple sites of action in target erythroid

```
                                                  10                  20
Ala-Pro-Pro-Arg-Leu-Ile-Cys-Asp-Ser-Arg-Val-Leu-Glu-Arg-Tyr-Leu-Leu-Glu-Ala-Lys-
                                                  30                  40
Glu-Ala-Glu-Asn-Ile-Thr-Thr-Gly-Cys-Ala-Glu-His-Cys-Ser-Leu-Asn-Glu-Asn-Ile-Thr-
                                                  50                  60
Val-Pro-Asp-Thr-Lys-Val-Asn-Phe-Tyr-Ala-Trp-Lys-Arg-Met-Glu-Val-Gly-Gln-Gln-Ala-
                                                  70                  80
Val-Glu-Val-Trp-Gln-Gly-Leu-Ala-Leu-Leu-Ser-Glu-Ala-Val-Leu-Arg-Gly-Gln-Ala-Leu-
                                                  90                 100
Leu-Val-Asn-Ser-Ser-Gln-Pro-Trp-Glu-Pro-Leu-Gln-Leu-His-Val-Asp-Lys-Ala-Val-Ser-
                                                 110                 120
Gly-Leu-Arg-Ser-Leu-Thr-Thr-Leu-Leu-Arg-Ala-Leu-Gly-Ala-Gln-Lys-Glu-Ala-Ile-Ser-
                                                 130                 140
Pro-Pro-Asp-Ala-Ala-Ser-Ala-Ala-Pro-Leu-Arg-Thr-Ile-Thr-Ala-Asp-Thr-Phe-Arg-Lys-
                                                 150                 160
Leu-Phe-Arg-Val-Tyr-Ser-Asn-Phe-Leu-Arg-Gly-Lys-Leu-Lys-Leu-Tyr-Thr-Gly-Glu-Ala-
                                            166
Cys-Arg-Thr-Gly-Asp-Arg
```

Fig. 8.22. cDNA-derived amino acid sequence of human erythropoietin. Underlined residues indicate possible glycosylation sites

cells (for more details see Goldwasser 1981, Browne et al. 1986, Spivak 1986).

In vitro studies have recently indicated that, apart from Epo, there is another class of mediators which appears to be important in stimulating erythroid progenitor cells. Previously, these factors have generally been referred to as burst-promoting activity (BPA) because they stimulate the growth of early erythroid precursors (BFU-E) which gives rise to colonies of up to 1000 hemoglobinized cells. Purification of a BPA from media conditioned by the Mo T-lymphoblast cell line infected with human T-cell lymphotrophic virus type II (HTLV-II) has been previously reported (for references see Cosson et al. 1985). The purified material, a glycoprotein of M_r 28,000, stimulated colony formation by more mature erythroid precursors (CFU-E), and therefore was referred to as erythroid potentiating activity (EPA). A human EPA cDNA encoding a 184 amino acid mature protein (Fig.8.23) has been published by Cosson and associates (1985). The EPA cDNA has also been expressed in monkey COS cells and the recombinant EPA obtained showed characteristic EPA activity.

In contrast to IL-3 which stimulates the growth of all hemopoietic precursors, EPA specifically stimulates the growth of peripheral and bone marrow-derived erythroid precursors from mouse and man. There is no homology between human EPA and mouse IL-3, and this is consistent with the observation that

```
                                   10                                  20
Cys-Thr-Cys-Val-Pro-Pro-His-Pro-Gln-Thr-Ala-Phe-Cys-Asn-Ser-Asp-Leu-Val-Ile-Arg-
                                   30                                  40
Ala-Lys-Phe-Val-Gly-Thr-Pro-Glu-Val-Asn-Gln-Thr-Thr-Leu-Tyr-Gln-Arg-Tyr-Glu-Ile-
                                   50                                  60
Lys-Met-Thr-Lys-Met-Tyr-Leu-Gly-Phe-Gln-Ala-Leu-Gly-Asp-Ala-Ala-Asp-Ile-Arg-Phe-
                                   70                                  80
Val-Tyr-Thr-Pro-Ala-Met-Glu-Ser-Val-Cys-Gly-Tyr-Phe-His-Arg-Ser-His-Asn-Arg-Ser-
                                   90                                 100
Glu-Glu-Phe-Leu-Ile-Ala-Gly-Lys-Leu-Gln-Asp-Gly-Leu-Leu-Met-Ile-Thr-Thr-Cys-Ser
                                  110                                 120
Phe-Val-Ala-Pro-Trp-Asn-Ser-Leu-Ser-Leu-Ala-Gln-Arg-Arg-Gly-Phe-Thr-Lys-Thr-Tyr-
                                  130                                 140
Thr-Val-Gly-Cys-Glu-Glu-Cys-Thr-Val-Phe-Pro-Cys-Leu-Ser-Ile-Pro-Cys-Gln-Leu-Gln-
                                  150                                 160
Ser-Gly-Thr-His-Cys-Leu-Trp-Thr-Asp-Gln-Leu-Leu-Gln-Gly-Ser-Glu-Lys-Gly-Phe-Gln-
                                  170                                 180
Ser-Arg-His-Leu-Ala-Cys-Leu-Pro-Arg-Glu-Pro-Gly-Leu-Cys-Pro-Trp-Gln-Ser-Leu-Arg-
                              184
Ser-Gln-Ile-Ala
```

Fig. 8.23. cDNA-derived amino acid sequence of the mature form of human "erythroid potentiating activity". Glycosylation sites are underlined

unlike IL-3, EPA does not stimulate pluripotent or myeloid precursor cells.

While no significant homology has been found between EPA and other known human proteins, EPA cDNA shows some homology to cDNA encoding a murine interferon-β-like protein. Since EPA has no interferon activity the significance of this structural relatedness is unclear at present.

The physiological function of human EPA in vivo remains to be elucidated. However, since human EPA stimulates both human and murine erythroid precursors, it may valuably contribute to studies aimed at elucidating in vivo effects of this modulator in the mouse (for information on a human erythroid differentiation factor see Eto and coworkers 1987).

More recently, a basic urinary fraction has been partially characterized which showed a potent erythropoiesis inhibitory activity (Neal et al. 1985). The activity was shown to be associated with an α-acid glycoprotein and to be a protein-prostaglandin $F_{2\alpha}$ complex.

(2) Erythrotropin. In 1983, Congote (1983) reported the isolation from fetal bovine intestine of two new polypeptide factors acting on erythroid cells, which were termed erythrotropin I (or A) and erythrotropin II (or B), the two having a slightly different amino acid composition and physicochemical characteristics.

Besides IL-3, erythropoietin and erythroid potentiating activity these new erythroid cell-stimulating factors now seem to represent a fourth class of molecules regulating erythropoiesis. As to their biological activities, both erythrotropins stimulate globin chain synthesis in fetal calf liver cells and act synergistically with erythropoietin on the stimulation of ^{3}H-TdR incorporation into acid-insoluble materials in rat liver cells. In comparison to IL-3 and erythropoietin, the approximately M_r 4,000 erythrotropins are low molecular weight substances. An erythrotropin I-like molecule was also isolated from fetal bovine serum (Congote 1984).

8.1.3.1.3. GROWTH FACTORS FOR THE LYMPHOCYTES

(1) T-lymphocyte growth-stimulatory activity. From media conditioned by PHA-stimulated lymphocytes, an activity was coseparated with GM-CSF that was characterized by its ability to increase the number of T-lymphocytes in suspension culture (Wu and Gallo 1976, Wu 1979). The putative factor thought to be responsible for this effect was termed T-lymphocyte growth-stimulatory activity (TL-CSA), which was assumed to be the product of a T-cell subpopulation distinct from that producing the coisolated GM-CSF.

TL-GSA was purified and characterized to some extent. It is assumed to be a polypeptide of M_r around 13,000, having biological and chemical characteristics distinct from those of other lymphokines. However, its relationship to some of the thymic factors awaits further clarification (Wu 1979).

TL-GSA was shown to be capable of supporting long-term growth not only of various functional T cells, but also of their progenitor cells in suspension culture. The effect of TL-GSA on progenitor cells raised the intriguing possibility that autoregulation of the proliferation-related differentiation of T cells might occur not only at the level of mature cells but also at that of progenitor cells.

(2) T and B cell growth factors. Since these factors play an essential role in the differentiation of T and B cells they are discussed in Chapter 7 as immunopeptides.

8.1.3.2. MAST CELL GROWTH FACTOR

A number of factors with a common capability of stimulating proliferation of mast cells, or mast cell-like cell lines, have been described. One of these was shown to be a product of an inducer T-cell line with a characteristic phenotype (Nabel et al. 1981) that promoted the growth of a histamine-producing mast cell-like cell line. The factor has been partially purified and estimated to have a M_r somewhere between 40,000 and 50,000, but further details on its chemistry have not been published.

Long-term in vitro growth of murine mast cells was found to be dependent on a mast cell growth factor (MCGF) present in media conditioned by mitogen-activated splenic leukocytes, or by various murine leukemic cell lines (Yung and Moore 1982). MCGF proved to be an about M_r 35,000 glycoprotein whose carbohydrate portion plays an active, if not an exclusive role in stimulating mast cell growth. MCGF is surely distinct from IL-2 or T-cell growth factor (Yung and Moore 1982), however, its relationship to G-CSF is not entirely clear. They share many properties, though a number of functional tests did indicate their separate entities. In vitro production of MCGF by normal spleen cells requires the presence of T-lymphocytes and the process is relatively macrophage-independent. However, it should be noted that, in recent publications MCGF activity is attributed to IL-3 and many investigators regard these two factors as identical entities. It should also be kept in mind that biological activities of IL-3 and those of the recently described IgG1-inducing factor/IL-4 (Lee et al. 1986, Noma et al. 1986, Yokota et al. 1986) shed a new light also on the MCGF problem (see Chapter 7).

8.1.3.3. GLIAL FACTOR

Although glial factor is often mentioned as a growth factor, it has no mitogenic activity and is a differentiation factor rather than a growth factor for neuroblastoma cells. Glial factor is exclusively produced by C-6 glioma cells in

culture (Monard et al. 1973). Chemically it may be a glycopeptide similar to those originating from neural membranes as described by Combos et al. (1972). Another C-6 glioma cell factor capable of supporting both the survival and the fibre formation of isolated chick sensory neurons has been described by Barde et al. (1978).

8.1.3.4. MIGRATION FACTOR

In media conditioned by SV28 cells (SV40 virus-transformed BHK cells), but not by normal cells, nor by cells transformed by other viruses, an about M_r 40,000 factor was isolated which enhanced the migration of 3T3 cells. It was termed migration factor (MF) by Burk (1973). MF stimulates DNA synthesis in 3T3 cells and induces their overgrowth to a higher density and, transiently at least, allows the survival of these cells in a serum-free medium. Due to the similarities between the biological properties of MF and transforming growth factors, MF is regarded by many as a molecule belonging to the family of tumor growth factors (for a review see Gospodarowicz and Moran 1976).

8.1.3.5. TRANSFORMING GROWTH FACTORS

It was first observed by DeLarco and Todaro (1978) and Todaro and DeLarco (1978) that when media conditioned by murine sarcoma virus-transformed cells were transferred to cultures of normal rodent fibroblasts, they have conferred the transformed phenotype on the normal cells. (The transformed phenotype is operationally defined by a set of criteria: loss of the density-dependent inhibition of the growth of cells with a consecutive overgrowth of the cells in monolayers; decreased serum and growth factor requirement of the cells in culture; reversible changes in the morphology of affected cells; acquisition by the normal, so-called indicator cells of the capability of growing anchorage-independently i.e., unattached to the plastic culture vessels in semisolid

medium such as soft agar, a feature that correlates well with tumorigenicity in vivo; Kahn and Shin 1979). It was subsequently demonstrated that a particular type of polypeptide growth factors, termed originally sarcoma growth factor (SGF), was responsible for the transforming effect Later, similar observations have been made on other types of transformed cells and thus the term SGF was replaced by the terms transforming growth factor or tumor growth factor both abbreviated as TGF. It was originally thought that TGF would be found only in malignant cells. However, subsequent studies have demonstrated that, at least some types of TGFs are also produced by normal cells on the one hand, and that some physiological growth factors, e.g., PDGF can also stimulate the anchorage-independent growth of normal cells in the presence of serum on the other (Kaplan and Ozane 1982, Anzano et al. 1986).

TGF was shown to be a heterogenous group of substances that could be classified into at least two broad categories depending on their structural relation to, and functional interaction with, EGF. Type α (TGF-α) or type-I (TGF I) or EGF-like TGF (eTGF) displays a certain degree of structural homology to EGF and compete with the latter for binding to cellular EGF receptors. SGF, a series of tumor-derived growth factors and EGF itself are α-type TGFs, though SGF was later shown to be composed of both TGF-α and TGF-β (Anzano et al. 1983). On the other hand, TGF-β or Type-II TGF (TGF II) a heterogenous group itself, is structurally unrelated to EGF and displays no measurable binding to EGF receptors. A third type of TGFs, termed TGF-γ or TGF III, has also been described, but it remains insufficiently characterized for the time being (for references see James and Bradshaw 1984).

(1) Transforming growth factor α. Partial N-terminal amino acid sequences of TGF-α derived from media conditioned by virally transformed rat and mouse fibroblasts and human melanoma cells suggested an identity of the rodent peptides and an about 88% homology of the latters to their human counterpart (Marquardt et al. 1983, 1984, Derynck 1986). In addi-

tion, these N-terminal sequences proved to be structurally related to the corresponding stretches of human and rat EGFs. The highly conserved nature of TGF-α originating from various cells of different species indicates the phylogenetic importance of this protein (Twardzik et al. 1983, Lee et al. 1985a, Tam 1985). The complete amino acid sequence of rat TGF-α (r-TGF), and a cDNA-derived sequence of the human peptide (h-TGFα) have been reported (Derynck et al. 1984, Marquardt et al. 1984) and are shown, together with a partial N-terminal sequence of the mouse TGF-α (mTGF-α) in Fig.8.24.

The sequences of hTGF-α and rTGF-α have been confirmed by chemical synthesis and various properties of the synthetic products proved to be indistinguishable from those of the natural proteins (Tam et al. 1984, 1986). Mature TGF is a single chain polypeptide with 50 residues and a calculated M_r of about 5,600. The three disulfide bridges present within the rTGF-α molecule link the cysteine residues at positions 8 and 22, 16 and 32, 34 and 43, respectively (Marquardt et al. 1984). Intact disulfide bonds are required for biological activity. The human TGF-α gene has been mapped to chromosome 2 close to the breakage point in Burkitt's lymphoma (Brissenden et al. 1985, Murray et al. 1986, Tricoli et al. 1986) suggesting a similar activation mechanism in certain neoplasias. The 50 amino acid TGF-α was demonstrated to be translated as a part of a 160 amino acid precursor whose structural analysis suggested its transmembrane protein character, and that TGF-α

```
                                                  10                                  20
h: Val-Val-Ser-His-Phe-Asn-Asp-Cys-Pro-Asp-Ser-His-Thr-Gln-Phe-Cys-Phe-His-Gly-Thr-
r: -   -   -   -   -   -   -   Lys -   -   -   -   -   -   -   Tyr -   -   -   -   -
m: -   -   -   -   -   -   -   Lys -   -   -   -   -   -   -   Tyr -   -   -   -   -
                                                  30                                  40
h: Cys-Arg-Phe-Leu-Val-Gln-Glu-Asp-Lys-Pro-Ala-Cys-Val-Cys-His-Ser-Gly-Tyr-Val-Gly-
r: -   -   -   -   -   -   -   -   Glu -   -   -   -   -   -   -   -   -   -   -   -
m: Xxx -   -   -   - (Gln) - (Glu) - Xxx - Xxx - Xxx - Xxx(Gly) -   -   Xxx-
                                                  50
h: Ala-Arg-Cys-Glu-His-Ala-Asp-Leu-Leu-Ala
r: Val -   -   -   -   -   -   -   -   -
m: Xxx-Xxx-Xxx-Xxx-Xxx-Xxx-Xxx-Xxx-Xxx-Xxx
```

Fig. 8.24. cDNA-derived amino acid sequence of human (h) transforming growth factor α, chemically determined amino acid sequence of rat (r) TGFα and a partial amino acid sequence of the mouse (m) protein. Dashes indicate residues that are identical to those in the human sequence. Tentative residues are in parentheses. X triplets indicate unidentified residues

is secreted from the cells by a proteolytic cleavage of the precursor at the cell membrane (Derynck 1986). hEGF/urogastrone is known to be functionally and structurally related to mEGF, 70% of their amino acids being in identical positions. On the other hand, these two peptides show 33% (mEGF) and 44% (hEGF) homology to rTGF-α.

Other structurally related molecules containing EGF-like and TGF-α-like sequences are the EGF precursor (Gray et al. 1983, Scott et al. 1983b), the vaccinia virus growth factor (VVGF; Stroobant et al. 1985) the low density lipoprotein (LDL)-receptor (Yamamoto et al. 1984) and several serine proteases (Patthy 1985). A number of larger proteins with TGF-α activity has also been detected in media conditioned by transformed cells (Derynck 1986). These larger proteins appear to be encoded by the same gene and are presumably derived from the same precursor (Derynck et al. 1984). Variations in the proteolytic processing may explain the different sizes of TGFs-α. A TGF-α-like factor derived from human mammary adenocarcinomas was termed mammary tumor factor (MTF; Zwiebel et al. 1982). Of its several activities, the NRK cell colony-stimulating and EGF-competing activities were copurified when MTF was prepared from the tumor itself, but the two activities were dissociated into a M_r 6,000 NRK colony stimulating activity and a M_r 60,000-70,000 EGF-competing activity, in media conditioned by the same tumor cells (for more information see Salomon et al. 1984). It should be mentioned, however, that TGF-α-like factors with M_rs lower than that of TGF-α have also been described (Kim et al. 1985).

TGF-α is produced by different malignant cells but not by normal adult cells (Ozane et al. 1980, Roberts et al. 1980, Todaro et al. 1980; see, however, Soondar et al. 1986). TGF-α probably is an embryonic form of EGF, which is inappropriately expressed in various malignancies.

As to its biological activities, TGF-α stimulates tyrosine-specific phosphorylation of the EGF receptor, the kinase activity of some membrane proteins (Pike et al. 1982), as well as DNA synthesis and cell multiplications in serum-deprived fibroblasts (Marquardt et al. 1984). TGF-α

alone only weakly stimulates soft-agar colony formation in serum-containing medium (Anzano et al. 1983) eliciting the formation of small colonies only. However, in a cooperation with TGF-β it elicits an enhanced colony formation (Roberts et al. 1981). The quantitative TGF requirement of cells for soft-agar growth depends on the assay system employed: some cells only require TGF-α for their growth in soft agar in the presence of serum, whereas others exhibit an additional EGF or TGF-β requirement. TGF-α is a powerful angiogenic agent (Schreiber et al. 1986), a potent inhibitor of gastric acid secretion (Rhodes et al. 1986) and it causes precocious eyelid opening and tooth eruption in newborn mice (Smith et al. 1985, Tam 1985) features that TGF-α shares with EGF. TGF-α may also play a role in tumor formation via an autocrine mechanism, whereby it helps sustaining the transformed character of the same cell population from which it is secreted (Ozane et al. 1980, Sporn and Todaro 1980, Sherwin et al. 1983, Twardzik et al. 1983, Rosenthal et al. 1986). TGF-α may also function as a membrane receptor (Todaro et al. 1985). Apart from the mature protein, the TGF-α precursor also has biological activities (Ignotz et al. 1986; for further information on TGF-α see Derynck et al. 1985a, Massague 1985a, Nestor et al. 1985, Todaro et al. 1985, Derynck 1986, Keski-Oja et al. 1987).

(2) β-type transforming growth factors. TGF-β exists in multimolecular forms (Massague et al. 1987). Of these, TGF-β1 and TGF-β2 have been structurally characterized (Fig.8.25). The cDNA-derived primary structures of human (Derynck et al. 1985b), murine (Derynck et al. 1986), porcine (Derynck and Rhee 1987) and simian TGFs-β1 (Sharples et al. 1987) are all consisted of 112 amino acids and show extensive sequence homologies between each other: the human and porcine peptides are identical while the rodent peptides differ in Ser(75) from the former two. The biologically active form of TGF-β1 is a disulfide-linked homodimer (M_r 25,000) consisting of two 112 amino acid monomers with a M_r of 12,500 each. The chemically determined amino acid sequence of human TGF-β2 derived from a pros-

```
                                    10                                  20
1 Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser-Ser-Thr-Glu-Lys-Asn-Cys-Cys-Val-Arg-Gln-Leu-
2 -   -   -   Ala-Ala -   -   -   Arg-Asn-Val-Gln -   -   -   -   Leu -   Arg -
                                    30                                  40
1 Tyr-Ile-Asp-Phe-Arg-Lys-Arg-Leu-Gly-Trp-Lys-Trp-Ile-His-Glu-Pro-Lys-Gly-Thr-His-
2 -   -   -   -   Lys-Arg -   -   -   -   -   -   -   -   -   -   -   -   -   Asn
                                    50                                  60
1 Ala-Asn-Phe-Cys-Leu-Gly-Pro-Cys-Pro-Tyr-Ile-Trp-Ser-Leu-Asp-Thr-Gln-Tyr-Ser-Lys-
2 -   -   -   -   Ala -   Ala -   -   -   Leu -   -   Ser -   -   -   His -   Arg
                                    70                                  80
1 Val-Leu-Ala-Leu-Tyr-Asn-Gln-His-Asn-Pro-Gly-Ala-Ser-Ala-Ala-Pro-Cys-Cys-Val-Pro-
2 -   -   Ser -   -   -   Ile-Ile -   -   Glu -   -   -   Ser -   -   -   -   Ser
                                    90                                  100
1 Gln-Ala-Leu-Glu-Pro-Leu-Pro-Ile-Val-Tyr-Tyr-Val-Gly-Arg-Lys-Pro-Lys-Val-Glu-Gln-
2 -   Asp -   -   Thr -   -   -   Leu -   -   Ile -   Lys-Thr -   -   Ile -   -
                                    110 112
1 Leu-Ser-Asn-Met-Ile-Val-Arg-Ser-Cys-Lys-Cys-Ser
2 -   -   -   -   -   -   -   Lys -   -   -   -   -
```

Fig. 8.25. cDNA-derived amino acid sequences of the monomeric forms of human transforming growth factor β1 (1) and β2 (2). Dashes indicate identities

tatic adenocarcinoma cell clone is shown in Fig.8.25 (Marquardt et al. 1987). TGF-β1 and TGF-β2 are functionally and structurally related molecules displaying an about 71% sequence homology. Bovine and porcine TGFs-β2 are structurally identical with their human counterpart. Although human TGF-β2 consists of two disulfide-linked, identical subunits (homodimer) a heterodimeric form with a TGF-β1.2. construction has also been isolated from porcine platelets (for references see Marquardt et al. 1987). Human TGF-β1 is also homologous to a remarkable extent to the β-chains (β_A and β_B) of porcine inhibin, the human Mullerian inhibitor substance (MIS) and to the decapentaplegic gene complex protein (DPP-c) of D r o s o p h i l a, while bovine cartilage-inducing factor-A (CIF-A), and the differentiation inhibitor of Buffalo rat liver (BRL)-cells are closely related to human TGF-β2 (for references see Ikeda et al. 1987, Marquardt et al. 1987).

These molecules appear to form a group of growth and differentiation regulators with multiple functions, and the high degree of the structural conservation of TGFs-β suggests a phylogenetically important role(s) for these peptides.

TGFs-β are widely distributed in different tissues and have been purified from placentas, kidneys, platelets and from cultured cells, both normal and neoplastic (for references see Keski-Oja et al. 1987). There are indications that TGFs-β released from cells are in an inactive (latent) form that are

subsequently activated by presently unknown mechanisms (Lawrence et al. 1984, Pircher et al. 1984, Moses et al. 1985a, Huang et al. 1988). Platelets have been the most common source for the purification of TGF-β1 due to their high TGF-β1 content (Assoian et al. 1983). The human and murine TGF-β1 genes have been localized to chromosomes 19 and 7, respectively (Fujii et al. 1986). Dissimilarly to TGF α acting through EGF receptors TGFs-β have their own specific membrane receptors. TGF-β1 receptors are highly conserved and are different both from the EGF or the PDGF receptors functioning as tyrosine-specific kinases (for more details see Keski-Oja et al. 1987 and references cited therein).

TGFs-β have a multifaceted role in cell regulation. Some of their biological activities are associated with growth regulation and only these will be surveyed here (for other activities of TGFs-β see the reviews listed at the end of this paragraph and the references cited therein). Most of our present knowledge on the activities of TGFs-β concerns TGF-β1. Therefore, whenever the term TGF-β is used in the following discussion, it should be interpreted as TGF-β1.

As to the growth-associated biological activities of TGF-β, it inhibits the growth of most normal, especially epithelial cells, and that of several transformed cell types (Moses et al. 1985b, Roberts et al. 1985, Shipley et al. 1985). On the other hand, TGF-β acts as a mitogen for mesenchymal cells, (Moses et al. 1981, Roberts et al. 1981, 1985, Leof et al. 1986), and stimulates the anchorage-independent growth of normal fibroblastic cells (Moses et al. 1981, Roberts et al. 1981). It enhances wound healing (Sporn et al. 1983).

Evidence has been presented (Tucker et al. 1984) showing that TGF-β and the growth inhibitor (GI) isolated by Holley and coworkers (1978, 1980,1983) from media conditioned by BSC-1 cells, a monkey kidney cell line, are similar molecules (see later) and that TGF-β also shares common properties with a tumor inhibitory factor, termed TIF-1. Both GI and TGF-β stimulate the anchorage-independent growth of AKR-2B cells, a fibroblastic cell line and inhibit DNA synthesis in BSC-1 and CCL-64 epithelial mink lung cells. It is a general experience

that the epithelial cells or carcinoma cell lines are either inhibited by, or show no response to, TGF-β under usual culture conditions. TGF-β is also a potent growth inhibitor for the secondary cultures of human foreskin keratinocytes, the primary cultures of human megakaryocytic and erythroid progenitor cells and it also inhibits the EGF-induced stimulation of DNA synthesis in primary cultures of rat hepatocytes and the FGF-stimulated proliferation of endothelial cells. The mechanisms by which TGF-β inhibits cell proliferation remains largely unknown for the time being. It is, however, highly possible that TGF-β primarily is an inhibitor for all cell types (hence the designation TGF-β/GI, where GI indicates growth inhibitor) and its mitogenic activity on fibroblastic cells is fortuitous through inducing the proto-oncogene c-sis and the autocrine activity of the PDG-like sis-protein, which is the direct mitogen (for references see Keski-Oja et al. 1987).

TGF-β stimulates DNA synthesis in quiescent monolayer cultures of fibroblastic AKR-2B cells without the presence of other growth factors in a completely defined medium, however, the stimulation has a delayed kinetics relative to the stimulations by other growth factors such as EGF, insulin, FGF, PDGF or serum. Stimulation with the latter growth factors results in a 12-14 hour lag phase before the onset of DNA synthesis which then peaks at 20-25 hours following stimulation. In contrast to this, TGF-β stimulation results in a 24 hour lag phase with a peak DNA synthesis between 30 and 35 hours. In subsequent investigations, the reason for this delayed kinetics was found in the indirect nature of TGF-β mitogenicity. Namely, when quiescent AKR-2B cells are stimulated by TGF-β, an early expression of c-sis proto-oncogene occurs with a consecutive increase of a PDGF-like protein (sis protein) in the medium. In addition, PDGF-regulated proto-oncogenes such as c-fos and c-myc are also stimulated by TGF-β with delayed kinetics relative to that seen with direct PDGF stimulation. The results suggest that mitogenicity of TGF-β for adherent cells is mediated by the induction of c-sis (PDGF) with a subsequent autocrine stimulation of c-fos and c-myc, other PDGF inducible genes, and of DNA synthesis (for references see Keski-Oja et al. 1987).

Recently it has been demonstrated that fibronectin plays a role in the anchorage-independent growth of NRK cells (Ignotz and Massague 1986): both fibronectin and procollagen production are enhanced by TGF-β, and fibronectin enhances soft agar growth of the indicator cells. Inhibition of fibronectin binding to its cell surface receptors results in an inhibition of the colony-forming effect of TGF-β, suggesting that the formation of pericellular matrix structures may be important for TGF-β actions (for references see Keski-Oja et al. 1987).

TGF-β induces a marked desmoplastic reaction manifested in an induction of angiogenesis and connective tissue formation, when injected in mice (Roberts et al.1986). Since TGF-β is abundantly present in platelets, its role in wound healing has been suggested (Sporn et al. 1983, Assoian and Sporn 1986).

Other activities of TGF-β not strictly associated with growth regulation include its effects on differentiation, fibroblast chemotaxis, immunomodulation, plasminogen activation and its potential role in neoplasia and other disease states (for recent reviews on TGF-β see Keski-Oja et al. 1987, Massague 1987, Massague et al. 1987, Moses et al. 1987, Sporn et al. 1987).

Biological significance of transforming growth factors

It is now becoming increasingly clear that polypeptide hormones and hormone-like growth factors play an important role in the control of growth of normal cells. A whole array of new polypeptides possessing growth-promoting activities has been recently recognized and characterized to varying extent (for reviews see Gospodarowicz and Moran 1976, Baserga 1981, Heldin and Westermark 1984, James and Bradshaw 1984). However, the growth of malignant cells is controlled differently from that of normal cells. This is manifested, for instance, in an often spectacular reduction in the amount of exogenous growth factors that are required for optimal growth of malignant cells compared to the similar needs of their normal counterparts. This is why it was suggested that the escape from normal growth control of transformed cells might somehow be causally

related to their reduced requirement for hormonal growth factors (Holley 1975). This was, in fact, demonstrated to be the case by Temin (1967) and Holley and Kiernan (1968). In addition, TGFs should be produced by the transformed cells themselves (Todaro and DeLarco 1978), and the transformed cells should have their own functional cellular receptors for these polypeptides, allowing phenotypic expression of the peptides by the same cells that produce them. Sporn and Todaro (1980) proposed the term autocrine secretion to describe this phenomenon of self-stimulation, whereby a cell secretes a substance for which the cell itself has functional external receptors. Such an endogenous production of growth-promoting polypeptides, associated with the autocrine mode of cellular response, provides a perpetuating stimulus for continued cell division, thereby allowing the peptide-producing cells to escape from the physiological control mechanism operating through hormone-like growth factors that are provided exogenously for normal cells. This concept of autocrine secretion provides a simple hypothetical model for interpreting certain aspects of malignant transformation, and also a utilizable working hypothesis for future research in this field. In the light of recent data suggesting an intricate relationship between "physiological" growth factors (e.g., PDGF) or their receptors (e.g., EGF) and the products of some cellular oncogenes (e.g., c-sis or c-erb B), the relationship between TGFs and the products of cellular oncogenes also awaits elucidation.

Since β-type TGFs are present also in normal cells and tissues, it is plausible to attribute some physiological role to them. These roles may include participation in various repair phenomena in response to tissue injury (Sporn et al. 1983) or in earlier evolutionary or embryogenetic development (Sporn and Todaro 1980, Twardzik et al. 1982), but these all have yet to be confirmed. TGFs may also be involved in the pathogenesis of proliferative diseases other than cancer, e.g., atherosclerosis, diseases of the connective tissue (Sporn and Harris 1981), and also in wound healing. The latter effect was substantiated by in vivo experiments (Sporn et al. 1983). Little

is known of the major site(s) of synthesis and storage of TGFs, but platelets containing up to 100 fold more TGF-β than other nonneoplastic tissues examined so far appear to be a major storage site of TGF-β (Assoian et al. 1983). The simultaneous occurrence of PDGF and TGF-β in platelets, and the fact that PDGF behaves as a competence factor while TGF-β is a poor mitogen suggest that TGF-β might play a unique role in those physiological and pathological processes where platelet-derived factors modulate cell proliferation.

In a recent publication (Assoian et al. 1984) evidence was presented indicating that a major effect of TGF-β is to control EGF receptor levels in responsive cells. This finding suggests that delayed biological effects of TGF-β resembling those of EGF may be indirect consequences of its ability to regulate EGF receptors and thereby amplify EGF-induced responses.

More recently, two types of leukemia-derived growth factor (LGF), LGF-I and LGF-II have been isolated from K-562 T1 cells, a human erythro-leukemia cell line (Mihara et al. 1987). LGF-I stimulates the proliferation of a wide range of human leukemia cell lines, whereas LGF-II contributes to the growth of fibroblasts. LGF-I was purified to homogeneity and partially sequenced: its 30 N-terminal residues are identical with those of ubiquitin (see Chapter 7). Available data indicate that LGF-I (M_r 20,000) is a new autocrine growth factor containing ubiquitin at the N-terminal end. (Those who are interested in more details and references on TGFs are referred to read the following papers and reviews: Assoian et al. 1985, Knowles et al. 1985, Lawrence 1985, Massague 1985a,b, Tam 1985, Sporn et al. 1986, Keski-Oja et al. 1987).

8.2. GROWTH INHIBITORY PEPTIDES

In sharp contrast to the ever increasing number of chemically defined endogenous growth stimulators, only a modest number of endogenous growth inhibitory substances have been structurally characterized to date. While looking for explana-

tions, the uncertainties inherently associated with the assay systems used for monitoring purification of growth inhibitors should be particularly stressed. Whilst it is relatively easy to design assay systems that reliably detect growth stimulation, it often proves to be extraordinarily difficult to find adequate test systems capable of distinguishing between a true inhibitory and an aspecific, e.g., cytotoxic effect of candidate growth inhibitors. Consequently, as was often the case in the past, an observed effect attributed to an inhibitor proved to be, upon a more precise analysis, the result of a cytotoxic effect. Nevertheless, owing to the introduction of the modern techniques of separation, molecular cloning and gene technology a remarkable progress is being witnessed which might ultimately result in the long expected breakthrough in this field of research (for past and more recent results concerning endogenous inhibitors of cell proliferation see the reviews by Lozzio et al. 1975, Marx 1986, Wang and Hsu 1986, Keski-Oja and Moses 1987).

8.2.1. INTERFERONS

Since the late 1950s when Isaacs and coworkers discovered interferon (IFN) a potent antiviral agent of endogenous origin (Isaacs and Lindenmann 1957, Isaacs et al. 1957), much has been learned of the structural and biological characteristics of these molecules, especially during the last few years. It became established knowledge that even within one species, IFN is produced in multiple molecular forms with distinct biological, chemical and physical properties and also with distinct biological activities. It has also been established that, although viruses are differentially susceptible to various IFN species, IFNs are generally not virus-specific. The original conception that the activity of IFNs is highly species-specific has also been challenged over the past few years. Until the late 1960s collection of reliable data on the composition, structure, as well as on the intrinsic biological activities of IFNs were seriously hampered by the lack of availability of sufficiently pure IFN preparations of adequate

quantity. However, recent progress in the purification of IFNs, as well as the successful application of the techniques of DNA recombination as a tool of IFN breeding, have finally provided sufficient material for chemical analysis and for biological and physical characterization.

Interferons (IFNs) are generally defined as nonspecific endogenous antiviral agents. In the old terminology still in use, IFNs are categorized according to their cells of origin. Since, however, different cells (e.g., leukocytes and fibroblasts) can produce the same IFN, and a given cell type can produce several types of IFN, the old terminology does not allow us to satisfactorily identify the type of IFN produced. Thus, for the sake of clarity, the introduction of a new terminology seemed to be highly desirable. The new and old terminology is comparatively shown in Table 8.2. The new terminology applies, first of all, to human and murine IFNs, since available information on IFNs from other species is less abundant than that on IFNs from these two species. However, some evidence indicates that the α, β and γ classes do not encompass all the human IFNs (Sehgal 1982, Wilkinsons and Morris 1983, Goeddel 1984).

As to the cellular origin of IFNs, α-type IFN is predominantly produced by buffy coat white cells but also by leukemic cells when exposed to viruses (e.g., Newcastle virus, Sendai virus etc.). IFN-β is predominantly generated when selected

Table 8.2. New and old terminology of human and murine interferons

new	old		type
	human	murine	
IFN-α	Leukocyte (Le)	fast (F), C	I.
IFN-β	Fibroblast (F)	slow (S), A, B	
IFN-γ	Immune (FII)	--------	II.

cultures of fibroblasts are exposed to synthetic double stranded RNA, such as poly(I) and poly(C). Finally, IFN-γ is generally produced in T-lymphocytes upon antigen or mitogen stimulation. Lymphoblastoid IFN is a particular type of IFN-γ which is produced by virus inoculation into the continuous cultures of lymphoblastoid cells, usually the Namalva cell-line or Burkitt lymphoma cells. This IFN species is heterogenous in composition consisting predominantly of IFN-β but IFN-α often comprises 5-10% of the total. The presented new terminology, however, should not overshadow the fact that there are subtypes of IFNs which differ in size, amino acid sequence, charge, biological activity and also in other characteristics.

Due to the intensive efforts in several laboratories in recent years, the primary structures of many IFN species have been elucidated, either directly using conventional methods of protein structure identification, or indirectly by deducing amino acid sequences from nucleotide sequences of cloned cDNAs. In a number of cases, the expression of cloned IFN cDNAs has also been accomplished by using appropriate expression vector systems.

Chemistry of human interferons

(1) α-type interferon. Partial and complete amino acid sequences have been obtained from several purified natural human IFNs-α (hIFN-α) and additional sequences have been determined through recombinant DNA technology. Sequence analysis of these molecules has clearly demonstrated that hIFN-α exists in several chemically distinct yet closely related forms. The established multiplicity of the genomic sequences predicted a multiplicity of the genes coding for hIFNs-α, that were assigned to chromosome 9 within the human genome (Owerbach et al. 1981). Dissimilarly to all other genes in higher eukaryotes, except histone genes, the genes encoding hIFNs-α are not interrupted by intervening sequences. The consensus sequence of hIFNs-α deduced from DNA sequences of cloned cDNAs or genomic fragments is shown in Fig.8.26 (for details see Langer and Pestka 1985).

```
                                                  10                  20
Cys-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Asn-Arg-Arg-Ala-Leu-Ile-Leu-Leu-Ala-Gln-
                                                  30                  40
Met-Gly-Arg-Ile-Ser-Pro-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-
                                                  50                  60
Glu-Glu-Phe-Asp-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Gln-Ala-Ile-Ser-Val-Leu-His-Glu-Met-
                                                  70                  80
Ile-Gln-Gln-Thr-Phe-Asn-Leu-Phe-Ser-Thr-Lys-Asp-Ser-Ser-Ala-Ala-Trp-Asp-Glu-Thr-
                                                  90                  100
Leu-Leu-Glu-Lys-Phe-Ser-Thr-Glu-Leu-Tyr-Gln-Gln-Leu-Asn-Asp-Leu-Glu-Ala-Cys-Val-
                                                 110                 120
Ile-Gln-Glu-Val-Gly-Val-Glu-Glu-Thr-Pro-Leu-Met-Asn-Glu-Asp-Ser-Ile-Leu-Ala-Val-
                                                 130                 140
Arg-Lys-Tyr-Phe-Gln-Arg-Ile-Thr-Leu-Tyr-Leu-Thr-Glu-Lys-Lys-Tyr-Ser-Pro-Cys-Ala-
                                                 150                 160
Trp-Glu-Val-Val-Arg-Ala-Glu-Ile-Met-Arg-Ser-Phe-Ser-Leu-Ser-Thr-Asn-Leu-Gln-Lys-
                                                166
Arg-Leu-Arg-Arg-Lys-Glu
```

Fig. 8.26. Consensus sequence of human α-type interferon deduced from nucleotide sequences of cloned cDNAs or genomic fragments

Most hIFNs-α consist of 166 amino acids, although species with only 165 amino acids have also been detected. It is of interest that a number of natural hIFNs-α have only 155 residues, lacking the 10 C-terminal amino acids. This truncation does not seem to have any functional consequences indicating that the C-terminal amino acids, though highly conserved, are not required for biological activity. Of the two disulfide bonds present in hIFN-α species only one is necessary for biological activity. Although there are natural hIFN-α species that are not glycosylated, O-glycosylated and N-glycosylated forms have also been detected. Under nonreducing conditions, estimates of the M_rs of various hIFN-α species generally range from 17,500 to 23,000 with one or two species having an M_r above 26,500. These differences in the molecular weights are due partly to different primary structures and partly to differences in the degree of glycosylation (for more details on human and nonhuman IFNs-α see Goeddel 1984, Langer and Pestka 1984, 1985, Pestka et al. 1987, Spiegel 1987 and references cited therein).

(2) β-type interferon. In contrast to the large number of hIFN-α species and genes, the number of hIFN-β molecules and genes seems to be much less. Until to date, two molecular forms of IFN-β, termed IFN-β1 and IFN-β2 have been described.

```
                                   10                                  20
Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Gln-Gln-Arg-Ser-Ser-Asn-Phe-Gln-Cys-Gln-Lys-Leu-
                                   30                                  40
Leu-Trp-Gln-Leu-Asn-Gly-Arg-Leu-Glu-Tyr-Cys-Leu-Lys-Asp-Arg-Met-Asn-Phe-Asp-Ile-
                                   50                                  60
Pro-Glu-Glu-Met-Lys-Gln-Leu-Gln-Gln-Phe-Gln-Lys-Glu-Asp-Ala-Ala-Leu-Thr-Ile-Tyr-
                                   70                                  80
Glu-Met-Leu-Gln-Asn-Ile-Phe-Ala-Ile-Phe-Arg-Gln-Asp-Ser-Ser-Ser-Thr-Gly-Trp-Asn-
                                   90                                 100
Glu-Thr-Ile-Val-Glu-Asn-Leu-Leu-Ala-Asn-Val-Tyr-His-Gln-Ile-Asn-His-Leu-Lys-Thr-
                                  110                                 120
Val-Leu-Glu-Glu-Lys-Leu-Glu-Lys-Glu-Asp-Phe-Thr-Arg-Gly-Lys-Leu-Met-Ser-Ser-Leu-
                                  130                                 140
His-Leu-Lys-Arg-Tyr-Tyr-Gly-Arg-Ile-Leu-His-Tyr-Leu-Lys-Ala-Lys-Glu-Tyr-Ser-His-
                                  150                                 160
Cys-Ala-Trp-Thr-Ile-Val-Arg-Val-Glu-Ile-Leu-Arg-Asn-Phe-Tyr-Phe-Ile-Asn-Arg-Leu-
                                  166
Thr-Gly-Tyr-Leu-Arg-Asn
```

Fig. 8.27. cDNA-derived amino acid sequence of human β-type interferon. Underlined amino acids represent a possible glycosylation site

Of these, hIFN-β2, also termed 26 kDa protein, a fibroblast-derived protein with both interferon and B-cell growth stimulatory activities, was shown to be identical to B-cell stimulatory factor-2 (BSF-2), a 184 amino acid protein characterized by Hirano and associates (1986), and to hybridoma growth factor (HGF) described by Van Damme and coworkers (1987; BSF-2 is discussed in Chapter 7 as an immunopeptide). hIFN-β1, the other known β-type interferon is a product of stimulated fibroblasts. Based on cDNA-derived amino acid sequence data, hIFN-β1 has been identified as a 166 amino acid protein (Fig.8.27) with an estimated M_r of about 20,000. Similarly to the hIFN-α gene, the hIFN-β1 gene has been assigned to chromosome 9 (Owerbach et al. 1981) whereas the hIFN-β_2 gene to chromosome 7 (Sehgal et al. 1986) within the human genome. Of the three cysteine residues present within the hIFN-β1 molecule, two are used for disulfide bond formation that is required for biological activity. hIFN-β1 is a glycoprotein in which the carbohydrate moiety is N-linked to the asparagine residues at position 80. The hIFN-β1 gene bears 40-50% homology with the IFN-α genes, but the homology is somewhat less at the amino acid level. There are no intervening sequences within the hIFN-β1 gene, a feature shared with the genes coding for hIFNs-α. The functional form of hIFN-β1 may be a dimer (Pestka et al. 1983). For additional structural

information on human and nonhuman IFNs-β1 see Langer and Pestka (1985) and Pestka et al. (1987).

(3) γ-type interferon. The main, and probably the only producer cells of hIFN-γ are stimulated T-lymphocytes, although B-cells were also suggested as producer cells (Taylor et al. 1984). Independently of the separation schemes used for purifying natural hIFN-γ they have unexceptionally yielded two active species with a M_r of 17,000-20,000 and 25,000, respectively. Amino acid sequences of these natural hIFNs-γ have been determined by Rinderknecht and associates (1984) and found to be identical irrespective of their molecular weights indicating that the observed differences in their M_r values cannot be ascribed to different primary structures of these molecules. From analysis of cloned cDNAs, a 146 amino acid mature protein could be predicted whose primary structure is shown in Fig.8.28. All hitherto analysed natural forms of hIFN-γ lack the first three N-terminal residues (Cys-Tyr-Cys) present in the cDNA-derived sequence and have blocked N-termini (pyroglutamate) corresponding to the glutamine residue at position 4 in the predicted sequence. The mechanism through which the natural proteins lose the three amino acids is unclear at present. Furthermore, the C-terminal ends of the natural proteins are heterogenous: the longest natural form hitherto recognized has 138 amino acids and a glycine residue corresponding to that at position 141 in the predicted sequence, as its C-terminus but forms with Gly(121), Lys(122), Arg(123) and Arg(125) have also been detected (Pan et al. 1987). hIFN-γ is a glycoprotein. The M_r 17,000-20,000 species are N-glycosylated only at Asn(28), whereas the M_r 25,000 species are glycosylated at both Asn(28) and Asn(100). Apart from a C-terminal heterogeneity, this differential glycosylation may also account for the molecular weight heterogeneity of hIFNs-γ. It is presently uncertain whether all C-terminally heterogenous forms of hIFN-γ are biologically active, as is the origin of the C-terminal heterogeneity. Available data indicate that the natural protein is present in an oligomeric form in vivo, most likely as a trimer or a tetramer. In con-

```
                                   10                                  20
Cys-Tyr-Cys-Gln-Asp-Pro-Tyr-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr-Phe-Asn-Ala-
                                   30                                  40
Gly-His-Ser-Asp-Val-Ala-Asp-Asn-Gly-Thr-Leu-Phe-Leu-Gly-Ile-Leu-Lys-Asn-Trp-Lys-
                                   50                                  60
Glu-Glu-Ser-Asp-Arg-Lys-Ile-Met-Gln-Ser-Gln-Ile-Val-Asp-Phe-Tyr-Phe-Lys-Leu-Phe-
                                   70                                  80
Lys-Asn-Phe-Lys-Asp-Asp-Gln-Ser-Ile-Gln-Lys-Ser-Val-Glu-Thr-Ile-Lys-Glu-Asp-Met-
                                   90                                 100
Asn-Val-Lys-Phe-Phe-Asn-Ser-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Glu-Lys-Leu-Thr-Asn-
                                  110                                 120
Tyr-Ser-Val-Thr-Asp-Leu-Asn-Val-Gln-Arg-Lys-Ala-Ile-His-Glu-Leu-Ile-Gln-Val-Met-
                                  130                                 140
Ala-Glu-Leu-Ser-Pro-Ala-Ala-Lys-Thr-Gly-Lys-Arg-Lys-Arg-Ser-Gln-Met-Leu-Phe-Arg-
                                  146
Gly-Arg-Arg-Ala-Ser-Gln
```

Fig. 8.28. cDNA-derived amino acid sequence of human interferon γ. Primary structure of the natural protein differs from the deduced structure partly in its N-terminus (pyroglutamate) whose position corresponds to the glutamine residue at position 4 in the deduced sequence, partly in the heterogeneity of its C-terminal end (see text). Underlined residues represent possible glycoslation sites

trast to the genes of hIFNs-α and hIFNs-β, that have no intervening sequences, the hIFN-γ gene assigned to chromosome 12 within the genome contains three introns (for additional information on the structure of hIFN-γ see the reviews by Langer and Pestka 1985, Pestka et al. 1987).

(4) δ-type interferon. In the light of recent findings one gets the impression that the list of IFN subtypes may not be exhausted with the IFNs hitherto described. Wilkinson and Morris (1983) reported a novel IFN species tentatively called IFN-δ which was produced when human peripheral leukocytes were treated with PHA and a tumor promoter. This novel IFN, while possessing all the important characteristics of a classical IFN, differs decisively from previously identified IFN types in its antigenicity and biological and physicochemical properties. The most conspicuous biological property of IFN-δ is that, of all cell types hitherto tested, it exhibits antiviral activity only in trisomy-21 human fibroblasts, a property not shared by any other IFN species analysed so far. No structural data are presently available on this unique IFN species.

(5) Hybrid interferons. Theoretically at least, the number of HuIFNs can be increased artificially without limit by fusing different IFN genes, or parts of them, by gene technology, thereby producing hybrid IFNs. This was already tested in practice when hybrids of HuIFN-α_1 and HuIFN-α_2 were produced by gene technology using plasmids containing fused cDNAs of the two IFN species. Under these conditions, the expression in E. c o l i of fused cDNAs yields the corresponding fused IFNs. Experience gained so far clearly shows that the biological activities of fused IFNs display remarkable differences as compared to the parent strains. This kind of genetic manipulation may prove to be a powerful tool by the aid of which modification of the activity spectrum and other properties of IFNs, and thus a construction of IFN subtypes superior to naturally occurring IFN species can be artificially achieved (for more details see Pestka 1983, Kirchner 1984a, Wagner 1984a).

Biological properties and clinical significance of interferons

Initially, when only crude or poorly purified IFN preparations were available, it was not certain whether the observed effects were due to interferons or to some contaminants in the crude concentrates. A comprehensive list of biological effects observed with differently purified IFN preparations was presented in Taylor-Papadimitrou's review (1980), showing that partially purified IFN preparations markedly influence a multitude of cellular functions, either by inhibition or by stimulation. In subsequent studies, most of these activities could be elicited with highly purified IFN preparations indicating that they represent genuine IFN activities. In the present discussion, however, where IFNs are treated as negative growth regulators we should concentrate on the antiproliferative capacity of IFNs. (Those who are interested in other activities of IFNs should consult the reviews by Taylor-Papadimitrou 1980, Pestka 1983, Wagner 1984b,c, Jacobsen 1986, Kirchner 1986.)

IFNs reversibly inhibit cell proliferation both in vivo and in vitro (Gresser et al. 1970, Balkwill et al. 1978) and this antiproliferative effect seems to be in direct proportion to the antiviral effect, at least for hIFN-α. Tumor cells are inhibited by all three IFN types in vitro, however, IFN-γ has a more marked effect than the other two. There is presently no indication that in vitro proliferation of tumor cells is more markedly inhibited by IFNs than are normal cells. The antitumor activity of IFNs seems to be independent of their antiviral effect, even in animal cancers whose viral etiology has been established. It should be noted, however, that antiproliferative activity varies according to the cell-lines tested: for example, IFN species that inhibit lymphoblastoid cell growth may be hardly active, or not at all, in blocking the growth of human astrocytoma cells. The existence of at least two distinct types of IFN receptors that respond differentially to each IFN species could account for differences in the molecular antiproliferative to antiviral activity ratios. It is conceivable, in fact, that a whole family of IFN receptors may exist, an assumption that is strongly supported by direct and competitive binding studies (for more detail on IFN receptors see Yonehara 1984, Borecky 1986).

Although the doses of IFNs required for a marked inhibition of proliferation are generally higher than those required for their antiviral effects, they are in a compatible range with, or even less than that of the known positive growth factors, e.g., EGF or FGF. However, IFNs can be cytotoxic if applied in high concentrations for a long period to cells in sparse culture.

The natural stimuli eliciting IFN production in animal organisms are foreign antigens, including viruses. The latters represent a strong stimulus for the production of IFN-α in several types of cells. IFNs-α generally are more effective inhibitors of viral replication than of cell proliferation. Even so, the levels produced by some viruses can be sufficiently high to elicit permanent damage to some organs in newborn animals, most likely through affecting cell proliferation and/or other cellular functions (Riviere et al. 1977).

IFNs-γ produced by lymphocytes in response to antigenic stimuli may participate in the immune surveillance mechanism of the host, due to their growth inhibitory effects on cells carrying the antigens, including viral and tumor ones. IFN-induced modulation of the proliferation of stimulated lymphocytes is probably an important means by which immune response is modulated.

Tumor growth inhibition by IFNs may be accomplished directly by inhibiting tumor cell proliferation, or indirectly by enhancing the functions of the immune cells and the expression of surface antigens on such cells. In vitro observations indicate that a direct effect is likely to play at least some role (Gresser and Bourall-Maury 1973, Balkwill et al. 1980, Taylor-Papadimitriou et al. 1980). On the other hand, the observation that the growth of some tumor cells is inhibited by IFN in vivo, but not in vitro, clearly indicates the potential importance of the indirect effect.

As to the value of IFNs as anti-cancer agents, the opinions changed sharply during the past few years. Initially IFNs were heralded as the most potent antitumor agent of the future. This euphoric opinion, however, was later transformed to a more resigning one. The present, more balanced general opinion appears to be more realistic maintaining that, although IFNs can by no means regarded as efficient remedies for tumors in general, they may prove to be useful therapeutic agents against a selected group of tumors such as hairy cell leukemia or non-Hodgkin lymphoma.

It would go beyond the scope of the present survey to list all the clinical trials undertaken so far, many of which included only a few patients. Making use of effective methods presently available for large scale production and purification of various types of IFN species, controlled clinical trials are now under way in many countries, whose results will hopefully lead to the final evaluation of the clinical value of IFN in human tumor therapy.

It should also be mentioned that IFN was applied with relative good results in patients with some benign tumors such as condyloma acuminata, warts, juvenile laryngeal papillomas,

probably because of their viral etiology (for more details on the clinical application of IFNs see Taylor-Papadimitriou 1980, Wagner 1984c, Beaupain et al. 1986, Boreczky 1986, Goldstein and Laszlo 1986, Nakamura et al.1986a, Ruff et al. 1986, Saito et al. 1986, Sherwin 1987).

Inhibition of the growth of normal cells in vivo by exogenously administered IFNs, and the possible role of IFNs in growth regulation of normal cells is an important area of current research (Friedman et al. 1986, Shearer and Taylor-Papadimitriou 1987; for more recent data on the biology of IFNs see Bocci 1985, Carter et al. 1985, Clemens and McNurlan 1985, Dianzani 1985, Heyns et al. 1985, Kushornaryov et al. 1986, Pessina et al. 1986, Pestka et al. 1987).

8.2.2. ANTIPROLIFERATIVE CYTOTOXINS: TUMOR NECROSIS FACTOR AND LYMPHOTOXIN

It has been known for years that stimulated lymphocytes and macrophages of animal and human origin can be activated to release substances that mediate growth inhibitory and lytic effects on mammalian cells in vitro. Materials with these activities were originally termed lymphotoxins (LT) and macrophage cytotoxins (MCT; for references see Granger et al. 1985). It has also been observed that sera from Bacillus Calmette Guerin (BCG)-primed and endotoxin treated animals produce material(s) that induces selective necrosis of various tumors when injected into tumor-bearing mice. The active component(s) in these sera were termed tumor necrosis factor. Available evidence indicates that all these activities are mediated by discrete but related proteins that preferentially affect transformed cells in vitro and induce tumor necrosis in murine model systems in vivo. These results suggested that both lymphocytes and macrophages may have a common mechanism involved in the control and destruction of transformed cells.

(1) Tumor necrosis factor. The first observation indicating that tumor necrosis occurs in human cancer patients challenged with various poorly defined bacterial toxins goes as far back as the turning of this century (for references see Beutler and Cerami 1986b). Subsequently it was established that lipopolysaccharide (LPS), also termed endotoxin or bacterial pyrogen, which is a major component of the cell wall of Gram negative bacteria, was especially active in inducing tumor necrosis. The appearance of a factor in the serum of BCG-treated and endotoxin-challenged mice which elicited hemorrhagic tumor necrosis in recipient animals has been described by Carswell and associates (1975).The active principle was referred to as tumor necrosis factor (TNF; Ruff and Gifford 1981a,b). In subsequent studies, a causal involvement of TNF in endotoxin-induced events was clearly demonstrated when mice proved to be fully protected from the lethal effect of endotoxin following immunization of the animals against TNF (Beutler et al. 1985c). Isolated macrophages were identified as the principal producer cells of this factor. (Hammerstrom 1982, Aggarwal et al. 1985a, Beutler et al. 1985b,d). Sera from primed, endotoxin-treated animals were shown to be capable of selectively lysing some, but by no means all, tumorigenic cells both in vitro and in vivo (Carswell et al. 1975, Helson et al. 1975). Although the exact mechanism underlying this selective cytolytic activity remains largely unknown, (for recent data see Flick and Gifford 1986), interest in TNF has been considerable mainly due to its possible use in anticancer therapy.

Following several years devoted to analysing biological activities of TNF, the human material finally was purified to homogeneity and partially sequenced (Aggarwall et al. 1985c). A full sequence deduced from cloned cDNA was reported by Pennica et al. (1984b) prior to this partial protein sequence. Subsequently, several investigators working independently have reported human-derived sequences that were identical or nearly so (Marmenout et al. 1985, Nedwin et al. 1985b, Shirai et al. 1985, Wang et al. 1985). Simultaneously murine (Fransen et al. 1985, Pennica et al. 1985, Semon et al. 1987) and rabbit

```
                                                  10                                                  20
h: Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala-Asn-Pro-
m: Leu -  -  -  -  Gln-Asn-Ser -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  His-
r:       -  Ala -  -  Ala-Leu -  -  -  -  Leu -  -  -  -  -  -  -  -
                                                  30                                                  40
h: Gln-Ala-Glu-Gly-Gln-Leu-Gln-Trp-Leu-Asn-Arg-Arg-Ala-Asn-Ala-Leu-Leu-Ala-Asn-Gly-
m: -  Val -  Glu -  -  Glu -  -  Ser-Gln -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
r: -  Val -  -  -  -  -  -  -  -  Ser -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
                                                  50                                                  60
h: Val-Glu-Leu-Arg-Asp-Asn-Gln-Leu-Val-Val-Pro-Ser-Glu-Gly-Leu-Tyr-Leu-Ile-Tyr-Ser-
m: Met-Asp -  Lys -  -  -  -  -  -  -  -  Ala-Asp -  -  -  -  Val -  -
r: Met-Lys -  Thr -  -  -  -  -  -  -  -  Ala-Asp -  -  -  -  -  -  -
                                                  70                                                  80
h: Gln-Val-Leu-Phe-Lys-Gly-Gln-Gly-Cys-Pro-Ser-Thr-His-Val-Leu-Leu-Thr-His-Thr-Ile-
m: -  -  -  -  -  -  -  -  -  -  -  -  Asp-Tyr    -  -  -  -  -  -  -  -  -  -  Val-
r: -  -  -  -  Ser -  -  -  -  Arg -  Tyr    -  -  -  -  -  -  -  -  -  -  Val-
                                                  90                                                  100
h: Ser-Arg-Ile-Ala-Val-Ser-Tyr-Gln-Thr-Lys-Val-Asn-Leu-Leu-Ser-Ala-Ile-Lys-Ser-Pro-
m: -  -  Phe -  Ile -  -  -  -  Glu -  -  -  -  -  -  -  -  -  Val -  -  -  -
r: -  -  Phe -  -  -  -  Pro-Asn -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
                                                  110                                                 120
h: Cys-Gln-Arg-Glu-Thr-Pro-Glu-Gly-Ala-Glu-Ala-Lys-Pro-Trp-Tyr-Glu-Pro-Ile-Tyr-Leu-
m: -  Pro-Lys-Asp -  -  -  -  -  -  -  -  Leu -  -  -  -  -  -  -  -  -  -  -  -  -
r: -  His -  -  -  -  -  Glu -  -  Pro-Met-Ala -  -  -  -  -  -  -  -
                                                  130                                                 140
h: Gly-Gly-Val-Phe-Gln-Leu-Glu-Lys-Gly-Asp-Arg-Leu-Ser-Ala-Glu-Ile-Asn-Arg-Pro-Asp-
m: -  -  -  -  -  -  -  -  -  -  -  -  Gln -  -  -  -  Val -  Leu -  Lys-
r: -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  Thr -  Val -  Glu -  Glu-
                                                  150                                 157
h: Tyr-Leu-Asp-Phe-Ala-Glu-Ser-Gly-Gln-Val-Tyr-Phe-Gly-Ile-Ile-Ala-Leu
m: -  -  -  -  -  -  -  -  -  -  -  -  Arg-Val -  -  - (156)
r: -  -  -  Leu -  -  -  -  -  -  -  -  -  -  -  -  -  - (154)
```

Fig. 8.29. cDNA-derived amino acid sequences of the mature forms of human (h) murine (m) and rabbit (r) tumor necrosis factor. Dashes indicate residues identical to those in the human sequence. Deletions (empty spaces) at position 73 in the murine sequence and at positions 1,2 and 73 in the rabbit sequence, are introduced for maximizing homology. Numbers in parentheses indicate the number of residues constituting the murine and the rabbit protein, respectively

(Haranaka et al. 1985b, Itoh, H. cited by Beutler and Cerami 1986a) cDNA-derived sequences have also been established. Predicted amino acid sequences of the monomeric forms of human, murine and rabbit TNFs are shown in Fig.8.29.

TNF is composed of M_r 17,000 subunits arranged in dimeric, trimeric or pentameric forms depending on the species investigated and the method of isolation. It is not yet decided whether the multimeric arrangement is required for biological activity. Although the mouse sequence contains possible glycosylation sites, there is presently no evidence for the glycoprotein character of TNF in any species. In comparison to

the 156 amino acid mouse protein, the rabbit material lacks two N-terminal residues and therefore has only 154 residues. On the other hand, human TNF is a 157 amino acid peptide owing to the presence of an additional histidine residue at position 73 (see Fig.8.29).

Both the human and the mouse precursors have unusually long leader sequences containing 76 (human) and 79 (mouse) amino acids, respectively. The mature human and mouse proteins are highly homologous (97%), as are the corresponding leader sequences (86%). The latter ones may have distinct biological functions (Fransen et al. 1985, Pennica et al. 1985). The TNF, molecule contains two cysteine residues per subunit which are involved in disulfide bridge formation between residues at position 69 and 101, respectively.

Invasive diseases, infectious or neoplastic, are frequently accompanied by serious metabolic derangements manifested in a variety of clinical symptoms such as wasting of the body (cachexia; Torti et al. 1985) and shock, which threaten the host integrity and may of themselves lead to death (Filkins 1985). Many of the aforementioned metabolic changes now are attributed to the products of cells of the reticuloendothelial system (RES), primarily macrophages, instead of the invasive stimuli themselves as previously suggested. Among these changes hypertriglyceridemia frequently occurs as a result of a deficient clearance of triglycerides caused by a systemic suppression of the enzyme lipoprotein lipase (LPL). Attempts to identify the molecular mechanism underlying this phenomenon led to the detection of a factor in sera of endotoxin-treated mice which specifically suppressed LPL activity in adipocytes in vitro, and LPL expression in adipose tissue in vivo (Kawakami and Cerami 1981, Beutler et al. 1985c). This particular factor was termed c a c h e c t i n referring to its presumed role in the induction of cachexia (see, e.g., Cerami and Beutler 1988). When the N-terminal sequence of mouse cachectin was compared to that of human TNF, a striking homology between the two could be demonstrated (Beutler et al. 1985a). It was also observed that highly purified cachectin possessed potent TNF activity in vitro suggesting that cachectin and TNF may be

related. The complete cDNA-derived primary structures of the mouse and human proteins (Fransen et al. 1985, Pennica et al. 1985, Wang et al. 1985) confirmed this, and showed that bioactivities of cachectin and TNF originate from a single, highly conserved protein. As a result, cachectin and TNF now are regarded as identical entities (Beutler et al. 1985a, Beutler and Cerami 1986a, Sappino and Alberto 1987).

There is another point affecting TNF nomenclature. Since TNF and lymphotoxin (LT) (see in the following section) share 30% sequence homology and have similar and/or identical biological activities in several test systems including in vivo tumor necrosis activity and in vitro cytolytic activity (Ruff and Gifford 1981a,b, Gray et al. 1984, Pennica et al. 1984b, Stone-Wolf et al. 1984, Kelker et al. 1985), it has been proposed that the classical macrophage-derived TNF should be designated as TNF-α and the classical lymphocyte-derived LT as TNF-β (Nedwin et al. 1985c). Thus TNF, TNF-α and cachectin are synonimic names as are LT and TNF-β.

A murine macrophage-derived acidic selfaggregating proteinaceous cytotoxin with M_r ranging between 70,000 and 55,000 has been described and proved to be a multimeric construction composed of M_r 15,000 monomers (Kull and Cuatrecasas 1984). This toxin has potent in vivo effects, some of which are reminiscent of n e c r o s i n, a necrotizing factor detected in inflammed tissue extracts in the early 1940s (for references see Kull and Cuatrecasas 1984). Although the authors kept the name necrosin for historical reasons, this material is obviously related to, or identical with, TNF. Similar results were obtained by Rubin and associates (1985b) who have purified both multimeric and monomeric forms of a factor with TNF activity from LukII cells, a human lymphoblastoid cell line, that was termed TNF(LukII).

The TNF gene resides on chromosome 6 in man and is closely linked to the LT gene (Nedwin et al. 1985a,b) suggesting a common ancestral origin and a tandem duplication event during evolution. The murine genes for TNF and LT were assigned to chromosome 17 (Nedospasov et al. 1986) where they are tandemly arranged similarly to their human counterpart.

Although TNF-binding plasma membrane receptors have not yet been isolated, several characteristics of the binding process and the receptors have been elucidated (Aggarwal et al. 1985a, Hass et al. 1985, Beutler and Cerami 1986a, Israel et al. 1986). Initially it was thought that TNF acts only on tumor cells. It has soon become clear, however, that certain nontumor cells possess TNF receptors (Kull et al. 1985, Rubin et al. 1985a) and may be targets for TNF-mediated biological activities which are distinct from those eliciting selective cytotoxic effects on tumor cells.

In spite of an extraordinarily wide scale of known biological responses elicitable by TNF, its physiological function remains elusive for the time being. This is mainly due to extremities of the activity spectrum of this monokine: while the effects elicited by low levels of TNF are obviously beneficial to the host (for example, activation of neutrophils and eosinophils, mobilization of the energy stores, etc.,) those manifested when TNF is released in large quantities provoke catastrophic metabolic derangements that may lead to the death of affected organisms. Such a deranged metabolic state is precipitable by an endotoxin challenge or by other invasive stimuli (Haranaka et al. 1985a). Since a detailed discussion of all biological activities of TNF would go beyond the scope of this section, in the present survey only the antiproliferative/antitumor properties of this monokine will be stressed, (readers who are interested in important metabolic effects of TNF are referred to the reviews by Beutler and Cerami 1986a,b, Old 1987).

TNF is capable of producing cytotoxicity to tumor cells in vitro and of causing hemorrhagic necrosis of certain tumors in vivo (Carswell et al. 1975, Green et al. 1976, 1982). It is of practical importance that TNF and interferon-γ are strongly synergistic (Williamson et al. 1983, Balkwill et al. 1986, Beutler et al. 1986, Fransen et al. 1986). Moreover, there is a multilevel interplay between TNF and other known immunomodulators such as interleukin-1 and -2 as well as γ-interferon reinforcing biological activity of TNF (Nedwin et al. 1985c, Bachwich et al. 1986, Libby et al. 1986, Philip and Epstein

1986, Stolpen et al. 1986). It is also important to note that in vitro cytotoxicity of TNF lacks species-specificity (Helson et al. 1975), while it displays tumor cell-specificity. The latter is supported by observations indicating that mouse TNF exerts either a cytotoxic or a cytostatic effect on a variety of TNF-sensitive, but not TNF-resistant, human tumor cells in vitro (Fung et al. 1985, Beutler and Cerami 1986a, and causes hemorrhagic necrosis in TNF-sensitive tumors in vivo (Williamson et al. 1983), whereas normal cells remain cytotoxically unaffected (for an opposing view see Sugarman et al. 1985). The in vivo tumor necrotizing effect of TNF may be mediated, partly through an indirect action affecting tumor vasculature and the coagulation system (Bevilacqua et al. 1986), partly through antiproliferative (Helson et al. 1975, Old 1976, Hahn et al. 1986, Kohase et al. 1986, Sato et al. 1986) and antitumor effects (Flick and Gifford 1986, Haranaka et al. 1986, Motoo et al. 1986, Urban et al. 1986, Watanabe et al. 1986) directed on the tumor cells themselves. As to its antiproliferative effect, TNF was demonstrated to act cell-cycle specifically on mouse L-cells inducing cycle arrest in the G_2 phase of the cell cycle (Darzynkiewicz et al. 1984). However, TNF was recently reported to be mitogenic for normal fibroblasts (Vilcek et al. 1987). TNF is a potent inhibitor of endothelial cell growth in vitro (Frater-Schroder et al. 1987) but is angiogenic in vivo (Sato et al. 1987).

Presently, there is no satisfactory explanation for TNF-sensitivity of a variety of tumor cells, and for TNF-resistance of others. Although initially it was hoped that TNF receptors might be correlated with TNF sensitivity, the detection of TNF receptors on a range of cell types, both TNF-sensitive and TNF-resistant, indicated that the basis for the selective action needs to be sought elsewhere.

Clinical trials aimed at using TNF as an antineoplastic agent now are in progress in several clinical departments. However, no consensus has emerged so far as to the efficacy of this monokine. The initial hope that isolated TNF, natural or recombinant, will enable oncologists to induce selective tumor necrosis without inducing the toxic effects regularly seen

following endotoxin-induced tumor necrosis, has faded away with the recognition that TNF/cachectin is the principal mediator of both the endotoxin-induced shock and the tumor necrosis. Fortunately, however, recent clinical reports do not support the fear, that TNF administered to patients would elicit intolerable side effects. Efforts aimed at modifying TNF molecule by chemical means to obtain a molecule retaining tumor necrotizing capacity but lacking nonselective toxic properties are in progress. It is hoped that a properly modified TNF molecule can ultimately be produced which, alone or in combination with other potent immunomodulators such as γ-interferon or interleukin-2, will provide oncologists with an antineoplastic agent(s) that can be more efficiently used in cancer therapy than those presently in use (for recent reviews on structure and biological properties of TNF see Beutler and Cerami 1986a,b, Fiers et al. 1986, Goeddel et al. 1986, Maury 1986, Koren 1987, Munker and Koeffler 1987, Old 1987, Ruddle 1987).

(2) Lymphotoxin. Lymphotoxin (LT), also called tumor necrosis factor-β (TNF-β) is a lymphokine with a preferential anticellular activity on various tumor cells in vivo and in vitro. It is a product of activated lymphocytes. Human LT (hLT) was demonstrated to be a glycoprotein with an M_r of 60,000-70,000, if measured by molecular sieving (Aggarwal et al. 1984a). It probably represents a polymeric structure. The monomer has a M_r of 25,000 (for references see Gray et al. 1984) and a strong tendency to aggregate to polymeric forms. The primary structure of hLT was determined both by protein structure analysis (Aggarwal et al. 1984a,b) and DNA recombinant technology (Gray et al. 1984; Nedwein et al. 1985a,b). Sequence analysis of the gene revealed a fairly large (34 amino acid) signal sequence of the precursor molecule. The primary structure of a genomic DNA-derived hLT is shown in Fig.8.30 (Nedwin et al. 1985a,b). The sequence chemically determined by Aggarwal and coworkers (1985b) differs only in a single residue [(threonine(26)] from the predicted sequence shown in Fig.8.30 (for data on the structure of the murine TNF-β gene see Gray et al. 1987).

```
                                                  10                  20
Leu-Pro-Gly-Val-Gly-Leu-Thr-Pro-Ser-Ala-Ala-Gln-Thr-Ala-Arg-Gln-His-Pro-Lys-Met-
                                                  30                  40
His-Leu-Ala-His-Ser-Asn-Leu-Lys-Pro-Ala-Ala-His-Leu-Ile-Gly-Asp-Pro-Ser-Lys-Gln-
                                                  50                  60
Asn-Ser-Leu-Leu-Trp-Arg-Ala-Asn-Thr-Asp-Arg-Ala-Phe-Leu-Gln-Asp-Gly-Phe-Ser-Leu-
                                                  70                  80
Ser-Asn-Asn-Ser-Leu-Leu-Val-Pro-Thr-Ser-Gly-Ile-Tyr-Phe-Val-Tyr-Ser-Gln-Val-Val-
                                                  90                  100
Phe-Ser-Gly-Lys-Ala-Tyr-Ser-Pro-Lys-Ala-Thr-Ser-Ser-Pro-Leu-Tyr-Leu-Ala-His-Glu-
                                                  110                 120
Val-Gln-Leu-Phe-Ser-Ser-Gln-Tyr-Pro-Phe-His-Val-Pro-Leu-Leu-Ser-Ser-Gln-Lys-Met-
                                                  130                 140
Val-Tyr-Pro-Gly-Leu-Gln-Glu-Pro-Trp-Leu-His-Ser-Met-Ile-His-Gly-Ala-Ala-Phe-Gln-
                                                  150                 160
Leu-Thr-Gln-Gly-Asp-Gln-Leu-Ser-Thr-His-Thr-Asp-Gly-Ile-Pro-His-Leu-Val-Leu-Ser-
                                                  170 171
Pro-Ser-Thr-Val-Phe-Phe-Gly-Ala-Phe-Ala-Leu
```

Fig. 8.30. Primary structure of human lymphotoxin. Underlined residues indicate a potential glycosylation site

Accordingly, the human protein consists of 171 amino acids and has a theoretical M_r of 18,664, as opposed to the value of 25,000 determined by SDS-PAG electrophoresis of the natural protein. The difference between the two M_r estimates probably is due to the carbohydrate moiety, which is most likely linked to residues at position 62-64 of the authentic (natural) molecule. Sequence analysis of a M_r 20,000 species previously detected (Aggarwal et al. 1984a,b) showed that this sequence lacked the N-terminal 23 amino acids of the larger form shown in Fig.8.30. This smaller form may be a degradation product, or it may point to the heterogeneity of hLT. Whatever is the case, the lack of the 23 N-terminal residues does not seem to influence biological activity of the molecule. Although IFN-γ, IL-2, IL-3 and LT are all secreted by induced lymphocytes or monocytes, no significant sequence homology was detected between the listed molecules and LT. Natural killer cells were also reported to secrete an anticellular factor.

The structure of the hLT gene and its localization on chromosome 6 has recently been reported by Nedwin et al. (1985b) while the cellular origin of hLT and its purification by Pichangkul et al. (1985). The gene structure of LT/TNF-β and TNF/TNF-α share some similarities, but they also display dis-

tinctive differences. The two genes are tandemly organized on chromosome 6 in human.

LT has an anticellular activity on neoplastic cell lines. It specifically inhibits tumor cell growth both in vivo and in vitro and is usually less cytostatic against non-tumorigenic cells of the same species (for references see Aggarwal et al. 1984a, 1985a, Gray et al. 1984). Besides a cytostatic effect, LT has a marked cytolytic activity on some target cells from different species in vitro (Kramer and Granger 1976), but the relative sensitivity varies greatly among target cells (Kolb and Granger 1970, Shacks et al. 1973, Papermaster et al. 1976). LT has been shown to inhibit the transformation of cells induced by chemical carcinogens or ultraviolet irradiation, and the ability of morphologically transformed cells to induce tumor in vivo (Evans et al. 1977, Evans and DiPaolo 1981). LT was also reported to act as an antitumor agent against human lymphomas, ovarian carcinomas and multiple myelomas (Khan et al. 1982). LT acts synergistically with IFN-α and IFN-γ both in vivo and in vitro, and the potent antitumor activity observed with natural preparations may be a result of this synergistic activity. Predictably, the availability of recombinant LT will be a great help in the biological characterization of LT (for a review on human LT see Aggarwal et al. 1984b).

8.2.3. MAMMARY-DERIVED GROWTH INHIBITOR

Although many polypeptide growth inhibitors have been extensively purified and characterized (see e.g., Balazs et al. 1980, Paukovits and Laerum 1982, Hsu et al. 1984, Hsu and Wang 1986, Sharifi et al. 1986) amino acid sequence information has not yet been presented for these inhibitors. Thus, the interferons, tumor necrosis factors α and β and the transforming growth factor-β1, a proliferation inhibitor for epithelial and endothelial cells remained the only structurally identified growth inhibitors factors up to now. However, in 1987 a M_r 13,000 protein has been isolated and sequenced from lactating

```
                                10                                  20
AcVal-Asp-Ala-Phe-Val-Gly-Thr-Trp-Lys-Leu-Val-Ser-Ser-Glu-Asn-Phe-Asp-Asp-Tyr-Met
                                30                                  40
  Lys-Ser-Leu-Gly-Val-Gly-Phe-Ala-Thr-Arg-Gln-Val-Gly-Asn-Met-Thr-Lys-Pro-Thr-Leu
                                50                                  60
  Ile-Ile-Ser-Val-Asn-Gly-Asp-Thr-Val-Ile-Ile-Lys-Thr-Gln-Ser-Thr-Phe-Lys-Asn-Thr
                                70                                  80
  Glu-Ile-Ser-Phe-Lys-Leu-Gly-Val-Glu-Phe-Asp-Glu-Thr-Thr-Ala-Asp-Asp-Arg-Lys-Val
                                90                                 100
  Lys-Ser-Ile-Val-Thr-Leu-Asp-Gly-Gly-Lys-Leu-Val-Gln-Val-Gln-Lys-Trp-Asp-Gly-Gln
                               110                                 120
  Glu-Thr-Ser-Leu-Val-Arg-Glu-Met-Val-Asp-Gly-Lys-Leu-Ile-Leu-Thr-Leu-Thr-Hys-Gly
                               130 131
  Thr-Ala-Val-Cys-Thr-Arg-Val-Tyr-Glu-Lys-Gln
```

Fig. 8.31. Amino acid sequence of bovine mammary-derived growth inhibitor

bovine mammary glands that acted as a reversible inhibitor of both DNA synthesis and mitotic division in different mammary carcinoma cell lines (Bohmer et al. 1987).

The inhibitor termed mammary-derived growth inhibitor (MDGI) was found to be a 131 amino acid protein (Fig.8.31). Although antisera raised against MDGI reacted with fibroblast growth regulator (FGR), a protein obtained from media conditioned by 3T3 cells, that probably is involved in the density-dependent inhibition of the proliferation of 3T3 cells, the highly different pIs of the two proteins contradict their identity. However, MDGI shows extensive sequence homologies to a number of polypeptides that are known, or are expected to carry hydrophobic ligands such as heart fatty acid-binding protein (H-FABP); p422, a differentiation-associated protein in adipocytes; p2, a protein constituent of the basic myelin fraction of peripheral nervous tissue having a capability of inducing experimental allergic neuritis; cellular retinoic acid-binding protein (CRABP), and cellular retinol-binding protein (CRBP; for references see Bohmer et al. 1987). Although little is known of the functions of these proteins, MDGI might also be able to carry hydrophobic ligands and an interaction of MDGI with as yet unknown hydrophobic ligands might play a functional role in the mechanism of its growth inhibitory action.

8.2.4. BSC-1 CELL GROWTH INHIBITOR

A pr tein isolated from serum-free media conditioned by BSC-1 African green monkey kidney epithelial cells was found to be a pote t inhibitor of the growth of a variety of cells in culture, and it could also inhibit the growth of a human mammary carcinoma grown in nude mice in vivo (Holley et al. 1983). Recently, a cDNA has been isolated and sequenced (Hanks et al. 1988) whose open reading frame encodes a 414 amino acid peptide. N-terminal sequencing of the purified inhibitor has indicated that the C-terminal 112 amino acids of the 414 amino acid precursor corresponds to the mature BSC-1 cell growth inhibitor (GI; Fig.8.32).

The BSC-1 cell GI is related both functionally and structurally to human platelet-type transforming growth factor (PGF; 80 of the 112 residues are in indentical positions) and is, by all indications, identical with the factors that have been called type β_2 TGF and cartilage inducing factor B. The authors proposed the name "polyergin" to designate this inhibitor whose abbreviation PRG is also appropriate to the term "polyfunctional regulator of growth".

```
                                10                                  20
Ala-Leu-Asp-Ala-Ala-Tyr-Cys-Phe-Arg-Asn-Val-Gln-Asp-Asn-Cys-Cys-Leu-Arg-Pro-Leu
                                30                                  40
Tyr-Ile-Asp-Phe-Lys-Arg-Asp-Leu-Gly-Trp-Lys-Trp-Ile-His-Glu-Pro-Lys-Gly-Tyr-Asn
                                50                                  60
Ala-Asn-Phe-Cys-Ala-Gly-Ala-Cys-Pro-Tyr-Leu-Trp-Ser-Ser-Asp-Thr-Gln-His-Ser-Arg
                                70                                  80
Val-Leu-Ser-Leu-Tyr-Asp-Thr-Ile-Asn-Pro-Glu-Ala-Ser-Ala-Ser-Pro-Cys-Cys-Val-Ser
                                90                                  100
Gln-Asp-Leu-Glu-Pro-Leu-Thr-Ile-Leu-Tyr-Tyr-Ile-Gly-Lys-Thr-Pro-Lys-Ile-Glu-Gln
                               110     112
Leu-Ser-Asn-Met-Ile-Val-Val-Ser-Cys-Lys-Cys-Ser
```

Fig. 8.32. Primary structure of BSC-1 cell growth inhibitor

8.2.5. MEMBRANE-BOUND PROLIFERATION INHIBITORS

One of the explanations aimed at interpreting the mechanism of density dependent growth regulation of cells in culture is based on the postulated accumulation of inhibitory substances in the medium of cells grown in monolayer culture. The assumption that the quiescent state of density dependently growing cells may be causally related to membrane-derived inhibitory agents was first confirmed by the observation that addition of a membrane preparation from density inhibited 3T3 cells to a culture of growing 3T3 cells reduced the rate of ^{3}H-TdR incorporation in these cells (Whittenberger and Glaser 1977, Whittenberger et al. 1978, 1979) indicating early G_1 as its point of attack. The activity seems to be tightly bound to the membrane, and may be carried by an intrinsic membrane component. As a supporting evidence, purified plasma membranes were shown to inhibit polypeptide growth factor-induced DNA synthesis in subconfluent 3T3 cells (Vale et al. 1984).

DNA synthesis- and cell division-inhibiting activities closely attached to the cell surface were also detected in quiescent BALB/C 3T3 cells (Natraj and Datta 1978a,b). The responsible agent of proteinaceous character was designated as fibroblast growth regulatory factor (FGRF). Circumstancial evidence collected with synchronized cultures indicated that the physiological effect of FGRF is to arrest cells in early G_1, thereby disenabling them to traverse the S phase and to initiate a new round of cell cycle (Datta and Natraj 1980). Whether the FGRF-mediated growth arrest point is identical to the restriction (R) point proposed by Pardee (1974) remains to be elucidated. Experimental evidence is compatible with the conception that glycosylation of some unknown cell surface acceptors with N-acetylglucosaminyl (GlcNAc) residues transforms the active FGRF into an inactive form (Natraj and Datta 1978a,b). Accordingly, the reversible transition of cells from a quiescent state to a proliferative one may be controlled by a glycosylation-deglycosylation reaction of the surface acceptors (for a more detailed account see the review by Datta 1981). More recently, growth inhibitory proteinaceous sub-

stances have been separated and partially characterized from membranes of cultured chick embryo fibroblasts (Yaoi 1984), senescent human fibroblasts (Pereira-Smith et al. 1985, Stein and Atkins 1986) lymphoid cells (Stallcup et al. 1984a,b), and from a membrane-bound glycoprotein with an antiproliferative activity from bovine cerebral cortex cells (Johnson et al. 1985). Isolated endothelial cell plasma membranes were recently shown to mediate inhibitory activity specifically on the proliferation of endothelial cells (Teitel 1986).

8.2.6. GROWTH INHIBITORS PRESENT IN, AND PRODUCED BY, CULTURED CELLS

Several past observations indicated the production of a variety of inhibitory factors by certain cells in culture. The majority of evidence supporting the existence of such factors were circumstantial so far, and none of these postulated inhibitors were characterized in a sufficient detail until recently.

Division of log phase Chinese hamster cells are delayed in cultures if the cells are grown adjacent to a confluent culture of the same cells under conditions allowing the interchange of humoral factors between the two cultures (Froese 1971). This was interpreted as indicating the production of some unknown inhibitory factor(s) by the cells grown to a confluent layer.

In experiments by Garcia-Giralt et al. (1970), the successive decrease in the division potential of WI38 human fibroblasts in culture was attributed to a progressive accumulation of a proliferation inhibitor(s) in the medium. This assumption received further support when, in the extracts of WI38 cells reaching saturation density, as well as in the used medium originating from monolayer cultures of these cells, an inhibitory activity was detected (Houck et al. 1972). The dialyzed, centrifuged and lyophilized used medium exerted a concentration-dependent and cell line-specific inhibition of ^{3}H-TdR incorporation into the DNA of WI38 cells in sparse culture. The

molecular weight of the assumed protein inhibitor was estimated to be somewhere between 30,000 and 50,000.

While studying the mechanism of density dependent growth regulation in BSC-1 cells, a monkey kidney epithelial cell line, Holley and associates (1978) have detected at least three inhibitors in the conditioned media of dense cultures of these cells. Of the three inhibitors, two were identified as lactic acid and ammonia, respectively, whereas the third remained temporaly unidentified. The latter was shown to be destroyed by heating or storing the medium in the absence of cells. In Holley's interpretation, this unidentified inhibitor was largely responsive for the observed inhibition, whereas lactate and ammonia were assumed to be less important. In subsequent experiments Holley and associates (1980) demonstrated that at least two inhibitors with a M_r of 15,000 and 10,000, respectively, could be isolated from the culture medium of BSC-1 cells that reversibly and cell line-specifically arrested the growth of BSC-1 cells at ng/ml concentrations. The growth inhibitory action of these factors on BSC-1 cells was counteracted by EGF from calf serum (Walsh-Reitz et al. 1984). One of these factors, termed BSC-1 growth inhibitor (see above) has been structurally defined.

A low M_r peptide inhibitor of lymphocyte DNA synthesis was described by Okâi (1984) in the serum-free culture medium of 3T3 cells, which lacked tissue specificity. Likewise, supernatants from stationary phase cultures of a human lymphoid cell line (802) were demonstrated by Djerassi (1983) to contain a less than M_r 5,000 inhibitor, probably a peptide, that lacked tissue specificity but displayed species specificity (for additional low M_r growth inhibitors see Chander et al. 1986, Koga et al. 1986).

A contact-inhibited (CI) amelanotic melanoma cell line with specific morphological characteristics was established from a highly malignant hamster melanoma cell line (RPMI No 1846). The CI cell line had a markedly reduced proliferative capacity and saturation density (Lipkin and Knecht 1974). A lyophilized Sephadex G-200 fraction obtained from media conditioned by this cell line caused a 55% decrease in saturation density of

the highly malignant cell line, whereas the corresponding fraction from media conditioned by a noncontact-inhibited cell line had no such effect. The effect was attributed to a melanocyte inhibitory factor (MCIF; Lipkin and Knecht 1975, 1976). A MCIF-like activity could also be prepared from media conditioned by human epidermal cells (Lipkin et al. 1978). A M_r 160,000 glycoprotein was the principal component of MCIF that acted similarly to plant glycoproteins known to function as mitotic inhibitors for transformed cells (for details see Knecht and Lipkin 1977).

Apart from TGF activity, a tumor inhibitory factor (TIF) has also been isolated from rat Novikoff ascites fluid (Levine et al. 1984). The TGF and TIF activities could be completely separated by reverse phase HPLC. TIF inhibited the anchorage independent growth of a more differentiated human colon carcinoma cell line, but left the less differentiated cells practically unaffected. The simultaneous presence of stimulatory (TGF) and inhibitory (TIF) activities in the same extract suggests that the relative concentrations of these factors may be important in the control of cell growth.

Two classes of TIFs, TIF-1 and TIF-2 have been demonstrated in media conditioned by A673 cells, a human rhabdomyosarcoma cell line (Fryling et al. 1985, Iwata et al. 1985), which also produces TGF-α. The partially purified TIF-1 (M_r 10,000-16,000) inhibited the growth of a variety of human tumor cell lines, but stimulated that of normal human fibroblasts and epithelial cells in monolayer cultures. On the other hand, TIF-2, also a polypeptide (M_r 18,000-22,000), shares many properties in common with TIF-1, but can be distinguished by its M_r, chromatographic properties, and its effect on certain target cells, indicating its distinct character. TIF-1 and TIF-2 may be members of two distinct classes of tumor cell inhibitory factors (for further details and references see Fryling et al. 1985, Iwata et al. 1985).

A polypeptide, termed oncostatin M, possessing the capability of inhibiting the proliferation of A375 melanoma and other human tumor cell lines, but not normal human fi-

broblasts, has been isolated from serum-free supernatants of U-937 histiocytic lymphoma cells previously induced to differentiate into macrophage-like cells by treatment with the phorbol ester, phorbol 12-myristate 13-acetate (PMA). No such activity was detected in untreated U-937 cells. The molecular weight of this particular protein was found to be 18,000 by gel chromatography and 28,000 by polyacrylamide gel electrophoresis. Some physicochemical properties and the sequence of the N-terminal 26 amino acids of oncostatin M have been determined and no significant sequence homology between this and other proteins was found indicating that oncostatin M may be a distinct cell growth regulator (for more details and references see Zarling et al. 1986).

A protein (M_r 57,000) described by Wang (1985a) was assumed to be associated with the inhibition of cell proliferation on the basis of indirect evidence. This protein is exclusively detectable in the nucleus of nonproliferating cells including senescent human fibroblasts in vitro. However, besides aged fibroblasts, young fibroblasts can also be induced to express this protein by spontaneous or artificial arrest of their proliferation. Because this protein disappears from the cells entering the cell cycle (Wang and Lin 1986), and its presence is strictly associated with the nonproliferating state of the cells, the name s t a t i n was coined to designate this protein. In full harmony with these observations, the presence of statin in the cell nucleus was demonstrated to be characteristic of many tissues composed of nonproliferating cells in vivo. On the other hand, the absence of statin was regularly noted in tissues composed of replicating cells such as the epithelium of the gastrointestinal tract, the kidney and the basal layer of epidermis. In a more recent paper (Wang 1985b) it was reported that expression of statin is rapidly turned off once the restriction of cell replication at the G_0/G_1 boundary is removed. The deactivation of statin expression generally preceedes the actual events of mitosis. It is worth recalling that statin appears to be a functional opposite of cyclin and dividin, two other nuclear proteins, which are synthesized only in cycling cells (see elsewhere in this

Chapter). The chemical structure of statin, its mechanism of action and its possible role in the control of cell proliferation remain to be explored.

In co-cultivation experiments, certain human fibroblast cell lines were shown to release proteinaceous substances having potent growth inhibitory effects on Burkitt's lymphoma and human melanoma cell lines (Wu et al. 1985). The active substance has not yet been chemically defined.

Mouse lung conditioned medium was shown to contain both growth stimulatory and inhibitory activities (Szaniawska et al. 1985). Upon separation of the medium, inhibitory activities were found both in the 3,000-5,000 and the 12,000-20,000 molecular weight fractions. The activities seemed to be mediated by substances of peptidic nature which inhibited the growth of both normal and neoplastic cells. The exact chemical nature of these inhibitory activities and their relation to other proteinaceous inhibitory substances remain to be determined.

Similarly, both stimulatory and inhibitory activities have been demonstrated in media conditioned by 3T3 cells grown to confluency (Harel et al. 1985). Although the inhibitory substances (M_rs 10,000 and 35,000) have not been precisely identified, they likely are polypeptides. The authors suggested that regulation of cell proliferation in vitro is determined by a balance between these antagonistic, stimulatory and inhibitory, autocrine growth factors.

8.2.7. PROLIFERATION INHIBITORS OF THE HEMATOPOIETIC CELLS

(1) Physiological inhibitors. The concentration of various blood cells within the circulation is normally regulated with high precision. This is due to the flexibility with which the bone marrow adapts in its rate of cell production to the actual demand of the organism. In ascertaining normal steady state conditions and adaptation to altered needs, the interaction of stimulatory and inhibitory factors probably play a critical role. Amongst the stimulatory factors, erythropoietin, a

stimulator of erythropoiesis, has been thoroughly studied and characterized. In recent years, the identification and characterization of colony-stimulating factors (CSF), which are thought by some investigators to play an erythropoietin-like role in the production of granulocytes and monocytes, have also been advanced to a considerable extent. However, a definitive evidence concerning the role of the inhibitory factors in normal granulopoiesis is still lacking, and even their existence is supported by circumstantial evidence only. Broxmeier and associates (1977b) described a factor in the crude extracts of normal neutrophils which inhibited CSF production by monocytes and macrophages. A possible involvement of these colony formation inhibiting factors (CIFs) in the regulation of granulopoiesis in vivo was suggested by Broxmeier and coworkers (1977a). In a subsequent paper, Broxmeier and associates (1978) assumed that CIF is identical with lactoferrin, which was shown to have a remarkable inhibitory capacity on CSF production by mononuclear blood cells. However, data presented so far on the assumed identity of the two agents should be interpreted with extreme caution, and need further confirmation.

Several data have been published on the inhibition of CFU-C colony growth by serum from various species. The inhibitory capability of serum was attributed to a lipoprotein serum component by Chan et al. (1971).

Numerous authors presented evidence on the existence of tissue-specific, species nonspecific proliferation inhibitors operating in various cells including hemopoietic cells. This particular group of assumed endogenous inhibitors, called chalones, are separately discussed in a subsequent section (for more recent information on the inhibitors of hemopoietic cells see Reichelt et al. 1987, Guigon and Najman 1988, Mitjavila et al. 1988, Paukovits et al. 1988).

(2) Pathological inhibitors.

(a) Leukemic inhibitors. The strongest argument for the existence of pathological inhibitors of hemopoietic cell proliferation has been collected from observations made in a few

but thoroughly studied cases of bone marrow failures. For instance, it has been assumed that inhibitors from leukemic cells may suppress the proliferation of normal hemopoietic clones during leukemic relapse (Chiyoda et al. 1975, Morris et al. 1975, Knudtzon and Mortensen 1976). One such inhibitor (K 562) was shown to be stable on heating at 56 °C for 30 min, and to have a M_r of 30,000 (Olofsson and Cline 1978).

(b) Immunologic inhibitors. It has recently been recognized that proliferation of the hemopoietic cells may be suppressed by immune responses, humoral or cell mediated. The inhibitory activity demonstrable under humoral immune responses probably resides in the immunoglobulin fraction, first of all in IgG and IgM type antibodies (Krantz 1974, Cline et al. 1976, Hoffman et al. 1979, Fitchen and Cline 1980). However, a truly conclusive proof of the asssumption that some cases of bone marrow failure result from antibody-mediated suppression of hemopoiesis is still lacking (for a more exhaustive discussion of this topic see the review by Fitchen and Cline 1981).

8.2.8. CHALONES

Saetren's observations (Saetren 1956) that liver and kidney extracts exert a dose-dependent inhibitory action on the proliferation of cells in their organ of origin in vivo, whereas proliferation in other organs remains unaffected, goes back to the mid-1950s. This, in fact, signaled the start of research on chalones, a much debated and still controversial group of endogenous proliferation inhibitors. In Saetren's observation, and especially in its interpretation, the existence of tissue-specific endogenous proliferation inhibitors has been implicated. Based on a theory put forward by Weiss and Kavanau in 1957, Bullough and Laurence (1960) as well as Iversen (1960a,b) simultaneously formulated a theory which stressed the importance of tissue- or cell line-specific inhibitors in the regulation of cell proliferation. The term chalone (from the Greek chalao meaning to loosen or to lower) was adopted by Bullough (1962) for designating these putative tissue-specific

antimitotic endogenous agents. Although chalone is defined differently by various authors, there is a general agreement on the most essential features a chalone molecule should have: (1) chalones are physiological substances of endogenous origin; (2) chalones are produced by the same cell lines (tissues) on which they selectively act; (3) chalones inhibit cell proliferation in a cell line(tissue)-specific manner; (4) chalone action is reversible and not based on cytotoxicity. As it can be seen, these criteria say nothing of the chemical character of chalones, partly because chalones do not seem to be a chemically homologous group of substances, partly because not a single chalone molecule has been fully identified so far. Nevertheless, the majority of the information collected up to now on the chemistry of purified chalone preparations indicate that chalones are oligo- or polypeptides, although some chalones also appear to have carbohydrate moieties in their molecule indicating their glycopeptide or glycoprotein character. The estimated molecular weight of the chalone preparations hitherto studied appears to range between a few hundred and a few-times ten thousand. However, some chalones were shown to preferentially form aggregates under the conditions of their purification, and this may have led to an overestimation of their true molecular weight. In support of this, and also due to the use of more advanced techniques of molecular weight determination, an increasing number of purified chalones has been shown to have a molecular weight significantly lower than formerly thought. This is why there is now a general feeling among scientists that chalones, in general, will turn out to be rather small molecules. There also are several indications that in the organism chalones may exist and function as composite molecules that consist of a small molecule (e.g., a small peptide) carrying the inhibitory activity, which does not necessarily need to act cell line-specifically, and which is coupled to a large carrier molecule (e.g., a glycoprotein), providing it with the capability of acting specifically.

Cell line (tissue)-specificity is a characteristic of paramount importance to chalone molecules. This means that one specific chalone exclusively acts on the same cell line by which it is produced. To prove cell line-specificity, a chalone from one cell line should be without effect on other cell lines, and chalones from another cell line should have no effect on the cell line on which the former chalone proved effective. This criterium obviously implies that each type of differentiated cells has to produce its own specific chalone. This assumption, although backed by an impressive body of evidence, needs further confirmation in well controlled experiments and the use of chemically identified chalone preparations to be produced in the future.

It is also assumed and supported by several observations that chalones act species nonspecifically. This means that a chalone prepared from a cell line (tissue) of one species is active on the same cell line of another species. This property, together with their cell line-specificity, makes chalones resemble hormones, and allows their isolation from animal sources, a favorable condition if a large scale preparation of a chalone is the aim.

Basic mechanisms concerning the site of attack of chalones in the cell cycle largely remain to be discovered. Nevertheless, the use of a combination of techniques in studies concerned with cell kinetics has already allowed the collection of information on the site of chalone action within the cell cycle. Due to methodical reasons, most information concern DNA synthesis and mitosis, that is the S and the M phase of the cell cycle, respectively. Experimental data indicate that chalones may affect the cell cycle at different points, and in different manners. They may prevent cells from entering one phase of the cell cycle into the next one; they may slow down the flow of cells through different phases thereby prolonging the cell cycle time, or may inhibit DNA polymerase, etc. Chalones acting by inhibiting cell entry from G_1 to S phase are called G_1 chalones or S factors, whereas those preventing cells from entering the M phase or mitosis are called G_2 chalones or M factors. There are cell lines in which both G_1

and G_2 chalones were shown to be operating.

Purely on theoretical grounds, chalones are supposed to function as mediators of a negative feedback system which, however, is integrated into more complex regulatory mechanisms operating with stimulators as well. Whether chalone-sensitive cells respond to the actual chalone concentration in the extracellular space, or to the rate of change in this concentration or to both, is undecided at present. Evidence presented on epidermal chalones supports the validity of the second alternative (Elgjo 1974).

Available evidence indicates that G_2 chalones are under the influence of various hormones, and their effect is mediated via the cAMP system. G_1 chalones, on the other hand, do not seem to have any relation to the cAMP system. However, the majority of experimental evidence suggests that both types of chalones act through cell membrane receptors, although the experiments published by Nakai and Gergely (1980) seem to invalidate such a generalization.

The relation of chalones to tumorigenesis, and the potential capability of chalones to cure malignant tumors, has been the matter of serious contradictions, and often of hot debates in the 1970s. The discussion in theoretical terms of chalones' relationship to cancer goes back to the still unresolved problem of the nature of tumorigenesis. There are many who maintain that malignancy is the result of a disturbed differentiation rather than of a disturbed proliferation, and that cellular properties characteristic of transformed cells are only secondary to differentiation disorders, and thus chalones, by being proliferation regulators, have little to do with the basic process of malignant growth (Iversen 1970, 1980). Others, however, also on theoretical bases, stress that whatever is the real cause of cancer, proliferation plays a decisive role in it, and chalones, by acting through slowing down the passage of cells through the cell cycle, prolong cell cycle time, and bring about a situation where cell gain is overcome by cell loss, which should necessarily lead to the regression of tumors, and thereby to the cure of them (Rytomaa et al. 1976, 1977, Rytomaa and Toivonen 1979). Experimental evidence

has been presented in remarkable numbers on the ability of tumor cells to produce the chalone of their parent cell line, and the ability of this chalone to reduce the proliferation of tumor cell. It has also been demonstrated that transformed cells are characterized by a low intracellular chalone level, and by a decreased sensitivity towards chalone effects in comparison to their normal counterparts. Moreover, it has also been repeatedly observed that cells actively proliferating have a much lower chalone content than those with a low proliferative activity. All these point to the conclusion that chalones may play a role in carcinogenesis, although evidence presently available on this line needs further confirmation (for more details on chalones in general and on specific chalones in particular see Houck 1976, Balazs 1979 Iversen 1981, 1985, Langen 1985).

Besides chalones, the number of the partially or insufficiently characterized peptides/proteins inhibiting normal and/or malignant growth is practically unlimited. The following citations only serve to give a few examples of such inhibitory peptides published in the last two years: Burzynski (1986), Komatsu et al. (1986), Sloane et al. (1986), Feltham et al. (1987), Huggett et al.(1987), Mashima et al. (1987), Nadal (1987), Rola-Pleszczynski (1987).

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CHAPTER 9

ENDOGENOUS PEPTIDES IN THE INTEGRATION OF CIRCULATORY AND RESPIRATORY FUNCTIONS AND THE FLUID-ELECTROLYTE BALANCE

P. SANDOR and A.Z. RONAI

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INTRODUCTION

In this Chapter an attempt is made to briefly summarize the established and probable functions of endogenously produced peptides in the regulation of cardiovascular and respiratory processes as well as in fluid-elecrolyte balance. Since the very same peptide may be involved in more than one type of these processes, it may appear in more than one sections or subsections, respectively.

Our apologies are offered to those authors who do not find their work in the present literature cited, but we are convinced that the significance of a truly valuable study never depends upon the skill or error of a reviewer.

9.1. THE CARDIOVASCULAR EFFECTS OF ENDOGENOUS PEPTIDES

9.1.1. SUBSTANCE P (SP)

It was first observed by von Euler and Gaddum (1931) that an intravenously (i.v.) administered SP concentrate produced a transient fall in the arterial pressure of anesthetized rabbits. The effect was attributed to a peripheral vasodilatation which differed qualitatively from that elicited by acetylcholine (Ach) and in mammals was not inhibited by atropine, ganglionic blocking agents, antihistaminic drugs, guanethidine (Pernow 1953, 1961, Beck 1965, Lofstrom et al. 1965) and by many other agents (Bunag and Walaszek 1963). SP-induced vasodilation and hypotension could also be elicited in other mammals (Tregear et al. 1971, Bury 1976, Dews 1974), including

human, where the hypotension was attributed to a vasodilation in the skin and muscle vessels (Liljedahl et al. 1958, Pernow 1963).

A number of data appeared to support the view that the vasodilator and hypotensive actions caused by peripherally administered SP are due to its direct effects on the vascular smooth muscle rather than to centrally mediated actions (Holton and Holton 1952, Haefely et al. 1962, Bunag and Walaszek 1963, Jaques 1963, Kato and Buckley 1965, Correa and Graeff 1975). In certain species and under certain circumstances, SP can elicit vasoconstrictor and hypertensive effects as well (Amin et al. 1954, Juan and Lembeck 1974, Bury and Mashford 1976) and an increase in venous tone was observed during i.v. infusions of rather large doses of SP in the human forearm (Pernow et al. 1975).

SP-immunoreactive perivascular nerves have been detected in various vascular areas in several mammals including man and, at least in a subpopulation of these neurons, SP coexists with calcitonin gene-related peptide (Edvinsson et al. 1981a, Jansen et al. 1986, McCulloch et al. 1986, Wharton et al. 1986; see also sections 5 and 6).

Although i.v. infusion or injection of SP generally causes a dose-dependent, vasodilation-related elevation of peripheral blood flow in the most varied vascular areas of unanesthetized dogs, small SP doses may be ineffective or elicit opposite effects on blood flow (Hallberg and Pernow 1975, Pernow and Rosell 1975, Burcher et al. 1977). On the other hand, intraarterial infusion of small doses of SP into the dog's gracilis muscle increased, while that of higher doses decreased the local blood flow concommitantly with arterial hypotension (Fisher et al. 1974). It is interesting to note that the C-terminal octa- and nonapeptide fragment of SP caused a more pronounced vasodilation and a larger increase in blood flow of the hind limbs of dogs than the native SP (Bury and Mashford 1976). In isolated dog hearts, SP increases coronary blood flow if injected directly into the coronaries, an effect that is subject to a marked tachyphylaxis (Losay et al. 1976).

In man, i.v. infusion of SP was shown to induce hypotension and tachycardia that were regularly accompanied by a bright red flush in the face which, however, disappeared upon continued infusion (Liljedahl et al. 1958). Arterial infusion of SP considerably increased skin and muscle blood flow in the human forearm (Lofstrom et al. 1965).

Upon i.v. infusion of SP blood flow increased in the carotid and femoral arteries in unanesthetized dogs but remained unaltered in the hypothalamus and cerebral cortex at normal arterial pressure. However, local blood flow increased also in these cerebral regions during hemorrhagic hypotension (Kovach et al. 1977). I.v. administered SP invariably induced a tachycardia-based increase in cardiac output in anesthetized dogs while the peptide proved ineffective in guinea pig and rabbit isolated heart preparations (Burcher et al. 1977). In man, continuous SP infusion resulted in a hypotension-induced tachycardia that probably was causally related to a moderate elevation in cardiac output observed under such conditions (Duner and Pernow 1960).

Intracisternally administered SP elicits a sodium-sensitive vasopressor effect with consecutive blood pressure elevation and decreased respiratory frequency (Fuxe et al. 1980, 1981, Unger et al. 1981b). The sodium sensitivity of the vasopressor effect may be based on the capability of sodium ions to interfere with the binding of peptide ligands to their receptors (Simantov et al. 1976). Evidence has been presented indicating that vasodepressor impulses through afferent vagus fibres, that inhibit activity in enkephalin interneurons in n. tractus solitarii, may modulate the central hypertensive action of SP (Fuxe et al. 1980). Indeed, SP and VIP immunoreactive sensory fibres have been identified in the vagus nerve (Lundberg et al. 1978) that innervate the n.tractus solitarii, n. commissuralis and n. motorius of the vagus nerve (Hokfelt et al. 1978), centres where enkephalin and SP immunoreactive nerve terminals have been observed in a large number. Furthermore, immunoreactive enkephalin-containing interneurons inhibiting the release of SP have been demonstrated both in substantia gelatinosa of the spinal cord and the n.tractus spinalis of

the trigeminal nerve. Experimental evidence suggests that a similar arrangement may exist within the n.tractus solitarii and adjacent nuclei (Bolme et al. 1978, Fuxe et al. 1980) indicating that enkephalin-SP interactions may play an important role in the regulation of central cardiovascular responses.

The distribution pattern of SP-containing nerve fibres suggest that SP may also affect intestinal blood flow (Furness et al. 1980) and that it may be involved in secretomotor, vascular and even in sensory functions in the vaginal epithelium (Bloom and Polak 1979).

Functional and morphological evidences (Olgart et al. 1977a,b) as well as the results of intraarterial SP infusions equally support the assumption that this peptide is also involved in vascular responses of the dental pulp.

A SP immunoreactive nerve plexus of trigeminal origin has been demonstrated around feline pial arteries, arterioles and veins (Edvinsson et al. 1981a) where SP coexists with calcitonin gene-related peptide (Uddman et al. 1985). This is consistent with the observations that isolated middle arteries from this species reacted with a slight relaxation to SP under resting conditions, but the response was more pronounced in arteries previously contracted with $PGF_{2\alpha}$ or substance K (SK; Janssen et al. 1986), and especially under in vivo conditions following perivascular administration. In vivo SP increased the venous calibre, too (Edvinsson et al. 1981b,c).

9.1.2. CALCITONIN GENE-RELATED PEPTIDES (CGRP)

CGRP and SP immunoreactivities usually are colocalized in perivascular nerves (Uddman et al. 1985a, McCulloch et al. 1986) and in rodent cardiac nerves (Wharton et al. 1986). CGRP is also a powerful vasodilator especially in arteries/arterioles of experimental animals (Jansen et al. 1986, McCulloch et al. 1986) but veins are less sensitive than are arterioles (Edwinsson et al. 1981b,c). CGRP exerts positive inotropic and chronotropic effects by a direct action (Tippins et al. 1984, Franco-Cereceda et al. 1985).

9.1.3. ENDOGENOUS OPIOID PEPTIDES (EOP): ENDORPHINS (EP), ENKEPHALINS (Enk) AND DYNORPHINS (Dyn)

Enkephalins and opiate receptors are present in the most varied structures such as brain cardiovascular centres (Atweh and Kuhar 1977, Fuxe et al. 1980, Lundberg et al. 1980), glomus caroticum (Lundberg et al. 1979b), adrenal gland and sympathetic chain ganglia (Costa et al. 1979, Hokfelt et al. 1980, Lewis et al. 1980, Viveros et al. 1980), peripheral nerves, blood vessels and the heart (Lundberg et al. 1980).

Although EOPs have been shown to exert a number of circulatory effects at the periphery, their central effect is of primary importance in the regulation of cardiovascular functions (for reviews see Fuxe et al. 1979, Holaday and Loh 1981, Unger et al. 1981a, Szekely 1982, Holaday 1983, McQueen 1983, Akil et al. 1984).

I.v. administered β-EP produces a maloxone- and serotonin-dependent hypotension in various animal models that is often followed by a mild hypertensive, and then a prolonged hypotensive reaction (Lemaire et al. 1978, Holaday et al. 1979). D-Ala^{2}, D-Leu^{5}-Enk, an enzyme-resistant Enk analog also lowered arterial blood pressure in anesthetized cats (Feldberg and Wei 1978).

Specific opiate/opioid receptors have been demonstrated in several vascular preparations. Met-Enk, Leu-Enk, several Enk analogs, and less potently, β-EP were shown to inhibit field electrical stimulation-induced vasoconstrictor responses acting through a neuronal Enk receptor described in an isolated rabbit ear artery preparation (Knoll 1976, Ronai and Berzetei 1978, Ronai et al. 1978). Met-Enk-Arg^{6}-Phe^{7} heptapeptide is rapidly transformed to Met-Enk by a catopril-sensitive enzyme in this preparation (Ronai et al. 1983, 1984).

Met-Enk inhibited SP-induced contractions in isolated rabbit aorta strip without having any intrinsic actions on this vessel (Moore 1979). SP and Met-Enk were supposed to act through a common receptor, although SP did not interact with opioid receptors in brain membrane preparations. Met-Enk also caused an increase in gastric mucosal blood flow in the dog

(Konturek et al. 1978) and a naloxone-dependent decrease in vascular resistance with a consecutive blood flow increase in the gut (Konturek 1980). Presynaptic opiate receptors capable of modulating serotonin-induced vein responses are present in the human hand dorsal veins (Sicuteri et al. 1980). The findings that $PGF_{2\alpha}$-contracted but not resting, cerebral middle artery and pial artery preparations were dilated by both Met-Enk and Leu-Enk indicated the presence of opiate receptors also in the cerebral vessel walls (Hanko and Hardebo 1978).

Although in Wahl's experiments (1981, 1982) β and δ opiate receptors did not seem to have played an important role in the adjustment of pial arterial resistance in anesthetized cats, Met-Enk applied by a micropipette technique caused a decrease in the cortical blood flow in awake freely moving cats (Sandor et al. 1982,) through an uncertain mechanism (Jhamandas et al. 1977, Palmer et al. 1978). I.v. injection of naltrexone, a potent opiate receptor blocking agent, into anesthetized cats reduced total brain oxygen consumption by almost 50%, with the hypothalamus and pons as the most susceptible areas (Grandison et al. 1982). The mechanisms involved in this action are rather unclear. On the other hand, in experiments applying natural opioid peptides and their specific inhibitors (naloxone and antisera raised against the opioid peptides used) it was concluded that central β-EPergic mechanisms play a major role in the autoregulation of cerebral blood flow (Sandor et al. 1986). The problem associated with the blood-brain barrier restriction against opioid and other neuropeptides, that emerged also in these experiments, is a topic of serious discussion (Kastin et al. 1976, Cornford et al. 1978; for reviews see Owman and Hardebo 1982, Pardridge 1983).

Although opiate receptors have been localized to the rabbit heart (Klee 1977), the centrally mediated opioid actions seem to be more important in the regulation of heart functions than are the peripheral actions. Morphine-induced bradycardia could be rapidly reversed by i.v. naloxone in anesthetized cats, an effect that was greatly reduced following cervical (C_2) transection. In depressed psychiatric patients, Catlin and associates (1980) have observed an increased heart rate during the

first 10-min period of i.v. β-EP infusion that was followed by a steady decline between 10 and 60 min. On the other hand, naloxone infusion practically was without any effect on the heart rate and the blood pressure (Lightman and Forsling 1980). However, healthy subjects reacted with a delayed increase in the heart rate and blood pressure upon naloxone administration, that were apparently associated with epinephrine release from the adrenals (Hernandez et al. 1985).

Intracisternally administered β-EP and stable Met-Enk analogs, but not Met-Enk itself, first induced a short-lasting tachycardia and hypertension in dogs and rats, that was followed by a prolonged period of centrally mediated naloxone-dependent bradycardia and hypotension (Laubie et al. 1977, Bolme et al. 1978). In anesthetized cats, ($D\text{-}Ala^2$, Leu^5)-Enk administered intracisternally or topically on the medulla near the obex lowered the blood pressure and caused bradycardia, while tachycardia or bradycardia occurred depending on the dose following intraventricular administration (Feldberg and Wei 1978). Met-Enk given intracisternally was almost inactive, but increased the blood pressure in conscious cats if administered intraventricularly (Delbarre et al. 1980). Met- and Leu-Enks as well as ($D\text{-}Ala^2$) Met-Enk exclusively produced bradycardia and hypertension, but in rats, tachycardia could be observed following intracerebroventricular administration of the drugs (Bolme et al. 1978, Simon et al. 1978). Both the hyper- and hypotensive responses proved to be naloxone sensitive. Intracisternal administration of Leu-Enk and α-EP elicited the highest pressor activity in unanesthetized rats, while β-EP and stable Enk analogs preferentially produced vasodepression (Fuxe et al. 1980). Vasoactivities of the opiate peptides could be abolished by blocking α-adrenergic receptors, and the effects of Leu- and Met-Enks on the blood pressure could be greatly reduced by β-receptor blocking agents (Simon et al. 1978).

Centrally administered dermorphin also elicited tachycardia without changing the blood pressure through an action on n. preopticus medialis in the hypothalamus (Feurstein and Faden 1983). Opiate receptors mediating bradycardia and hypotension

from the fourth ventricle, but not the third, in conscious dogs are likely to be interlinked with medullary circulatory structures (Arndt et al. 1980). Morphine-like agents appear to decrease the sympathetic and increase the vagal tone acting on the medulla oblongata and they also facilitate the baroreceptor reflex at the level of the nucleus of the solitary tract (Laubie et al. 1974, 1977). Exogenous opiates attenuate the baroreflex activity probably acting on μ receptors in the CNS (Petty and Reid 1980).

In a series of carefully conducted experiments Petty and coworkers (Petty and De Jong 1982, Petty et al. 1982, De Jong et al. 1983, Petty and De Jong 1983) have investigated the role of n. tractus solitarii in mediating the central cardiovascular effects of Met-Enk and β-EP. It was concluded from these experiments that at least two distinct endorphin systems are involved in cardiovascular control mechanisms at the level of n. tractus solitarii, one being depressor (β-EP-like) and the other pressor (Met-Enk-like) in nature.

The observations indicating that ACTH secretion is under the inhibitory control of endorphins (Volavka et al. 1979), EP and Enk inhibit catecholamine release via a presynaptic action (Bellet et al.1979), and that Met-Enk inhibits spontaneous Ach release from rat cerebral cortex (Jhamandas et al. 1977) point to the interactions existing between opioid peptides and various neurotransmitters, hormones and other peptides involved in cardiovascular control mechanisms. Further examples of such interactions are those existing between opioid peptides and catecholamines in neuroendocrine regulation, between opioid peptides and SP in blood pressure regulation (Fuxe et al. 1980), between central endorphin and serotonin systems in cardiovascular regulation (Sicuteri et al. 1980, Yao et al. 1982) and between brain circuits involved in central blood pressure regulation. As to the latter, the locus ceruleus, an important region of central blood pressure regulation that is richly supplied with opiate receptors, receives noxious sensory input and the activity of this region is inhibited by EPs and exogenous opioids (Delbarre et al. 1980). δ and μ receptors were both suggested to mediate cardiovascular and respiratory ef-

fects of opiates and opioid peptides (Ganten et al. 1981, Faden and Feurstein 1983). Central administration of μ receptor agonists (Petty and Reid 1982) and of a stable enkephalin analog (Ito et al. 1983) resulted in a depression of cardiovascular reflexes. Indirect evidence on the participation of opiates in the physiological blood pressure regulation has also been presented in the literature in a significant number (Dashwood and Feldberg 1978, Farsang and Kunos 1979, Delbarre et al. 1980, Hernandez et al. 1985).

9.1.4. ANGIOTENSIN II (AT-II)

AT-II, the most effective hypertensive substance hitherto recognized, influences cardiovascular functions by both peripheral and central actions (for a review see Phillips 1987). AT partly acts directly on the vascular smooth muscle and partly increases the sympathetic vasoconstrictor outflow from the autonomic nervous system by increasing activity of the brain vasoconstrictor centres (Severs et al. 1966), by stimulating the sympathetic ganglia (Lewis 1968), potentiating norepinephrine output (Panisset and Bourdois 1968) and by stimulating catecholamine release from the adrenal medulla (Feldberg and Lewis 1964, Douglas 1970).

The AT-induced pressor effect can be counteracted neither by sympathectomy, adrenalectomy or by spinal cord transection nor by α-adrenergic and ganglionic blockade or by atropine. Precapillary vessels are more susceptible to the effect of AT than are the postcapillary ones and the veins, and vessels of the kidney, splanchnic region and the skin are affected more by AT than are those in the brain, heart, skeletal muscle and the lung (Douglas 1970). In humans both direct and indirect actions have been observed (Henning and Johnson 1967). AT significantly reduces blood flow through vascular beds whose precapillaries are preferentially affected by this peptide. AT is a potent constrictor of middle cerebral arteries in several species (Conde et al. 1981, Hanko et al. 1981) and increases cerebrovascular resistance with a consecutive reduction in

blood flow in several brain areas in anesthetized rabbits (Reyniel-Rebuffel et al. 1981).

AT-II increases contractility of the cardiac muscle in vitro, but not in vivo (Koch-Weser 1964). Cardiac output remains practically unaffected, the end diastolic pressure rises and bradycardia occurs following AT-II injections. The latter is secondary to the baroreceptor reflexes evoked by the systemic pressure elevation. Tachycardia is seen in vitro and, following baroreceptor inactivation, also in vivo due to the AT-induced increase in the sympathetic neural outflow (Farr and Grupp 1967).

The brain has its own renin angiotensin system having special tasks (for a review see Phillips 1978, 1987, Phillips et al. 1979, Saavedra and Plunkett 1985; see also Chapter 5 and section 9.2 of this Chapter), but AT-II acts also at several CNS sites involved in cardiovascular regulation.

Intraventricularly administered AT-II-induced blood pressure elevation is mainly due to an increased sympathetic activity, but it also increases vasopressin release (Severs and Daniels-Severs 1973, Falcon et al. 1978). The mechanisms underlying AT-induced suppression of the baroreceptor-mediated reflex inhibition of heart rate under hypotension is discussed by Vallotton (1987), and the CNS sites involved in the actions of AT by Phillips (1978, 1987), Haywood et al. (1980), Camacho and Phillips 1981, Miselis (1981), Mangiapane et al. (1982), Thrasher et al. (1982), Ferrario (1983), Mangiapane and Brody (1983), Mangiapane et al. (1983), van Houten et al. (1983), Healy and Printz (1984), Lind et al. (1985) and by Nelson and Johnsson (1985).

9.1.5. VASOACTIVE INTESTINAL POLYPEPTIDE (VIP)

VIP has been identified in cholinergic neurons, in the cardiovascular CNS centres and VIP immunoreactive nerve terminals have been demonstrated around the blood vessels in many different regions in the body such as the digestive, urinogenital, respiratory and the cardiovascular systems and the endocrine glands (Lundberg et al. 1980).

I.v. administration of VIP elicits peripheral vasodilation and a strong, prolonged hypotension and stimulates cardiac output (Said and Mutt 1970). Femoral and splanchnic blood flow as well as the blood flow in the hepatic and superior mesenteric arteries and in the portal vein increase significantly following i.v. VIP infusion and VIP might have a physiological role in the regulation of blood flow in the digestive organs. VIP was suggested to be the transmitter released from enteric vasodilator nerves in the cat (Fahrenkrug et al. 1978; for the localization of these nerves see Furness et al. 1980). Intestinal and colonic vasodilation known to be mediated by noncholinergic and nonadrenergic mechanisms may be due to the local release of VIP (Bloom and Polak 1979).

VIP present in the presumably cholinergic neurons innervating exocrine glands (Lundberg et al. 1979a, 1980, Hokfelt et al. 1980) may be released concomitantly with Ach and cooperate to cause the secretory response when Ach stimulates secretory cells and VIP increases the blood flow.

VIP may play a significant role in the regulation of cerebral blood flow. VIP nerves are present throughout the cerebral circulation (Larsson et al. 1976, Edvinsson et al. 1979, Owman and Edvinsson 1979) and VIP levels are high in cerebral arteries that correlate with the vasodilator response (Heistad 1981). All this indicates that VIP may be an important neurontransmitter in cerebral resistance vessels. This is also supported by the observation that cerebral cortical blood flow increases following VIP administration (Heistad et al. 1979). VIP was also shown to induce vasodilation in the feline middle cerebral arteries and to increase the calibre especially of the smaller pial arteries (Edvinsson et al. 1979, McCulloch and Edvinsson 1979). Intra- and extracerebral blood vessels dilate, and blood flow to several important brain regions increases following VIP administration in anesthetized dogs (Wilson et al. 1979). However, cerebral vessels are unresponsive to blood-born VIP probably due to the blood-brain barrier. VIP may mediate noncholinergic cerebral vasodilation (Duckles and Said 1982).

VIP infusion can induce vasodilation also in man (for references see Said 1979). VIP immunoreactive material was detected in nerves supplying human temporal arteries and VIP potently relaxes these vessels if previously contracted by $PGF_{2\alpha}$, but does not affect the basal tone.

VIP immunoreactivity is present in lower brain stem nuclei and in the intermediolateral nucleus of tractus solitarii, CNS territories containing VIP in the highest concentration (Palkovits 1981). VIP administered intracerebroventricularly causes an acute blood pressure elevation.

9.1.6. PEPTIDE HISTIDINE METHIONINE 27 (PHM-27)

PHM-27, which is the human equivalent of peptide histidine isoleucine 27 (PHI-27; Itoh et al. 1983) is equipotent with VIP in relaxing $PGF_{2\alpha}$-contracted human temporal arteries (Jansen et al. 1986).

9.1.7. GLUCAGON

Glucagon, a member of the "glucagon-secretin family of peptides" (Tatemoto and Mutt 1981; see also Chapter 6), was shown to reduce peripheral vascular resistance and arterial blood pressure by eliciting vasodilation in several vascular beds in various mammalian organisms (for a review see Farah 1983).

The effects of glucagon on cardiac muscle largely resemble those of β-adrenergic agents, and among others, it elicits positive inotropic and chronotropic effects in the heart. However, glucagon effects are not blocked by pure β-adrenergic blocking agents but are blocked by mixed adrenergic agonist-antagonist agents (for the mechanism of action of glucagon on cardiac muscle see Farah 1983).

9.1.8. BOMBESIN/GASTRIN-RELEASING PEPTIDE (GRP)

Bombesin originally described in nonmammalian organisms (see Chapter 13), causes renal vasoconstriction and through a consecutive activation of the renin-angiotensin system elicits a hypertensive response (Polak and Bloom 1979).

GRP is structurally related to bombesin and may be the human equivalent of the latter. GRP immunoreactive nerve fibres have been demonstrated around the pial vessels (Uddman et al. 1983).

9.1.9. CHOLECYSTOKININ (CCK)

CCK immunoreactive neurons have been identified in cerebral cortical neurons of rodents that are in a close association with blood vessels (Hendry et al. 1983).

9.1.10. NEUROPEPTIDE Y (NPY)

NPY and norepinephrine coexist in a significant proportion of peripheral sympathetic nerves in several mammals including man (Jansen et al. 1986; for reviews see Haynes 1986, Heym and Lang 1986, Hokfelt et al. 1986) and are coreleased upon nerve stimulation (Lundberg et al. 1986). In man, natural activation of the sympathetic system results in a marked elevation of circulating NPY (Lundberg et al. 1985b).

NPY causes contractions of arterioles in the cat (Emson and Dequidt 1984) and those of mesenteric veins and some arteries in man (Pernow et al. 1987). Norepinephrine-induced vascular reponses are almost invariably potentiated by NPY (Ekblad et al. 1984, Wahlestedt et al. 1985, Jansen et al. 1986). Acting presynaptically, NPY reduces nerve stimulation-induced norepinephrine release from isolated human mesenteric veins (Pernow et al. 1987).

9.1.11. NEUROTENSIN (NT)

NT is a potent hypotensive agent that causes a visible dilation of the small vessels in the skin (Carraway and Leeman 1973). The extent of hypotension depends upon the starting pressure level and exhibits acute tachyphylaxis.

I.v. or vertebral arterial injection of NT brought about a similar fall in blood pressure in anesthetized dogs (Rosell et al. 1976). The peptide caused a delayed vasoconstriction in denervated subcutaneous adipose tissue probably through an indirect effect, whereas vasodilation, increased blood flow and subsequently a slight variable vasoconstriction could be observed in anesthetized dogs following i.v. administration of NT. There was either no change or a transient decrease in the skin blood flow under similar conditions (Rosell et al. 1976). It is worth mentioning that NT induces pronounced effects in the gut at doses that are too low to affect blood pressure or cardiac performance significantly (Rosell 1980).

NT was reported to contract smooth muscles in the rat portal vein following cholinergic and β-adrenergic blockades (Helle and Serck-Hanssen 1979), and the peptide proved to be a potent vasoconstrictor for rat, but not pig and rabbit, coronary vessels (Quirion et al. 1978). Heart rate remained practically unaltered upon i.v. infusion of NT in anesthetized dogs but was transiently decreased upon a single large dose of the peptide (Rosell et al. 1976). Studies performed in other species have revealed that cardiac effects of NT are species-dependent to a significant extent (Quirion et al. 1978).

Cerebral blood vessels are innervated by NT-containing nerve fibres (Chan-Palay 1977). Intracerebroventricular administration of NT (Rioux et al. 1981) and NT congeners (Quirion et al. 1981) produced a rapid and short-lasting decrease in arterial blood pressure in anesthetized rats and acut tachyphylaxis may develop to this hypotensive effect.

9.1.12. ADRENOCORTICOTROP HORMONE (ACTH)

ACTH acts indirectly on the vascular bed by increasing glucocorticoid secretion from the adrenal cortex. In the absence of glucocorticoids, catecholamines are unable to elicit arterial pressure responses due to the unresponsiveness of the vascular smooth muscle to catecholamines (Ganong 1979). In adrenocortical insufficiency and in adrenalectomized animals, cerebral blood flow is seriously impaired.

ACTH effects on the cardiac muscle are also accomplished indirectly by increasing both glucocorticoid and mineralocorticoid secretion from the adrenal cortex, both of which, and also aldosterone exert a positive inotropic effect in vitro (Ballard et al. 1960, Ganong 1979). This is consistent with the observation that the contractile force of the heart is impaired and cardiac output decreases following adrenalectomy (Brown and Remington 1955).

The centrally mediated cardiovascular actions of ACTH are also indirect and are relayed by the limbic system (for details see Bohus 1975, De Wied 1979).

9.1.13. MELANOCYTE-STIMULATING HORMONE (MSH): MELANOTROPIN, INTERMEDIN

β-MSH was shown to cause blood pressure elevation both in rabbits and rats (Dyster-Aas and Krakau 1965, Sakamoto 1966). After i.v. administration of α-MSH, blood flow decreased in most brain areas in conscious rats, except the occipital cortex (Goldman et al. 1976a,b).

9.1.14. VASOPRESSIN: ANTIDIURETIC HORMONE (ADH)

The hypertensive effect of ADH was first described at the end of the last century (Oliver and Schafer 1895). The pressor effect depends on the actual reactivity of the baroreceptor system: in states associated with an impaired baroreceptor

function, small doses of ADH produce a significant blood pressure elevation (Wagner and Braunwald 1956, Brazeau 1971, Cowley et al. 1974, 1980).

ADH causes general vasoconstriction acting preferentially on the venules and capillaries by a direct effect on the vascular smooth muscle. The ADH-induced vasoconstriction results in a decreased blood flow in several vascular beds including the skin, limbs, splanchnic area and the cardiac muscle (Ruskin 1947, Slotnick and Teigland 1951, Brazeau 1971), and the vasoconstriction is also extended to the middle cerebral arteries (Conde et al. 1981, Hanko and Hardebo 1981).

ADH sensitizes the isolated rat aorta strip to catecholamines by a cAMP-dependent mechanism (Bartelstone and Naismith 1965). Small vasopressin doses potentiate the response to norepinephrine and significantly alter the biomechanical properties of the large arterial vessel walls in vitro, and the distribution of cardiac output in vivo (Monos et al. 1978a,b).

Vasopressin may be involved in cerebral vasoconstriction induced in cats by stimulating supraoptic nuclei in the hypothalamus (Teplov et al. 1978). A transient increase in cerebral blood flow was registered following intracarotid injection of vasopressin (Kozniewska et al. 1981). The oxytocin-increased renal blood flow decreased significantly upon small doses of ADH indicating a modulatory capability of this peptide (Brooks and Pickford 1958).

ADH induces a decrease in the coronary blood flow and a secondary myocardial ischemia (Melville 1938), in which reflexly induced changes in sympathetic and vagal tones evoked by the blood pressure elevation are also involved (Brazeau 1971).

Although vasopressin-containing fibres/terminals have been demonstrated in several brain areas (Buijs 1978, Nilaver et al. 1980), data available on cardiovascular responses to centrally administered vasopressin remain contradictory (for references see Ganten et al. 1979, Morris et al. 1986).

9.1.15. OXYTOCIN (OT)

OT in large doses produces a marked but transient decrease in the systolic, and especially in the diastolic pressure that can be particularly marked during anesthesia. The blood pressure changes are accompanied by reflex tachycardia and increased cardiac output. During continuous infusion, the transient hypotension is followed by a moderate and sustained increase in the systemic blood pressure. Flushing and an increase in limb blood flow can be observed during the short-lasting hypotensive response elicited by large doses of OT (Kitchin et al. 1959).

OT has a direct relaxing effect on the vessel walls and the vasodilation can be counteracted by vasopressin (Kitchin et al. 1959, Pickford 1961, Andersen et al. 1965).

Renal blood flow is greatly increased upon OT infusion which can be potentiated by hypophysectomy and blocked by ADH (Pickford 1961, Andersen et al. 1965). However, OT proved to be a vasoconstrictor for isolated feline middle cerebral arteries (Hanko and Hardebo 1981).

Although oxytocin-immunoreactive fibres and terminals are present in several brain areas, centrally administered OT causes no appreciable changes in the cardiovascular functions (Buijs 1978, Nilaver et al. 1980).

9.1.16. THYROTROPIN-RELEASING HORMONE (TRH): THYROID-STIMULATING HORMONE-RELEASING HORMONE (TSH-RH)

TRH may cause vasodilation indirectly in most body tissues by stimulating TSH and thereby thyroid hormone secretion. However, TRH was shown to decrease cerebrovascular resistance and to increase brain blood flow through a direct action (Yamada et al. 1979). Following central administration, TRH elicits a pressor response unrelated to vasopressin in a number of species (Horita et al. 1976, Horita and Carino 1977, Koivu-

salo et al.1979, Eriksson and Gordon 1981, Yvonne an Wei 1982) that is counteracted by ganglionic blockade and by spinal cord transection at C_{1-8}.

9.1.17. SOMATOSTATIN (SS): GROWTH HORMONE-INHIBITING HORMONE (GIH)

Although SS was shown to induce vasoconstriction in feline middle cerebral arteries in vitro (Hanko and Hardebo 1981), SS analogs elicited a decrease in the systemic blood pressure in rats (Vale et al. 1979), and SS was ineffective in other vessels (Helle and Serck-Hanssen 1979).

SS-immunoreactive substances were demonstrated throughout the CNS (Fuxe et al. 1979, Palkovits 1981) and SS was suggested to take part in the central control of blood pressure and peripheral sympathetic activity by preventing the release of catecholamines from adrenal medulla through a central action resulting in hypotension in normal animals (Weitzman et al. 1979, Fisher and Brown 1980).

9.1.18. PLASMA KININS: KALLIDIN AND BRADYKININ (BK)

Plasma kinins belong to the most potent vasodilator substances known, surpassing about ten times a similar effect of histamine on a molar basis (Schachter 1964, Erdos 1966, Melmon and Cline 1967). Their dilator effects are based on a direct action on vascular smooth muscle in several vascular beds, resulting in a sharp fall both in the diastolic and the systolic blood pressure and in an increase in the heart rate and the cardiac output, respectively (Erdos 1966, Douglas 1970, Ganong 1979). On the other hand, BK proved to be a constrictor for isolated feline middle cerebral arteries (Hanko and Hardebo 1981), the umbilical vessels and the ductus arteriosus, while dilating the fetal pulmonary vascular bed (Melmon et al. 1968, Ganong 1979). Apart from this type of involvement in fetal circulatory adjustment, plasma kinins may also participate in

the induction of local vasodilation in active tissues and in thermoregulatory vascular adjustment. The effects of BK on circulatory homeostasis are accomplished through increasing adrenaline output from the adrenal medulla (Feldberg and Lewis 1964).

Both arterial blood pressure and heart rate increase following central administration of BK (Lambert and Lang 1970, Correa and Graeff 1975, Unger et al. 1981b).

9.1.19. PHENYLALANYL-METHIONYL-ARGINYL-PHENYLALANIL AMIDE (FMRFamide)

FMRFamide was detected in, and isolated from, nonmammalian species (Greenberg et al. 1973, Price and Greenberg 1977) as a mediator of cardioreactivity unassociated with any neurotransmitters identified so far. FMRFamide has positive inotropic and chronotropic effects and induces rhythmic activity in quiescent heart preparations (Greenberg and Price 1980; see also Chapter 13).

9.2. MISCELLANEOUS CIRCULATION-RELATED EFFECT OF ENDOGENOUS PEPTIDES

VIP, as a vasodilatory substance may play an important role in the process of vascularization (Polak and Bloom 1979).

Large doses of i.v. injected NT cause a dose-related, visible cyanosis in rats which is not associated with changes in the partial pressure of arterial O_2 and CO_2 (Carraway and Leeman 1973).

(For the role of various growth factors in promoting the proliferation of vascular smooth muscle and capillary endothelial cells see Chapter 8.)

9.3. THE ROLE OF ENDOGENOUS PEPTIDES IN FLUID-ELECTROLYTE BALANCE

In this context, three main systems operating through peptide mediators should be considered. These include ADH, the renin-angiotensin-aldosterone system and the recently discovered atrial natriuretic peptides/factors (ANP/ANF). Some other, possibly related peptide factors are only vaguely characterized at present, or contribute to the fluid-electrolyte balance (FEB) in a more restricted way by modulating drinking behavior, affecting secretion and vascular permeability. Since most issues related to these regulatory peptide systems are well documented by text books and recent reviews (e.g., for a review on the renin angiotensin system see Vallotton 1987) only some recent developments in these subjects will be mentioned, except ANPs that will be discussed in more detail.

9.3.1. VASOPRESSIN: ANTIDIURETIC HORMONE (ADH)

Besides the CNS's thirst controlling mechanism ADH provides the most important factor in the control of osmotic pressure and volume of the body fluids. Experimentally, Ach or prostigmin injections into the hypothalamic supraoptic and paraventricular nuclei, and the electrical stimulation of these areas, stimulate ADH secretion, whereas body dehydration and stimuli acting on the volume receptors in the atria and pulmonary veins directly affect the neurons of the mentioned nuclei.

Elevated ADH plasma levels increase permeability of the distal tubules and collecting ducts of the kidney to water resulting in an excessive reabsorption of water and a diminished output of urine with a markedly elevated osmotic pressure (Brazeau 1971, Guyton 1981). ADH also increases the permeability of brain capillaries to water, especially following intraventricular administration of the drug (Raichle et al. 1979).

9.3.2. ANGIOTENSIN II AND III (AT-II, AT-III)

AT exerts both indirect (aldosterone) and direct effects (kidney and brain) on the composition and volume of body fluids. As an indirect effect, AT-induced aldosterone secretion results in an excessive sodium retention in the tubules and in antidiuresis. The direct effect is accomplished in two phases: first, electrolyte and water secretion is reduced and this is followed by an opposite effect, the final result being dependent on many factors, but the usual response in man is a prompt antidiuresis (Douglas 1970).

At the periphery, AT-II generation starts with a cleavage of a AT-I decapeptide, from angiotensinogen (a liver-derived circulating protein substrate) by renin (an enzyme) produced mostly in the juxtaglomerular cells in the kidney but also in several extrarenal tissues (for discussion see Vallotton 1987 and also Chapter 5).

Renin secretion is mainly controlled by hemodynamic, neurogenic and humoral signals (for details see Fray 1980, Vallotton 1987) including peptides such as VIP, parathyroid hormone, glucagon (stimulators), AT-II and ANF (inhibitors). In contrast to a majority of endocrine cells, juxtaglomerular cells secrete renin when hyperpolarized or when free calcium is lowered intracellularly (Churchill 1985, Vallotton 1987).

The AT-I decapeptide is converted to AT-II octapeptide by angiotensin-converting enzyme (ACE) mainly produced in the lung but also in extrapulmonary tissues (Favre et al. 1974; for reviews see Soffer 1976, Stewart et al. 1981). ACE can also generate AT-III heptapeptide directly from des-Asp-AT-I (for AT-II receptor and for the mechanism of action of AT-II see Capponi et al. 1985a,b, Smith et al. 1984, Vallotton 1987). In contrast to previous suggestions assuming that AT-III is the final angiotensin mediator on the adrenal cortex (Chin and Peach 1974, Lohmeir et al. 1975, Devinck et al. 1977, Ganong 1979), more recent evidence supports the view that AT-II rather than AT-III is the major natural mediator eliciting adrenocortical responses (Braley et al. 1983).

The brain has its own complete renin-angiotensin system independent of the periphery (for recent reviews see Saavedra and Plunkett 1985, Phillips 1987 and also Chapter 5), but a bidirectional interplay seems to exist between the peripheral and central systems. Blood-born AT-II does not cross easily the blood-brain barrier but can reach the AT receptors present in the circumventricular organs having no blood-brain barrier.

Aldosterone freely enters the brain where it may modify the function of local renin-angiotensin system and vice versa.

Stimulation of water intake (thirst) is the most unequivocal central effect of angiotensin (Wagner and Braunwald 1956, Daniels-Severs et al. 1971, Fitzsimmons 1980). Drinking can be elicited both by peripheral and central administration of AT-II suggesting that hypovolemia-induced production of AT-II at the periphery may result in a series of AT-mediated events, both peripheral and central. The circumventricular organs (CVO), especially the subfornical organ (SFO), are the likely targets for the elevated plasma AT-II levels (Phillips 1978, 1987, Simpson 1981, Saavedra and Plunkett 1985).

Besides eliciting thirst, central injection of AT-II also produces sodium appetite (Fitzsimmons 1980), what peripheral AT infusion does not do unless applied in extremely high doses leading to elevated aldosterone levels and this enhances central AT-II induction of sodium appetite by increasing the number of brain AT receptors (Fluharty and Epstein 1983, Phillips 1987).

The AT-induced ADH secretion is related to the centrally rather than to the peripherally generated AT-II (Keil et al. 1975, Hoffman et al. 1977, Padfield and Morton 1977, Simonnet et al. 1979, Sterling et al. 1980). The site of the ADH secretion-inducing action of the centrally administered AT-II is still uncertain.

AT-II has some central actions that oppose its above discussed effects serving fluid and sodium conservation. For instance, it can produce natriuresis probably by inhibitng peripheral aldosterone secretion (Brooks and Malvin 1980) or by releasing natriuretic hormone (Buckalev and Gruber 1984). Intracerebroventricularly administered AT-II inhibits also renin

secretion via releasing ADH (Eriksson and Fyhrquist 1976, Lokhandwala et al. 1978, Malayan et al. 1979).

The brain renin-angiotensin system appears to be sensitive to actual sodium concentrations (Kapsha et al. 1979, Mann et al. 1980, Mendelsohn et al. 1982), and central AT-II may function to regulate brain sodium levels.

Central AT-II also facilitates ACTH release partly by inhibiting CRF release (Spinedi and Negro-Vilar 1983), partly at anterior pituitary level (Keller-Wood et al. 1986).

9.3.3. ADRENOCORTICOTROP HORMONE (ACTH)

ACTH indirectly affects both volume and composition of the body fluids by increasing both mineralo- and glucocorticoid secretions from the adrenal cortex. Thus, the final effects of ACTH on the fluid/electrolyte balance is determined by the largely opposite effects exerted on the kidneys by the two types of corticoids (for details see Sayers and Travis 1970, Ganong 1979).

9.3.4. ATRIAL NATRIURETIC FACTOR(S)/PEPTIDE(S) (ANF/ANP)

The chain of events leading to the discovery of ANPs started in the 1940s (for a historical overview see De Wardener and Clarkson 1985), but it was the pioneering experiment of de Wardener and Clarkson (1982) that has suggested that changes (actually a fall) in tubular sodium reabsorption might be due to a change in the concentrations of a circulating substance other than aldosterone. De Bold (1979) with his coworkers (1981) have demonstrated first that profound diuresis and natriuresis could be produced by i.v. infusion of atrial extracts in rats. This has been confirmed also in other species including human (Sonnenberg et al. 1982, Grammer et al. 1983, Nemeth and Gilmore 1983). Several structurally related peptides have been identified to account for these activities and designated as cardionatrin I (Flynn et al. 1983), atriopeptins

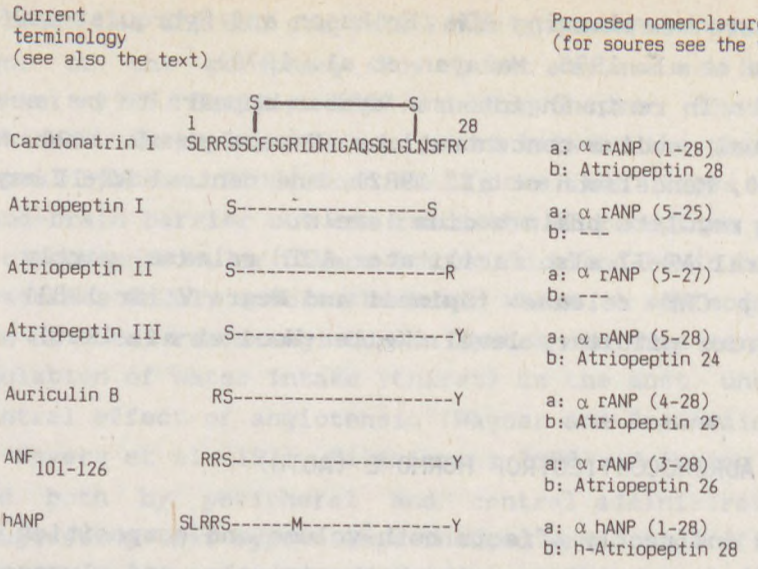


Fig. 9.1. Amino acid sequences and terminology of atrial natriuretic peptides /after Kanagawa et al. 1984 (a) and Needleman and Greenwald 1986 (b)/. The one letter amino acid symbols are used

I-III (Currie et al. 1984, Geller et al. 1984), atrial natriuretic factor(101-126) (Seidah et al. 1984), auriculin B (Atlas et al. 1984), α-rat atrial natriuretic polypeptides (α-rANP; Kanagawa et al. 1984), α-human atrial natriuretic factor (α-hANP; Kanagawa and Matsuo 1984), respectively (Fig.9.1).

As it is apparent from the listing, the currently used terminology is rather heterogenous. A simple comparative terminology has been proposed by Needleman and Greenwald (1986) and another version taking into account also the C-terminally shorter peptides, by Kanagawa et al. (1984) both demonstrated in Fig. 9.1. Human, rat and mouse cDNAs encoding the larger molecular weight precursor to the various ANPs have been cloned, sequenced and their deduced amino acid sequences determined (Greenberg et al. 1984, Kanagawa et al. 1984, Maki et al. 1984a,b, Nakayama et al. 1984, Nemer et al. 1984, Oikawa et al. 1984, Seidman et al. 1984a,b, Yamanaka et al. 1984, Zivin et al. 1984). The human, porcine and bovine mature C-terminal 28 amino acid peptides (which are identical) differ

from their rat and mouse counterparts in that Met is substituted for Ile at position 12 (see Fig.9.1).

The main production sites of ANPs in the mammalian organisms are the atrial myocytes, but natriuretic extracts have also been prepared from the plasma, urine and a number of organs of normal human and animals and from the plasma and urine of patients with chronic renal failure. The structure of NAPs of nonatrial origin is poorly characterized and also their function differ from those of NAPs (Trippodo et al. 1984).

ANPs are stored mainly in a prohormone form in the perinuclear granules of atrial myocytes (Needleman et al. 1985). The major circulating form appears to be ANP(1-28) but minor amounts of ANP(5-28) have also been detected (Schwartz et al. 1985). The basal plasma levels are low but increases can be elicited by volume expansion-induced atrial stretch (Lang et al. 1985), vasoconstrictor agents elevating atrial pressure (Manning et al. 1985), immersion in water (Katsube et al. 1985), atrial tachycardia (Schiffrin et al. 1985) and by high salt diets. Specific ANP receptors have been localized in glomeruli of the kidneys and in the zona glomerulosa of the adrenal gland (Lynch et al. 1986).

Most ANPs have overall effects opposite to those of angiotensin II (for reviews see Mills 1984, Sagnella and McGregor 1984, Needleman and Greenwald 1986). They increase glomerular filtration and enhance natriuresis, potassium excretion and diuresis by direct renal actions, inhibit aldosterone secretion (Briggs et al. 1982, Atlas et al. 1984, Campbell et al. 1985, Cantin and Genest 1985), cause vasodilation by a direct action on the vascular smooth muscle (Currie et al. 1983, Garcia et al. 1984, Hintze et al. 1985, Wakitani et al. 1985) and counteract the action of nonadrenaline, angiotensin II and histamine (Baines et al. 1983, Atlas et al. 1984).

Immunoreactive ANPs (Tanaka et al. 1984, Jacobowitz et al. 1985, Saper et al. 1985, Zamir et al. 1986) and specific ANP receptors (Quirion et al. 1984, Lynch et al. 1986, Saavedra et al. 1986) are present in the brain, especially in the hypothalamus where ANP levels can be altered by a high salt diet (Tanaka et al. 1984) or by dehydration (Samson 1985).

Centrally administered ANPs stimulate diuresis, natriuresis K^+-uresis and inhibit water intake, salt appetite (Fitts et al. 1985, Matsotto and Negro-Vilar 1985, Nakamura et al. 1985, Israel and Barbella 1986) and induce ADH release (Samson 1985). (For recent reviews on APNs see Buckalew and Gruber 1984, de Wardener and Clarkson 1985, Needleman and Greenwald 1986.)

9.3.5. SODIUM TRANSPORT/Na^+ K^+-ATPase INHIBITORY ENDOGENOUS SUBSTANCES

In whole plasma, and in plasma, urine, kidney and brain extracts, sodium transport-inhibiting activities can be detected that often are accompanied by natriuretic activities (for a recent review see de Wardener and Clarkson 1985).

One major group of endogenous substances having such activity consists of the digoxin/oubain-like substances. The term refers to their capability of crossreacting with antibodies raised against cardiac glycosides and inhibiting Na^+K^+-ATPase. Most of these substances partially purified from the brain, heart, adrenal gland and urine of mammals are of low molecular weight (1,000-2,500) and non peptidic in nature. In contrast, the majority of Na^+K^+-ATPase inhibitory endogenous materials other than the digoxin/oubain-like ones have peptide-like characteristics (for discussion see de Wardener and Clarkson 1985).

9.3.6. SUBSTANCE P (SP)

Available data indicate that SP, a powerful vasodilator, may participate in the regulation of the fluid-electrolyte balance by its apparent capability of altering the composition and volume of the body fluids through increasing vascular permeability (Pernow and Rosell 1975, Lembeck et al. 1977), stimulating glandular secretion (Haefely and Hurlimann 1962, Vogler et al. 1963), and by acting as a thirst inhibitor, through a central mechanism (DeCaro et al. 1978).

9.3.7. CALCITONIN GENE-RELATED PEPTIDE (CGRP)

CGRP coexisting with SP in perivascular nerves potentiates tachykinin-induced plasma extravasation (Gamse and Saria 1985).

9.3.8. OPIOID PEPTIDES: ENDORPHINS (EP), ENKEPHALINS (Enk) AND DYNORPHINS (Dyn)

In contrast to previous views assuming that opioids cause antidiuresis via stimulating vasopressin release (de Bodo 1944, Duke et al. 1951), more recent results proved to be conflicting. Previous results have indicated that EP and stable Enk analogs induce an elevation in plasma vasopressin levels in various species with all of its functional consequences (Weitzman et al. 1977, Bisset et al. 1978, Firemark and Weitzman 1978). Now there is convincing evidence that exogenous and endogenous opioids may either decrease or increase vasopressin release (Aziz et al. 1981), indicating that the vasopressor release-inhibiting, i.e. diuretic effects are, at least as important as the antidiuretic ones (Kamoi et al. 1979). Opioid peptides directly inhibit vasopressin release in vitro (Iversen et al. 1980) and there is now considerable evidence that opoid peptides predominantly inhibit the release of vasopressin also in man (for discussion see Grossman and Rees 1983) acting probably through the relatively naloxone-insensitive κ-receptors (Lightman et al. 1981; for discussion see Cowan and Gmerek 1986) in the neurohypophysis but also in the periphery (Blackburn et al. 1985).

At present it is difficult to reconcile the more recent data with the earlier reports on opioid-induced vasopressin release and antidiuresis. As a tentative explanation it may be proposed that opioids induce an effect centrally which independently of vasopressin and hemodynamic changes, alters the

renal function to cause antidiuresis through as yet unknown mechanisms. In certain species, opioids modulate drinking behavior (see Chapter 5). It is also worth mentioning that in the powerful antidiarrhoeal effect of opioids (Miller et al. 1978, 1980), besides their well-documented actions on intestinal motility, their intestinal antisecretory properties may also be important (Powell 1981).

9.3.9. VASOACTIVE INTESTINAL POLYPEPTIDE (VIP)

Intestinal secretion, and consequently, water and electrolyte secretion both from the small and the large intestines of dogs and rats are strongly affected by VIP (Barbezat and Grossman 1971, Schwartz et al. 1974, Makhlouf 1977, Said 1979).

9.3.10. BOMBESIN

Bombesin was shown to produce renal vasoconstriction, thereby activating the renin-angiotensin system and causing antidiuresis (Polak and Bloom 1979).

9.3.11. NEUROTENSIN (NT)

NT can cause a marked increase in vascular permeability in the limbs (Carraway and Leeman 1973), but not in the adipose tissue (see Rosell et al. 1976). This is consistent with the excessive increase in arterial hematocrit observed after i.v. injection of NT in rats. Being a vasodilatory substance, NT was suggested to be one of the as yet unidentified peptides that plays a role in neurogenic vasodilation (Carraway and Leeman 1973).

9.3.12. PLASMA KININS

Plasma kinins are among the most potent substances known to increase vascular permeability acting on small venules rather than on the capillaries, where this peptide separates junctions between the endothelial cells. Due to this effect, local bradykinin injection can cause a marked antihistamine-resistant edema in the tissues (Majno et al. 1961).

9.4. THE ROLE OF ENDOGENOUS PEPTIDES IN PATHOLOGICAL CIRCULATORY PROCESSES

9.4.1. HYPERTENSION

(1) The renin-angiotensin (AT)-aldosterone system

Since renal hypertension accompanying renal artery constriction was first observed, it was suspected that hypertension is due to an increased renin secretion. Indeed, conditions connected with unilateral renal artery stenosis are sometimes connected with elevated renin and AT levels. However, in many experimental situations, lowering of the arterial pressure also increased renin secretion, and in turn, circulating levels of renin and AT are not always elevated in chronic renal hypertension.

The activity of the renin-AT system changes in many pathological (and physiological) conditions not associated with hypertension. These include sodium loosing conditions, congestive heart failure with hyponatremia, cirrhosis, nephrosis, and Bartter's syndrome. On the other hand, high renin levels can usually, but not always, be detected in renal hypertension, while renin levels are commonly normal, or of low levels, in essential hypertension and in hypertension accompanying primary aldosteronism.

The brain AT-II may play a role in hypertension because it controls three major central mechanisms of blood pressure regulation such as sympathetic activation, vasopressin release

and inhibition of the baroreflex at the level of n. tractus solitarii (for a review see Phillips 1987). In spontaneously hypertensive rats (SHR) used as an experimental model for hypertension, increased AT-II levels in the cerebrospinal fluid (Ganten et al. 1975), increased AT-II binding to brain receptors (Stamler et al. 1980), increased sensitivity of neurons to AT-II (Felix and Schelling 1982), blunting of the baroreflex (Casto and Phillips 1984) and elevated levels of angiotensin-converting enzyme (ACE) in the intermediate and anterior lobes (Chevillard and Saavedra 1983) have been described. Brain AT-II receptors are up-regulated also in low renin hypertension models whereas in high renin hypertension models the high circulating AT-II may also reach receptor areas in the CNS otherwise inaccessible to AT-II. Angiotensin receptor antagonists (Phillips et al. 1977, Phillips 1979) and ACE inhibitors (Elijovich and Krakoff 1982, Phillips 1984) have been successfully applied to combat hypertension both in experimental models and in the clinical practice (for reviews see Heel et al. 1980, Antonaccio 1982, Johnston 1984, Mendelsohn 1984), though the mechanism of action of ACE inhibitors remains largely unknown for the time being (for additional considerations see Benuck and Marks 1979, McGeer and Singh 1979, Swerts et al. 1979, Yang et al. 1981, Ronai et al. 1983).

(2) Vasopressin

In SHRs there is a decrease in the CNS concentrations of Arg-vasopressin in a number of brain regions (Morris and Keller 1982, Mohring et al. 1983), and responses to centrally administered vasopressin are reduced (Izdebska et al. 1982). Plasma vasopressin levels are high both in malignant hypertension in man and in low renin hypertension animal models, whereas in human benign essential hypertension both decreased plasma levels and enhanced urinary Arg-vasopressin excretion have been reported to occur (Khokkar et al. 1974, Mohring et al. 1976, Padfield et al. 1976).

In rats with malignant or renal hypertension, a causal role was suggested for vasopressin (Kovacs et al. 1964, Melissinos et al. 1973, Mohring et al. 1976), whereas in man, the changes in the plasma levels of vasopressin were interpreted as consequences, rather than causal factors of hypertension (Padfield et al. 1976).

(3) Substance P (SP)

Intracerebroventricular injection of SP produced a three times stronger pressor reflex in SHRs than in normotensive controls, and the involvement of a central component of the baroreflex arc in the enhanced response to SP has been suggested in SHRs (Unger et al. 1981b).

(4) Opioid peptides

Reduced pain sensitivity observed in renal and low renin hypertension models as well as the restoration of normal pain sensitivity by naloxone suggested the involvement of nociception-related opioid mechanisms in these hypertension models (Zamir and Segal 1979, Zamir et al. 1980).

The ability of EPs to modulate painful and noxious stimuli affects cardiovascular homeostasis, due to the interaction existing between brain circuits involving central blood pressure regulation and pain perception (Terenius 1978, Holaday et al. 1979).

Reduced naloxone binding, altered levels of Met-Enk-like immunoreactivity and changed opioid binding observed in some brain regions of SHRs (Nakamura and Hayashi 1982), as well as elevated levels of Enk-like substances in the neurohypophysis (Morris et al. 1981) and the elevated responses to centrally administered Leu-Enk seen in SHRs, and finally the indications that central Enk-like peptides may attenuate the baroreceptor reflex (Schaz et al. 1980) all suggest the involvement of central opioid mechanisms in this experimental hypertension model.

At the periphery, a decreased content of Met-Enk immunoreactivity was found in the celiac and superior cervical ganglia, adrenal medulla and in the salivary glands of SHRs

(Di Giulio et al. 1979).

As a gross approximation it can be said that Enk-related peptides appear to participate in central cardiovascular regulatory processes ultimately with a pressor result, whereas β-EP seems to mediate hypertensive responses under both physiological and pathological conditions (De Jong et al. 1983; see also sections 9.1 and 9.3.2).

(5) Vasoactive intestinal polypeptides (VIP)

SHRs have been reported to have significantly lower VIP levels in the hippocampus and spinal cord, but higher levels in the midbrain, in comparison to normotensive controls (Lewis et al. 1986).

(6) Bradykinin (BK)

SHRs display enhanced pressor responses to centrally administered BK as compared to normotensive controls, and BK may be one of the endogenous peptides which impair baroreflex function (Unger et al. 1981a,b).

9.4.2. CIRCULATORY SHOCK

(1) Opioid peptides

A number of studies has convincingly demonstrated the involvement of EP and the therapeutic potential of opiate receptor blocking agents in various types of shock across a variety of species, such as hemorrhagic shock (Faden and Holaday 1979, Gurll et al. 1980a,b,c, Vargish et al. 1980), endotoxin shock (Holaday and Faden 1978, Faden and Holaday 1980, Reynolds et al. 1980) and spinal shock (Holaday and Faden 1980, Elam et al. 1980). Hypophysectomy was shown to counteract the therapeutic effects of naloxone in endotoxic and hypovolemic shock (Holaday and Faden 1979).

Although the mechanism of the beneficial effects of naloxone in experimental shock is largely unclear, the observations of Sandor and associates (1985) have shed some light on the mechanisms involved. As it was demonstrated, subcutaneous morphine pretreatment resulted in a decrease, whereas naloxone

pretreatment in an increase in the bleeding volume. While intracerebroventricular administration of Met-Enk and dynorphin was ineffective, the bleeding volume was markedly reduced by intracerebroventricularly administered β-EP and, contrarily, it was significantly elevated by microinjection of specific β-EP antisera into the lateral cerebral ventricle.

(2) Substance P (SP)

SP infusion failed to affect the survival rate of rats following hemorrhagic shock. In dogs with normotension, SP infusion produced an elevation in the hypothalamic and cerebral cortical blood flow. During bleeding-induced hypertension SP still produced an increased blood flow in the carotid but not in the femoral artery and increased the blood flow both in the hypothalamus and cerebral cortex (Kovach et al. 1977).

(3) Vasoactive intestinal polypeptide (VIP)

Hemorrhagic shock (Sakio et al. 1979), intestinal ischemia (Modlin et al. 1978) and acute respiratory acidosis (Said 1979) are accompanied by an increased VIP release from the gastrointestinal tract increasing VIP levels mainly in the portal circulation, but VIP release may also be provoked from the brain by increased arterial blood pCO_2 and shock.

(4) Vasopressin: antidiuretic hormone (ADH)

Severe blood loss results in an increase in ADH secretion and this, in turn, in an increase in water retention by the kidneys and also in blood pressure elevation based on arteriolar vasoconstriction (for references see Guyton 1981). By all indications, ADH may play a much greater role in blood pressure regulation than was previously thought.

(5) Plasma kinins

Increased kinin formation may contribute to hypotension and may participate in the pathomechanism of anaphylaxis, burn shock and mechanical trauma.

9.4.3. MISCELLANEOUS CIRCULATION-RELATED DISORDERS

(1) Migraine

Vasomotor responses may significantly contribute to the pathophysiology of migraine (Olesen 1985) and several agents including endogenous peptides have been implicated, but none have been proved responsible so far for vasomotor response and the headache associated with migraine. Nerve fibres containing vasoactive peptides such as SP, CGRP, VIP and NPY have been demonstrated around extra- and intracranial blood vessels in several species including man (Edvinsson 1985, Jansen et al. 1986), and a BK-like substance has also been suggested as a possible factor in migraine. Intermittent opiate receptor subsensitivity postulated by Sicuteri et al. (1980) may contribute to the development of spontaneous pain and in the poor analgesic effect of morphine during migraine attacks.

(2) Ischemic cerebrovascular diseases

Although naloxone, an opiate peptide antagonist, was reported to reverse neurological deficits and improve local blood flow folowing cerebral ischemia in dogs, gebrils and humans (Faden et al. 1982, Hosobuchi et al. 1982, Faden 1983a,b), the role of opioid peptides in the pathogenesis of stroke requires further investigations (Holaday and D'Amato 1982). Mostly because naloxone, apart from being a specific opiate antagonist, may also have nonopiate receptor-mediated effects on the cerebrovascular smooth muscle cells (Sawynok et al. 1979, Faden 1983a,b, Badawy et al. 1983, Sasaki et al. 1984).

(3) Inflammatory and allergic reactions

Histamine and bradykinin are implicated in the mechanism of acute allergic reactions and in various aspects of the inflammatory response, but SP may also be involved in the development of some pathological conditions mediated by these compounds. Because kinins can produce pain, swelling, redness and leukocyte migration, their involvement in inflammation seems likely (Douglas 1970). SP can cause the development of the

four major features of the inflammatory reactions: local vasodilation, increased capillary permeability, pain and migration and accumulations of leukocytes (Bisset and Lewis 1962). SP is more potent than bradykinins in causing the release of histamine from mast cells (Johnson and Erdos 1973). It should be stressed that available evidence ties SP neurons to the pain modality (Euler and Gaddum 1931, Hokfelt et al. 1975, 1979, Olgart et al. 1977a,b).

NT is a vasodilatory peptide having the capability of inducing changes in vascular permeability (Carraway and Lebieman 1973). Therefore, NT may be one of the as yet unidentified peptides that play a role in the inflammatory response.

(4) Varia

Common intestinal diarrhoeal conditions may be due to excessive local VIP release, because VIP is capable of inducing extreme vasodilation and secretion in the intestine (Polak and Bloom 1979).

Kinins appear to be responsible for episodes of vasodilation in patients with carcinoid tumors (Ganong 1979).

Due to its hypotensive actions NT may play a significant role in the pathophysiology of the dumping syndrome (Polak and Bloom 1979).

Enkephalins were identified in tumors causing cardiovascular symptoms (Sullivan et al. 1978).

Hypothalamic and hypophyseal tumors can destroy brain regions associated with ADH secretion which results in the development of diabetes insipidus. Lack of ADH secretion is characterized by a rapid, enormous increase in urinary output and by a constant thirst caused by the loss of fluids through the kidneys (Guyton 1981).

The role of VIP and "VIPomas" in the development of the watery diarrhoea syndrome (Verner-Morrison syndrome, WDHA or WDHH syndrome) is discussed in Chapter 6.

Syndrome of inappropriate ADH secretion (SIADH) characterized by decreased extracellular sodium concentrations with only a small increase in body fluid volume develops when tumors, especially bronchogenic carcinomas invade the hy-

pothalamus resulting in an excess of ADH secretion (Schwartz-Bartter's syndrome).

9.5. THE RESPIRATORY EFFECTS OF ENDOGENOUS PEPTIDES

The respiratory depressant effects of opiates have long been known. Respiratory frequency and tidal volume were shown to be depressed by intraventricularly administered β-EP, D-Ala^2-Met-Enk and Met-Enk in cats (Florez et al. 1978), but the three peptides affected the frequency and depth-generating mechanisms differently. It was suggested that the interaction of these peptides with opioid receptors at the suprapontine level may elicit stimulatory behavioral effects which modify the direct action of the peptides upon the respiratory centre.

Intracisternal injection of β-EP caused a marked respiratory depression in lightly anesthetized dogs (Moss and Friedman 1978). Iontophoresis of morphine or Met-Enk reduced the peak, but not the basal, discharge frequency of respiration-related units in the n. tractus solitarii, n. ambiguus and n. parabrachialis medialis of the cat brain (Denavit-Saubie et al. 1978). The chemosensitive structures of the medulla oblongata are especially sensitive to the respiratory depressant effects of opioid (Moss and Friedman 1978, Florez et al. 1980, Zobrist et al. 1981).

While intravenously administered β-EP was without any respiratory effects in man (Catlin et al. 1980). Met-Enk, Leu-Enk and less potently, morphine and β-EP depressed carotid chemoreceptor activity in experimental animals predominantly through a μ receptor-mediated mechanism (McQueen and Riberi 1980).

Although endogenous opioid peptides and their receptors are present at most strategic sites both in the CNS and the periphery, including peripheral chemosensors experiments (Wharton et al. 1980, Hansen et al. 1982) aimed at assuming a physiological role for endogenous opioids in respiratory regulation remained inconclusive (Stephen et al. 1976, Chernick and Russel 1978, Lawson et al. 1979, Moss and Scarpelli 1979, Willer

et al. 1979, Fleetham et al. 1980; for discussion see McQueen 1983).

Results obtained with intracerebroventricularly administered substance P in rabbits and cats remained contradictory (Euler and Pernow 1954, Haefely et al. 1962), though a slight decrease in the respiratory frequency was registered following intracisternal injection of SP in rats (Fuxe et al. 1979).

Thyrotropin-releasing hormone powerfully increases the respiratory rate and minute volume without affecting tidal volume in a variety of species (Andry and Horita 1977, Myers et al. 1977, Hedner et al. 1981).

Systematically administered VIP exerts an excitatory action on respiratory chemoreceptors (Said and Mutt 1970).

β-melanocyte-stimulating hormone induces hyperpnoea in rabbits and rats (Dyster-Aas and Krakau 1965, Sakamoto 1966).

9.5.1. THE POSSIBLE FUNCTIONS OF ENDOGENOUS PEPTIDES PRESENT IN THE RESPIRATORY TRACT

Several types of endogenous peptides have been detected in neural and secretory elements of the respiratory tract where they may partake in a variety of functions (Polak and Bloom 1984, Lundberg and Saria 1987).

(1) Vasoactive intestinal polypeptide (VIP)

VIP-like immunoreactivity has been localized to local ganglion cells and nerve fibres associated with airway smooth muscle, submucosal glands and bronchial vessels (Uddman et al. 1978, Dey et al. 1981, Laitinen et al. 1985). VIP is present in, and may be coreleased with Ach from airway cholinergic nerves (Laitinen et al. 1985). Immunoreactive VIP can be released by nerve stimulation from tracheobronchial preparations (Matsuzaki et al. 1980, Said 1984). Specific VIP receptors coupled to adenylate cyclase are present on airway smooth muscle glands, epithelium and vascular smooth muscle (Robberecht et al. 1981, Carstairs and Barnes 1986a).

VIP is the most potent endogenous bronchodilator discovered so far under in vitro conditions (Ito and Takeda 1982, Palmer et al. 1986a) and is a promising transmitter candidate for the nonadrenergic inhibitory nerves in tracheobronchial smooth muscle (Cameron et al. 1983, Said 1984). VIP reverses the serotonin-induced bronchoconstriction in the cat (Diamond et al. 1983). In man, dose limitations due to the circulatory effects of VIP make it difficult to demonstrate its bronchodilator effect in vivo (Barnes and Dixon 1984, Palmer et al. 1986c).

In animals, VIP is also a potent stimulant of mucus secretion (Peatfield et al. 1983) and of ion transport (Nathanson et al. 1983) and is a powerful vasodilator also in the bronchial circulation (for references see Barnes 1987, Lundberg and Saria 1987).

(2) Peptide histidine isoleucin (PHI)
and peptide histidine methionine (PHM)

PHI and PHM, the human equivalent of PHI, are structurally related to VIP, and VIP and PHM are contained within the very same propeptide (Itoh et al. 1983). Immunoreactive VIP and PHI likely coexist in nerves of airway smooth muscle (Lundberg et al. 1984b). PHI is approximately equipotent to VIP in relaxing the airway smooth muscle (Lundberg et al. 1984b, Palmer et al. 1986a) but is less potent as a vasodilator (Lundberg et al. 1984b, Barnes 1987). Both peptides may be involved in the noncholinergic vagal control of blood flow in the tracheobronchial smooth muscle layer and mucosa (Lundberg and Saria 1987).

(3) Substance P (SP)

SP-like immunoreactivity has been demonstrated in neural elements of lung, particularly in nonhuman mammalian species (Nilsson et al. 1977, Wharton et al. 1979). At least part of the SP-like immunorecativity resides within primary sensory neurons of nonvagal origin, the cell bodies of which being located in the nodose ganglion (Terenghi et al. 1983, Polak and Bloom 1984). In the guinea pig, tachykinin-containing sensory ganglion cells of the upper thoracic ganglia have been shown

to project to the lower airways via sympathetic pathways (Saria et al. 1985). SP-immunoreactivity was shown in the rat phrenic nerve (Malthe-Sorenssen and Oktedalen 1982). Recent evidence suggests that not only SP but the whole family of related tachykinins such as substance K, neuropeptide K and a not yet fully identified, eledoisin-related peptide, are present in nerves of the lung. SP-containing nerves are found in close association with the bronchial epithelium and around the blood vessels, and also within the tracheobronchial smooth muscle layers and around local ganglion cells (Lundberg et al. 1984c, 1985a, Polak and Bloom 1984, Hua et al. 1985, Lundberg and Saria 1987).

SP, substance K (Saria et al. 1984) and an eledoisin-related material (Lundberg and Saria 1987) can be released from isolated perfused guinea pig lung by a number of chemical stimuli and also by electrical stimulation of the distal end of cut vagal nerves. Tachykinin receptors have been detected in the smooth muscle of the entire bronchial tree (Carstairs and Barnes 1986b).

Tachykinins contract bronchial smooth muscle (Andersson and Persson 1977, Lundberg and Saria 1982a, Lundberg et al. 1983a, Finney et al. 1985) and might be the transmitter of noncholinergic bronchoconstrictor nerves in guinea pigs (Andersson and Grundstrom 1983). Tachykinins cause vasodilation in nasal mucosa (Lundblad et al. 1984) as well as in the trachea and enhance bronchial mucus secretion. They participate also in the mediation of protein extravasation. Tachykinins, particularly SP, are potent inducers of protein extravasation by increasing the permeability of postcapillary venules (Lembeck and Holzer 1979, Lundberg and Saria 1982b, Persson et al. 1985).

(4) Calcitonin gene-related peptide (CGRP)

CGRP-immunoreactivity is present mainly in the sensory nerves of the lung (Lundberg et al. 1985a, Lundberg and Saria 1987). CGRP-immunoreactivity coexists with tachykinins both in the cell bodies of sensory ganglia and in the projections to the airways (Lundberg et al. 1985a, Lundberg and Saria 1987).

CGRP is also a potent vasodilator but an uncertain bronchoconstrictor (Andersson and Grundstrom 1983, Lundberg et al. 1985a, Palmer et al. 1985), CGRP potentiates extravasation by tachykinins in the airway mucosa (Brain and Williams 1985, Gamse and Saria 1985).

(5) Neuropeptide Y (NPY)

NPY, in coexistence with norepinephrine and galanin colocalized with VIP have been detected in human airway nerves (Polak and Bloom 1986). Neither of these neuropeptides appears to affect bronchial smooth muscle directly; NPY may have a role in the regulation of bronchial blood flow.

(6) Bombesin (BOM)

Bombesin has been detected in the largest concentrations in fetal and neonatal lung, particularly in humans, but also in experimental animals (Polak and Bloom 1984). Bombesin is present in secretory cells of bronchial mucosa; the number and the bombesin content of cells fall rapidly in the perinatal period. Bombesin may have a trophic/growth promoting role in the lung (Oie 1983, Polak and Bloom 1984).

(7) Varia

ACTH and immunoreactive cholecystokinin, gastrin-releasing peptide and somatostatin have also been found in lung extracts (Polak and Bloom 1984, 1986) but the precise localization and the possible function(s) of these peptides have not yet been elucidated.

9.5.2. THE POSSIBLE ROLE OF PEPTIDE FACTORS IN THE PATHOMECHANISM OF ASTHMA AND OTHER PATHOLOGICAL PROCESSES

9.5.2.1. ASTHMA

Several lines of indirect evidence suggest that airway neuropeptides, particularly tachykinins and CGRP and, possibly, VIP and PHM may have a role in the pathomechanism of asthma (Barnes 1984, 1986). Processes stimulated by tachykinins and, to a lesser extent, CGRP such as bronchoconstriction, bronchial mucus secretion and protein extravasation appear with exaggerated intensity in asthmatic state. C-fibre afferents in the lung, which contain tachykinins and CGRP, can be activated by local irritation of the airway mucosa by chemicals, by mediators released during allergic reactions and by local tissue damage (Lundberg and Saria 1987). Capsaicin, the pungent agent of hot peppers is a widely used experimental tool in studying peptide-containing sensory nerves (Jancso et al. 1968, 1977, Szolcsanyi 1984). Capsaicin appears to selectively affect a population of C-fibre afferents; acute administration of relatively low doses release tachykinin and CGRP from isolated, perfused lung preparation whereas upon exposure to a high dose tachykinin- (Lundberg et al. 1983a) and CGRP-immunoreactive nerves (Lundberg et al. 1985a) largely disappear from the airways. Histamine, known to get released upon mast cell degranulation, produces protein extravasation in rat trachea and bronchoconstriction in the guinea pig; both effects are significantly reduced after "depleting" capsaicin treatment (Lundberg and Saria 1982a, 1983a, Martling et al. 1984). Histamine has been shown to release tachykinin-like immunoreactivity from guinea-pig lung in vitro. SP-depletion by capsaicin reduces bronchoconstriction in response to allergen in sensitized animals (Saria et al. 1983).

Bradykinin, a peptide generated during local tissue damage/inflammatory processes is one of the tachykinin-releasing substances in vitro. Protein extravasation caused by bradykinin in the rat trachea can be greatly reduced by capsaicin

pretreatment (Lundberg and Saria 1983). Bradykinin inhalation induces bronchoconstriction in asthmatics but it has very little effect in normal subjects (Simonsson et al. 1973).

The postulate that VIP and PHM may be involved in the pathomechanism of asthma (Barnes 1986) is highly speculative. Although both peptides are potent bronchovasodilators in vitro, it proved to be difficult to demonstrate this effect unambigously in vivo. It is reasonable to assume that VIP and PHM may represent endogenous substances which contribute with their bronchodilator effect to a balance of numerous mechanisms providing for the physiological tonic-phasic activity of bronchial smooth muscle. If pathological events, resulting in the development of asthma would create conditions causing a functional deficiency in the effect of these peptides (e.g., promoting their enzymic inactivation), this would shift the balance toward the preponderance of bronchoconstrictor mechanisms (Barnes 1987).

9.5.2.2. OTHER PATHOLOGICAL PROCESSES

Opioid peptides are likely to get released during hypoxemia if it is of adequate intensity to produce respiratory depression, especially in young animals (Stephen et al. 1976, Chernick et al. 1980, Grunstein et al. 1981). The opiate antagonist naloxone has a respiratory stimulant effect even in the absence of hypoxemia in new-born rabbits up to four to five days after birth (Hazinski et al. 1981); this effect of naloxone is virtually abolished thereafter.

These findings have raised the question whether the respiratory depression phase of the biphasic response of human neonates to hypoxic conditions is due to the mobilization of endogenous opioids. Levels of β-endorphin-like materials in the umbilical cord have been found elevated in hypoxemic infants (Wardlaw et al. 1979). However, clinical approaches based on the possible involvement of endogenous opioids in pathological respiratory processes in neonates, have proved to be discouraging so far (see e.g., Chernick 1981).

Naloxone improves respiration in rats following electroconvulsive shock (Holaday et al. 1978) or shock associated with spinal cord transsection (Holaday and Faden 1980) by a centrally mediated action.

Bombesin may have a trophic/growth promoting role in the lung (for discussion see Polak and Bloom 1984). The bombesin content of hypoplastic lungs from children with the respiratory distress syndrome has been found markedly decreased (Polak and Bloom 1984). High bombesin concentrations have been reported to occur in the rapidly growing small cell carcinoma of the lung (Moody et al. 1981, Erisman et al. 1982, Sorenson et al.1982) and bombesin has been shown to exert a growth enhancing effect in endocrine tumor cultures of the lung (Oie 1983).

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CHAPTER 10

ENDOPEPTIDES IN THE INTEGRATION OF BLOOD COAGULATION. COAGULATION DISORDERS

D. Bagdy

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10.1. PHYSIOLOGICAL FACTORS

To our present knowledge, blood coagulation involves a reaction chain of 13 plasma proteins (Fig.10.1). In addition, one tissue protein, phospholipid membrane surface, platelets and calcium ions interact during the clotting cascade. The most characteristic features of the endopeptides involved in blood clotting and fibrinolysis can be summarized as follows.

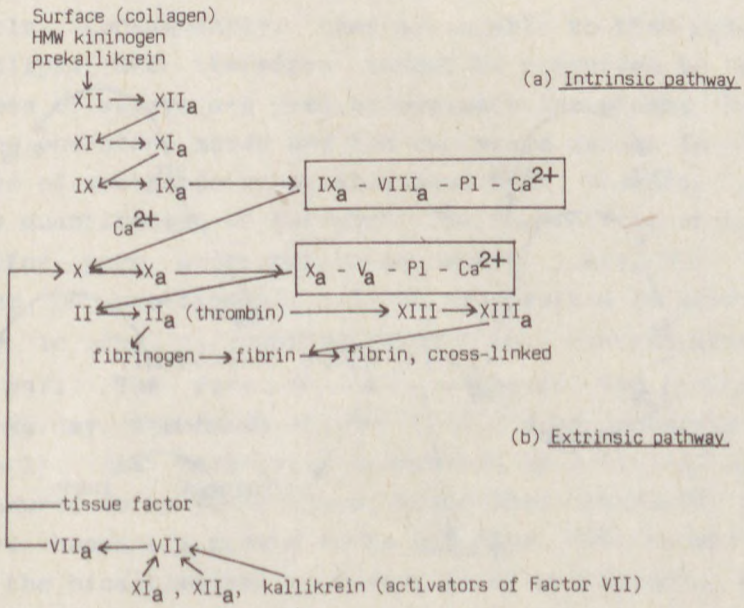


Fig. 10.1. Simplified scheme of the coagulation chain reactions. The "a"-subscripts refer to the activated form of coagulation factors circulating in blood as precursors; Pl indicates phospholipid.

10.1.1. FIBRINOGEN

Fibrinogen (Factor I, or simply I) contains about 3,000 amino acid residues. The primary structure of different species shows large sequence homologies as well as moderate to marked sequence differences. Fibrinogen of primates was found to give cross reactions against each other (Bagdy and Szilagyi 1953). Molecular weight of I was found to be 340,000. The fibrous molecule consists of six disulfide linked polypeptide chains, 2 A or α (M_r 64,000), 2 B or β (M_r 57,000) and 2 γ chains (M_r 48,000). A and B represent the two fibrinopeptides cleaved by thrombin (Lorand 1952). There are species differences in N-terminal amino acid residues (Laki 1968). I contains about 2-4% carbohydrate, which consists of hexoses, glucosamine and sialic acid residues (Szara and Bagdy 1953). There is a great variety of methods for assaying I. Liver cells synthesize about 1.5-5 g per day. Its normal level in circulating blood scatters between 1 and 5 g/l in various spe-

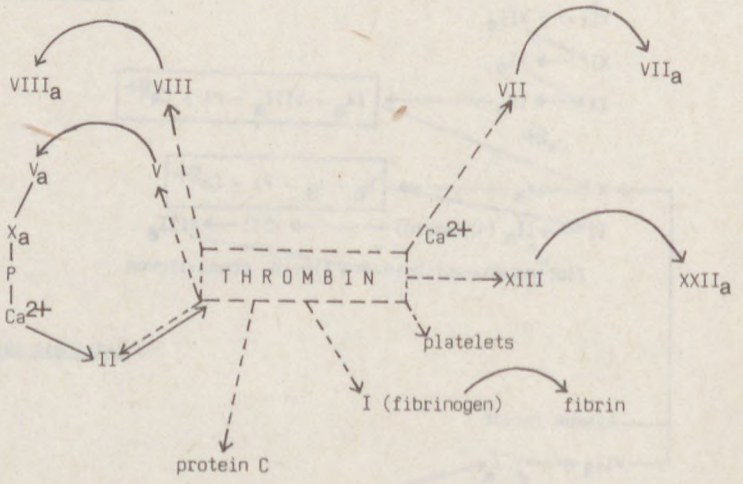


Fig. 10.2. The central position of thrombin in blood clotting

cies. The ratio of distribution between intra- and extravascular space was found to be 70(80):30(20) per cent. Its half-life was measured to be 2-6.5 days (Collen et al. 1972). Soluble I is converted by thrombin to fibrin monomer, which polymerizes to form the fibrin clot (Fig.10.2).

10.1.2. PROTHROMBIN

Prothrombin (Factor II, or II) isolated from various species has physical and chemical properties very similar to each other, except rat II. The amino acid composition of bovine and human II was found to be in reasonable agreement (Mann 1976, Mann et al. 1981). Vitamin K dependency of normal prothrombin is due to the presence of 10 γ-carboxy-glutamic acid residues (Gla) in the N-terminal part of the single polypeptide chain glycoprotein, the molecular weight of which has been established as 72,500 (Stenflo et al. 1974). In contrast, dicoumarol-prothrombins contain only 6,4 or only 2 Gla-s, respectively. Consequently, they are unable to bind calcium and phospholipid and therefore cannot be converted to thrombin. Two types of assays are used to evaluate the plasma level of II : the one-stage assay and the two-stage assay. In addition, a number of synthetic substrates have been prepared recently for the quantitation of thrombin. The theoretical specific activity for pure prothrombin is about 1,600 NIH thrombin units/mg of prothrombin. II is synthesized in liver and is present in the circulating blood in concentrations of 70-100 mg/l. The rate of its synthesis was found to be 2.4 mg/kg/day. About two-thirds of II can be detected intravascularly. Its half-life determined by a biological method was measured to be 40-60 hours, while that measured with ^{125}I labelled human II proved to be 2-8 days. The thrombin formed during the bioactivation by factor Xa of prothrombin has two polypeptide chains (49 amino acid residues in chain A and 265 in chain B) connected with one disulfide bridge.

10.1.3. FACTOR V

Factor V (V, accelerator globulin, proaccelerin, labile factor) is a glycoprotein with about 10-20% carbohydrate. This polysaccharide consists of about 8.7% neutral sugars and sialic acid ranging from 2 to 12%. Hydrolysis of the sialic acid residues with neuraminidase increases V activity by about 50%. On the other hand, its oxidation by galactose oxidase results in complete loss of activity. Molecular weight of human factor V was given as 330,000. Its concentration in human plasma is about 7 mg/l. The one-stage method is routinely employed in clinical measurements of factor V activity, however, a two stage method using highly purified reagents has also been developed. The half-life of factor V was found to be about 20 hours. Factor V appears to be synthesized in the liver, its catabolic fate, however, is not yet known. Factor V interacts with factor Xa, phospholipids and calcium ions. Its activated form, Va, binds prothrombin immobilized on Sepharose. Factor V is an accessory protein in prothrombin activation (Colman and Weinberg 1976).

10.1.4. FACTOR VII

Factor VII (VII, proconvertin, serum prothrombin conversion accelerator, SPCA) is a glycoprotein containing a single peptide chain with a molecular weight of 48,500. Its N-terminal sequence shows homology with that of other vitamin K-dependent (K-dependent) clotting factors (II, IX and X). The carbohydrate component (1.3%) consists of galactose, mannose, N-acetylglucosamine and N-acetylneuraminic acid. Factor VII may be assayed by its ability to correct the clotting time of factor VII deficient plasma upon the addition of thromboplastin tissue factor and calcium. Among K-dependent coagulation factors the concentration of VII in bovine plasma is the lowest: it amounts to about 1 mg/l. Factor VII is the first component of the extrinsic pathway of coagulation. It is the precursor of the proteinase which activates factor X. On the

other hand, factor VII may be a substrate for factor Xa. It can be activated by Xa or factor IXa in the presence of phospholipid and calcium, and by thrombin or factor XIIa without additional cofactors (Broze and Majerus 1981).

10.1.5. FACTOR VIII

Factor VIII (VIII, antihemophilic factor A, antihemophilic globulin: AHG, platelet cofactor I), a plasma protein absent in patients with classic hemophilia, is a large glycoprotein. At present, however, very little is known of its chemistry. The factor VIII coagulant activity and the von Willebrand platelet-aggregating activity are closely associated in the course of plasma fractionation (Legaz et al. 1975). Recent studies indicate that factor VIII has a high molecular weight carrier protein with platelet-aggregating activity and a low molecular weight subunit with factor VIII coagulant activity (Weiss et al. 1972). This latter was found to be present only after activation by thrombin of factor VIII. The inhibition by DFP of thrombin-activated factor VIII suggests that a serine protease is formed from a precursor protein. Factor VIII activity is routinely measured by a one-stage method (kaolin-activated partial thromboplastin test) using human factor VIII deficient plasma. AHG is synthesized in liver, spleen and bone marrow. Experiments carried out with normal and hemophilic dogs suggests that the spleen is of importance in the storage of AHG and the reticuloendothelial system may be the site of its synthesis. The half-life of human AHG has been given as 6-9 hours. According to our present knowledge, AHG is an accessory protein that participates with proteinase factor IXa in the activation of factor X via the intrinsic coagulation pathway.

10.1.6. FACTOR IX

Factor IX (IX, Christmas factor, hemophilia B factor, antihemophilic factor B, plasma thromboplastin component, PTC) is a single chain glycoprotein. It contains 74% protein and 26% carbohydrate. The molecular weight of human and bovine factor IX was found to be 55,400 and 57,000, respectively (Di Scipio et al. 1977). Factor IX activity is usually determined by the kaolin partial thromboplastin time using human factor IX deficient plasma as substrate. IX is synthesized in the liver, its biosynthesis is dependent on vitamin K. The plasma level of factor IX amounts to 5 mg/l, its half-life is 24-30 hours. Factor IX is a circulating precursor to proteinase which activates factor X in the intrinsic pathway of coagulation.

10.1.7. FACTOR X

Factor X (X, Stuart-Prower factor, prothrombinase, autoprothrombin III) is a glycoprotein composed of a light and a heavy chain. The heavy chain (M_r 39,300) of the bovine molecule contains 307 amino acid residues and also two carbohydrate chains linked to Asn(35) and Thr(300). The carbohydrate content of bovine factor X amounts to about 10%, that of the human one to about 15%. The light chain (M_r 16,500) contains 12 γ-carboxyglutamic acid residues. The heavy chain of human factor X contains an additional 15-20 residues on the N-terminal end of the chain (Fujikawa et al. 1972).

The amino acid sequences of the heavy and light chains of bovine factor X show a great deal of homology between factors X, IX and II (Titani et al. 1975). The assay of factor X is based on its specific activation by a component of Russel's viper venom. Factor X is a stable protein that persists in the serum. Its half-life is 48 hours. Factor X plays a central role in blood coagulation. Its activation, which is characterized by the cleavage of an internal peptide bond between

Arg(51) and Ile(52), represents the point at which the intrinsic and extrinsic pathways of clotting converge. Factor X is the precursor of a serine proteinase that converts prothrombin to thrombin.

10.1.8. FACTOR XI

Factor XI (XI, plasma thromboplastin antecedent, PTA) is composed of two identical polypeptide chains with a M_r of about 60,000 each.

It is a glycoprotein (M_r about 130,000) containing about 5% carbohydrate which consists of hexoses, hexoseamines and neuraminic acids. A complete analysis of amino acids and carbohydrates of human and bovine factor XI was published (Kurachi and Davie 1977). Factor XI activity is routinely measured by the one-stage kaolin-activated partial thromboplastin time using bovine factor XI deficient plasma as substrate. The half-life of XI in the circulation is about 60 hours. Factor XI is converted to XIa by factor XIIa. This activation is characterized by the cleavage of internal peptide bonds in each of the precursor chains (Arg-Ala and Arg-Val, respectively). In vitro coagulation systems, the protein substrates for XIa include factor IX, VII and XII, as well as plasminogen.

10.1.9. FACTOR XII

Factor XII (XII, Hageman factor) is a single chain glycoprotein with a M_r of 74,000. (Fujikawa et al. 1977. It contains 16.8% carbohydrate that consists of hexoses, hexoseamines and N-acetylneuraminic acids. The average concentration of factor XII in human plasma determined by the radial immunodiffusion method is 29 µg/ml. Factor XII activity can be measured for its ability to activate isolated plasma kallikrein or to clot factor XII deficient plasma. Hageman factor is the precursor of an enzyme which proteolytically activates factor XI. The conversion of XII to XIIa by plasma kallikrein

is characterized by the cleavage of a specific internal Arg-Val peptide bond as in most cases of the coagulation endopeptides. Factor XIIa is composed of a heavy chain (M_r about 52,000) and a light chain (M_r about 28,000) held together by a specific disulfide bond(s). The light chain contains the active site of the enzyme, while the heavy chain is responsible for the binding of the molecule to negatively charged surfaces.

10.1.10. FACTOR XIII

Factor XIII (XIII, Laki-Lorand factor, fibrin-stabilizing factor, FSF) isolated from plasma has a heterologous (ab) protomeric structure (Lorand 1972). The molecule of factor XIII consists of two "a" subunits (plasma and platelet forms with a M_r of 75,000) and two "b" subunits (plasma form only with M_r 80,000). Thus the M_r of the tetrameric plasma form was found to be 320,000 and that of the dimeric platelet form to be 160,000. The subunits are proteolytically cleaved by thrombin to form the activated form, XIIIa, an active transglutaminase with a reactive cysteinyl thiol. The assay methods for XIII can be divided into two groups (Curtis and Lorand 1976, Lorand et al. 1981). An extensively used method utilizes the incorporation of labeled synthetic amine substrates into a protein acceptor, such as casein. Other methods based entirely on synthetic substrates are employed mainly for kinetic studies in vitro. The half-life of factor XIII has been given as 3-5 days. Factor XIII is the circulating precursor of plasma transglutaminase (fibrinoligase) which catalyses the fusion of fibrin units within the clot network by forming intermolecular ε-(γ-glutamyl)-lysine bridges.

10.1.11. PLASMA PREKALLIKREIN

Plasma prekallikrein (Fletcher factor) is the precursor protein of kallikrein, a specific protease that liberates kinins from plasma kininogens. Human and bovine plasma prekallikreins are single chain glycoproteins, each with a minimum molecular weight of about 82,000 as determined by sedimentation equilibrium centrifugation (Heimark and Davie 1979). There are two ways for assaying prekallikrein in plasma. The first involves the conversion of prekallikrein to kallikrein and the determination of the rate of kinin release by bioassay or RIA. A direct immunochemical assay serves as a second method for measuring the amount of prokallikrein protein. Prekallikrein is converted to kallikrein by activated Hageman factor (XIIa) or its proteolytic fragment (XIIf large activator).

10.1.12. HIGH MOLECULAR WEIGHT KININOGEN

High molecular weight kininogen (HMW kininogen, Flaujeac-, Fitzgerald-, Williams-factor, contact activation factor) is a single chain glycoprotein containing about 13% carbohydrate. Its molecular weight determined by sedimentation equilibrium centrifugation was found to be 76,000 for bovine HMW and 108,000 for human HMW kininogen (Kato et al. 1981). HMW kininogen comprising about 20% of the total is a good substrate for plasma kallikrein, while the low molecular weight kininogen (LMW kininogen comprising about 80% of the total) is not. The cleavage of HMW and other kininogens in plasma by kallikrein releases a nonapeptide, bradykinin, and several other peptides of various molecular weights. To our present knowledge, HMW kininogen accelerates the activation of factor XII, prekallikrein, and of factor XI as cofactor, and thus it is an essential accessory protein for the XIIa catalyzed reactions.

10.1.13. PROTEINS C, S AND Z

In the course of the isolation of factors II, VII, IX and X three new vitamin K-dependent proteins: protein C, protein S and protein Z have been identified in the plasma.

P r o t e i n C is a plasma glycoprotein (M_r 60,000) that consists of a heavy (M_r 41,000) and a light (M_r 21,000) chain held together by disulfide bond(s). It contains 11 γ-carboxyglutamic acid residues present in the light chain of the molecule. The human protein C contains 77% protein and 23% carbohydrate including a number of galactose, mannose, glucosamine and sialic acid molecules (Kisiel and Davie 1981). Activation of protein C by thrombin (or by trypsin and the protease from Russel's viper venom, respectively) results in a serine protease having anticoagulant activity in the presence of phospholipid, Ca^{2+} ions and protein S. The activation is enhanced by thrombomodulin, a protein localized on the surface of the endothelial cells of vessel walls and represents a high affinity thrombin receptor. Protein Ca (activated protein C) specifically inactivates factor Va and factor VIIIa by limited proteolysis.

P r o t e i n S was found to be a single chain plasma protein (M_r 64,000) containing 10 residues of γ-carboxyglutamic acid in the N-terminal region. It is present in the blood partly in a free form, partly in a complex with C4b-binding protein which is involved in the regulation of the rate of complement activation.

The possible biological role of p r o t e i n Z has not been established so far.

10.1.14. THROMBOPLASTIN

Thromboplastin (III, tissue thromboplastin, tissue factor, thrombokinase) is a lipoprotein, in which both lipid and protein are required for coagulant activity, however, the specific activity is due to the protein component. The apoprotein contains a significant amount of carbohydrate, which consist

of fucose, hexoses, hexoseamines and sialic acid. The homogenous apoprotein prepared from human brain microsomes requires recombination with phospholipids for full activity. Brain, lung and placental tissue factors have similar specific activities, while the specific activity of kidney, liver and spleen tissue factors is only 5-10% of that of the brain. The clot-promoting activity of thromboplastin preparations can be determined in a recalcified clotting time assay or by the direct activation of factor X in the presence of factor VII and calcium. The tissue factor markedly shortens the time required for clot formation (Pitlick and Nemerson 1976). It initiates the extrinsic pathway of coagulation.

10.1.15. PLASMINOGEN

Plasminogen (fibrinolysin) is the plasma-protein precursor of the fibrinolytic enzyme, plasmin, which functions not only in the fibrinolytic system but also in other physiological systems. Plasminogen can readily be isolated from plasma by affinity chromatography on lysine agarose and its separation is achieved by gradient elution with ε-amino caproic acid into two separate forms which differ in their sialic acid content. They are single chain proteins containing 22 disulfide bonds. Native human plasminogen (M_r 92,000) has Glu as N-terminal and Asn as C-terminal amino acid residues. It contains a single Arg(560)-Val(561) bond that is split during its activation to plasmin. The light chain of plasmin (561-790) coming from the C-terminal part of plasminogen is responsible for the proteolytic activity, while the heavy chain (76-560) takes part in the plasmin(ogen)-fibrin affinity as well as in the reaction between plasmin and α_2-plasmin-inhibitor. Plasminogen activation can be catalyzed by urokinase (UK), tissue plasminogen activator (t-PA) and streptokinase (SK). In addition, several serine proteases including kallikrein and an activator from the vessel wall catalyze this reaction.

10.1.16. INHIBITORS OF COAGULATION PROTEINASES

Some characteristics of the physiological proteinase inhibitors involved in blood clotting are summarized in Table 10.1. Among these inhibitors antithrombin III (AT III) is a protein with broad specificity toward the serine proteases of the coagulation system (M_r 64,000). The physiological significance of AT III is well established by the observed thromboses occurring in patients with inherited or acquired AT III deficiency. AT III inactivates thrombin through its reactive arginine residues linked to the active serine site of thrombin. Thus, the physiological inactivation of thrombin and that of the other serine proteases of clotting represents protein-protein interactions. This interaction can be enhanced by heparin administered parenterally. According to our present knowledge, heparin acts as a catalyzator in the interaction between thrombin and AT III. It does not change the equilibrium of the reaction between enzyme and its inhibitor. The molar stoichiometry of the reaction is 1:1 both in the absence and in the presence of heparin.

Table 10.1. Some characteristics of the physiological proteinase inhibitors involved in blood clotting

Name	Origin	Structure	Enzymes inhibited
Antithrombin III (human)	Liver Endothelial cells	1 chain mw 62,000	Thrombin, Kallikrein, XII_a, XI_a, X_a, IX_a, II_a
α_2-macroglobulin	Liver	4 chains mw 725,000	Thrombin, Kallikrein, II_a
α_1-proteinase inhibitor	Liver	1 chain mw 54,000	Thrombin, Plasmin, Kallikrein, II_a, XI_a
C1-inhibitor	Liver	1 chain mw 104,000	Kallikrein, XI_a, XII_a

α_2-Macroglobulin (α_2 M, human: M_r 725,000) acts as a molecular trap for proteinase molecules. Having been enclosed within the macroglobulin molecule, thrombin is unable to act upon its physiological substrates. A great number of serine proteinases, cysteine proteinases, metalloproteinases and also aspartic proteinases are entrapped and thus inactivated by α_2-macroglobulin.

α_1-Proteinase inhibitor (α_1-PI, α_1-trypsin inhibitor), the major proteinase inhibitor in plasma, apparently acts as a general scavenger for tissue serine proteinases. Among others it inhibits also kallikrein, thrombin and plasmin.

α_2-Plasmin inhibitor (primary plasmin inhibitor, antiplasmin, primary fibrinolysis inhibitor) represents the fast-acting plasmin inhibitor in human plasma. (Before its identification α_2-macroglobulin and α_1-proteinase inhibitor were thought to be the major inhibitors of plasmin in plasma). α_2-plasmin inhibitor is a single chain glycoprotein (M_r 65,000-70,000) stabilized by 3 disulfide bridges.

Carbohydrate analysis showed about 11-14% carbohydrate (10 mol sialic acid, 30 mol hexose and 7 mol glucosamine per mole of α_2-antiplasmin). α_2-Antiplasmin forms an enzymatically inactive complex with plasmin very quickly. This presumably is its only physiologically important reaction, although it reacts with many other enzymes (trypsin, chymotrypsin, kallikrein, factor X, urokinase) in vitro. The active center of plasmin plays a role in the formation of the reversible enzyme-inhibitor complex. Physiologically, the plasma concentration of α_2-antiplasmin amounts to about 70 mg/l. Turnover studies with radiolabeled α_2-antiplasmin in humans indicated a half-life of 2.6 days. The half-life of the plasmin-α_2-antiplasmin complex was found to be half a day. α_2-Antiplasmin plays an important role in the regulation of fibrinolysis. A few patients with α_2-antiplasmin deficiency have been reported. Decreased values were measured in individuals with liver disease and with severe intravascular coagulation.

10.2. FACTORS INVOLVED IN BLOOD COAGULATION DISORDERS

10.2.1. HEMOPHILIAS A AND B

Hemophilia A is due to the deficiency of factor VIII, hemophilia B to that of factor IX. Both types may be severe (factor VIII and IX contents, respectively, are less than 1% of the normal), moderate (VIII or IX content varies between 1-4% of the normal) and mild (VIII or IX content amounts to 5-25% of the normal). The affected members of a hemophilic family always have hemophilia of the same type and severity. Different forms have been reported both in hemophilia A and B (Brown et al. 1970, Hoyer and Breckenridge 1970).

10.2.2. VON WILLEBRAND DISEASE (VWD)

VWD is characterized by an abnormal bleeding tendency which is due to partial factor VIII deficiency (60%), prolonged bleeding time and to decreased platelet adhesiveness. VWD represents the lack of a plasma protein which is present not only in normal, but also in hemophilic plasma. Data on its structure are still controversial (Holmberg and Nilsson 1975).

10.2.3. HEMOPHILIA C

Hemophilia C is due to factor XI deficiency. It is a rare congenital disease that occurs in both females and males and manifests itself as a mild hemorrhagic diathesis probably transmitted as a recessive autosomal character (Rosenthal et al. 1953).

10.2.4. HAGEMAN'S DISEASE

Hageman trait (Hageman's disease) is described by an abnormal prothrombin consumption and an abnormal thromboplastin generation test. All other coagulation factors are normal. Patients with the lack of factor XII show no increased bleeding tendency (Ratnoff and Davie 1962).

10.2.5. CONGENITAL PROTHROMBIN DEFICIENCY

It is caused by a recessive gene and is not sex linked. The prothrombin content of the patient's plasma was found to be about 10% of the normal. Moderate bleeding symptoms have been observed (Girolami et al. 1970).

10.2.6. CONGENITAL FACTOR V DEFICIENCY

This is an extremely rare disease. It is characterized by a prolonged one-stage prothrombin time which can be corrected by the addition of fresh prothrombin free-plasma, but not by addition of stored plasma (Owren 1947).

10.2.7. CONGENITAL FACTOR VII AND FACTOR X DEFICIENCY

Congenital factor VII and factor X deficiencies have common characteristics with regard to the prolonged one-stage prothrombin time and the decreased thrombotest time. They may be distinguished by the Stypven test and the thromboplastin generation test. Patients with factor VII or factor X deficiency have the same clinical bleeding symptoms and the defects are equally common among females and males (Marder and Shulman 1964, Kurz et al. 1969).

10.2.8. CONGENITAL AFIBRINOGENEMIA AND HYPOFIBRINOGENEMIA

The complete absence of fibrinogen is due to the lack of its synthesis in the liver. Patients with this disorder have incoagulable blood, however, coagulation cascade is otherwise normal. Fibrinogen infused to patients with bleeding symptoms had a normal survival. Congenital hypofibrinogenemia (0.1-1 g/l of plasma) causes only mild bleeding symptoms or none at all. Recently, most of the previously reported cases of afibrinogenemia and hypofibrinogenemia are supposed not to be deficiencies at all, but rather conditions due to abnormal fibrinogen (Jackson et al. 1965). A low fibrinogen content determined by coagulation assay and a normal content measured by immunological methods were found as common characteristics in these cases. Clotting of the patient's plasma required an abnormally large amount of thrombin, yet coagulation factors were normal and there was no increased fibrinolysis. The fibrinogen defects seem to be inherited as an autosomal dominant trait. The functional consequence of the defect manifested in most cases as a delayed aggregation of fibrin monomer. A specific amino acid replacement (Arg-Ser) has been shown in fibrinogen Detroit (Blomback et al. 1968).

10.2.9. FACTOR XIII DEFICIENCIES

Congenital deficiency of factor XIII is transmitted as an autosomal recessive trait. It is characterized by an abnormal thrombelastographic picture and the solubility of the plasma clot in 5 M urea or in 1% monochloracetic acid. The coagulation cascade, the fibrinolytic system and also the platelet function are normal, however, the platelets, unlike the normal ones, do not contain factor XIII (Stefanini et al. 1972).

Acquired factor XIII deficiency in the plasma was reported in patients with liver and renal diseases, pernicious anemia, lead poisoning, etc. In these cases factor XIII content of platelets was normal (Lorand et al. 1972).

10.2.10. ANTITHROMBIN III DEFICIENCY

Egeberg (1965) first described a family in which frequent thromboses were associated with a deficiency of thrombin inactivation and heparin cofactor activity. This observation was confirmed by Van der Meer et al. (1973) and Ambruso et al. (1980). In all of these families the degree of congenital antithrombin III (AT III) deficiency measured by both coagulation tests and immunologic methods was similar. Sas et al. (1974) reported a unique family with a high incidence of spontaneous thromboembolic episodes in which the affected members had a deficiency of progressive thrombin inactivation and heparin cofactor activity, but normal levels of immuno-reactive AT III. This thrombophilic family with an abnormal AT III molecule was thoroughly investigated (Sas et al. 1975). A detailed analysis of clinical and laboratory data of 15 patients with confirmed congenital deficiency of AT III or abnormal AT III activity was published by Nagy et al. (1979). The observed clinical symptoms consisted of recurrent thromboembolic manifestations of early onset and a tendency to grave recurrent complications. Three types of AT III deficiency have been described. In Type I, both quantity and function of AT III are decreased. In Type II, AT III is normal in quantity but abnormal in function. In Type III, AT III is quantitatively normal and also its function seems normal as far as its basic activity is concerned, but its abnormality is manifested in the presence of heparin both in vitro and in vivo. AT III deficiency is inherited as a dominant trait. It was demonstrable in 44 of the 60 family members of the 15 patiens, and 23 of them had also clinical symptoms.

10.2.11. DISSEMINATED INTRAVASCULAR COAGULATION (DIC)

DIC (defibrination syndrome, intravascular coagulation with fibrinolysis syndrome, consumption coagulopathy) is a complex pathological syndrome characterized by the conversion of prothrombin to thrombin and that of plasminogen to plasmin

(Colman et al. 1979). Formation of thrombin in circulation may be induced by several factors, e.g., by local denudation of the vessel wall and interaction of the subendothelial collagen with platelets, local arrest of circulation, decrease of AT III in plasma, release of neutral proteases present in polymorphonuclear leukocytes into the blood stream. Its formation results in subsequent activation and consumption of coagulation factors. It converts fibrinogen into fibrin monomer. Thrombin binds to platelet membranes and initiates the shape change, aggregation and release of many biologically active compounds. Among these, platelet factor 3, a phospholipoprotein is needed for the interaction of factors IXa-VIII and Xa-V-II. Thrombin activates factors V, VII, VIII and II, and this way a significant amplification in the cascade reaction is realized. In addition, thrombin converts factor XIII to an active transglutaminase that crosslinks fibrin, thus a fibrin polymer more resistant to fibrinolysis is formed. The interaction of thrombin with fibrinogen results in the liberation of fibrinopeptides A and B. Consequently, the decrease in platelet count, in fibrinogen content, as well as in the content of factors II, V, VIII and XIII in acute DIC can be due first of all to thrombin.

On the other hand, the symptoms of DIC are influenced by the fibrinolytic system. The activation of plasminogen to plasmin may be induced by different ways resulting in digestion of fibrin. Release of the plasminogen activator from the endothelial cells may cause an enhanced fibrinolysis, too. As a consequence of tissue injury, both plasminogen activators and thromboplastic materials are liberated. Thus, fibrinolysis usually accompanies the formation of thrombin. The degree of thrombosis and/or hemorrhage manifested clinically is determined by the equilibrium between these two enzymes. In contrast, fibrinogenolysis is independent of blood coagulation. Plasmin digestion of fibrinogen and fibrin, respectively, produces a series of fibrin degradation products which are decisive for the pathogenesis and diagnosis of DIC. The regulatory role of fibrinolysis in DIC has been experimentally proved.

10.2.12. INITIATION AND INACTIVATION OF BLOOD COAGULATION BY NEUTRAL PROTEASES OF HUMAN POLYMORPHONUCLEAR GRANULOCYTES

The possible role of polymorphonuclear granulocytes in local fibrinolysis was supposed on the basis of their intimate reactions with fibrin deposits in inflammatory processes (Barnhart 1965). On the other hand, the implantation of various fibrin products used as local hemostatic agents into experimental animals induces a marked leukocytosis without any signs of inflammation (Bagdy et al. 1963). In both cases the granulocytes release some proteolytic enzymes into the circulation and tissues. Elastase and the chymotrypsin-like enzymes are the primary neutral proteases responsible for the fibrinolytic activity of leukocytes which is independent of the plasminogen system. In addition, collagenase and cathepsin D in human granulocytes were reported. Depending upon the concentration of these enzymes released into the blood, activation and/or inactivation of the coagulation factors could be observed. For example, low or medium doses of granulocyte elastase activates factor V and factor VII, respectively, while high doses inactivates (digests) them completely. The proteases exert their effect even in the presence of plasma proteinase inhibitors (Egbring and Havemann 1978).

Endotoxin-induced coagulation disorders seem to have the same mechanism of action via the release of granulocyte neutral proteases. Recently, an increasing amount of evidence suggests that the presence of released endotoxin(s) may cause coagulation disorders in patients with different diseases. Endotoxinemia and coagulation disorders were observed in patients with acute leukemia and septicemia (Egbring et al. 1977).

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CHAPTER 11

ENDOGENOUS PEPTIDES IN THE INTEGRATION OF THE FUNCTION AND METABOLISM OF SKELETAL MUSCULATURE

I. SZIKLAI

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INTRODUCTION

During the past decade, data have gradually accumulated on some endogenous peptides that seem to be involved in the regulation of electric and contractile activity, as well as in the metabolism of skeletal muscles.

Skeletal muscle fibers are specialized in respect to fatiguability, contractile velocity, as well as to contraction cycle duration. According to a simplified classification based on these properties (though other assortments of characteristics also exist), the skeletal muscles can be classified as slow twitch and fast twitch type. With regard to certain characteristics of metabolic specialization, which can also be assigned to these functional properties and may be related to work performance and activity pattern, the conclusions are: without exception, slow twitch muscles rely on aerobic, while fast twitch muscles mainly on glucolytic metabolism for their ATP supply.

The coordination between the functionally and metabolically specialized muscles is mediated by the motoneurons, that is, by the central nervous system via the regulation of the contractile mechanism, in respect to locomotion; and by the autonomic nervous system via the regulation of the vascular tone, in respect to the "fuel" supply. Advances in the clarification of these two regulatory mechanisms are limited by the shortcomings of the methodology available for exploring the roles that endogenous peptides might play in the integrative mechanisms of the skeletal musculature, and in the overall protein metabolism, as well as in locomotion, especially in human subjects. According to our present knowledge, biologi-

cally active peptides play a modulatory role in the electric and in the contractile activity, as well as in the vascular supply of skeletal muscles.

It should be noted that the forthcoming discussion is strictly confined to a few peptides that are known to regulate or modulate the neuromuscular transmission and contractile function of skeletal muscles. This implies that structural proteins identified in an ever increasing number in the past few years, are not included in the present discussion irrespective of their possible direct involvement in the process of muscle contraction.

11.1. MODULATORY PEPTIDES IN THE JUNCTIONAL CHOLINERGIC TRANSMISSION

11.1.1. NEUROPEPTIDES

The role of neuropeptides in signal transmission at the sympathetic ganglia and in the tone and contractility of the smooth muscle has been amply documented in the literature (for references see Wali 1985). However, only scattered information is available concerning the effects of neuropeptides on neuromuscular transmission and muscle contraction (Steinacker 1977, Akasu et al. 1983). This may be explained by the widely accepted view which regards the neuromuscular junction as a simple cholinergic synapse where signal transmission occurs through a single neurotransmitter, e.g., acetylcholine (ACh). However, it has been recognized recently that, apart from ACh, there may be other neuroactive substances, for example circulating neuropeptides, that may modify neuromuscular transmission. It is conceivable that changes in the level of such circulating peptides may result in characteristic alterations in the neuromuscular function. Neuropeptides can modify junctional signal transmission by acting on transmitter release, acetylcholinesterase, postjunctional membrane and on intracellular Ca^{2+} concentrations.

(1) β-Endorphin (β-EP; see Chapter 5) is a specific inhibitor of the 16S oligomeric form of acetylcholinesterase (EC 3.1.1.7, AChE), which occurs exclusively in the motor endplate regions of adult rat skeletal muscle (Hall 1973). Regarding enzyme inhibition, there are species differences in the peptide action: the inhibitory activity of bovine β-EP is identical to that of the rat peptide on rat 16S AChE, while porcine β-EP acts weakly (Haynes and Smith 1982). The recognition site for 16S AChE inhibition probably is in the C-terminal region, as Met-enkephalin and C'-fragment (see Chapter 5) do not show a similar inhibitory action. β-EP loses its opiate and enzyme inhibitory-activity by cleaving off the C-terminal tetrapeptide sequence (Smyth et al. 1979). β-EP causes a selective inhibition of 16S AChE in a 10^{-9} mol/l concentration, and increases the contractile response to ACh of denervated rat skeletal muscle. It is presently unknown whether β-EP is released at the motor endplate. The inhibition of the enzyme is unlikely due to interaction with catalytic sites, because no similar effect could be observed on the 10S and 4S AChEs (Haynes and Smith 1982). Collagenase digested 16S AChE is not inhibited by β-EP, although enzyme activity remains unchanged, supporting separate sites for peptide binding catalytic center.

The AChE inhibitory effect of β-EP restricted to peripheral sites provides the possibility of its use in clinical practice as a potent anticholinesterase drug, without any central influence.

(2) α-Aspartyl-alanine. It was firstly isolated from pig brain, but it also occurs as part of other natural peptides (vasoactive intestinal peptide, ACTH(18-39), δ-sleep-inducing peptide, eledoisin). The peptide has proved to be a physiological modulator of neuromuscular transmission (Lim et al. 1981). A high concentration (10^{-5} mol/l) is necessary for its effect, and the long duration of its action (60 min after washing out from the incubation fluid) shows that it is a relatively weak modulator of cholinergic transmission, and is a slowly but long-acting dipeptide. In vitro, it enhances the

amplitude of the miniature andplate potentials (MEPP) in neuromuscular preparations, and induces giant MEPP populations. The simultaneous administration of d-tubocurarine decreases the MEPP amplitude. The peptide has no anticholinesterase activity.

(3) Angiotensin II and substance P. Animal studies have indicated that, in physiological concentrations, angiotensin II (AT II) modifies neuromuscular transmission in different species (Wali 1984a,b,c), probably acting through both pre- and postjunctional mechanisms. As a presynaptic effect, AT II increases the duration of the action potential suggesting that more calcium entered into the nerve terminals allowing an additional release of the transmitter substance. Available evidence also suggests that AT II may affect the postjunctional membrane: AT II does not only produce a small contracture in the skeletal muscle, but also reduces the contractures elicited by depolarizing agents such as ACh and tetraethylammonium (TEA). These actions may be interpreted to mean that AT II reduces the sensitivity of the postjunctional membrane to depolarizing agents and this is most probably accomplished via a direct effect on the skeletal muscle by raising the intracellular Ca^{2+} concentrations and producing a contracture.

Substance P (SP) produces similar effects as AT II except that SP elicits a direct contracture response in the skeletal muscle. In low concentrations, it slightly increases the TEA- and ACh-induced responses. SP may also act through both pre- and postjunctional mechanisms. In high doses, SP reduces both the amplitude and the frequency of the MEPPs, the amplitude of the evoked endplate potential and the quantal content of transmitter, but at no concentrations produces any change in the resting membrane potential in the frog muscle. The initial phase of SP-induced depression is Ca^{2+}-dependent (Steinacker 1977). Obtaining similar results, Akasu and coworkers (1983) suggested that SP depresses the sensitivity of the postjunctional membrane to ACh.

(4) Oxytocin and vasopressin. Dissimilarly to AT II and SP, oxytocin (OT) and vasopressin (VP) depress all contractile responses produced in the skeletal muscle of various species independently of the applied stimulus (Wali 1984d). Both peptides reduce the amplitude and the duration of the frog sciatic nerve compound action potential, the uptake of labeled choline as well as the contractures evoked by ACh and TEA. OT and VP produce similar effects on the compound action potential of the rabbit superior cervical ganglion (Wali 1983). At the neuromuscular junction, the effects of OT and VP could be slightly reversed by raising the external Ca^{2+} concentration.

The results presented above strongly suggest that neuropeptides can modify neuromuscular transmission and muscle contraction in various species. Their actions at the neuromuscular junctions are reminiscent of their effects at the presynaptic cells of the rabbit superior cervical ganglion (Wali 1983, 1984d), indicating that neuropeptides may play a functional role at the neuromuscular site. However, the exact role what neuropeptides may play in the neuromuscular system and in its diseases remains to be elucidated.

11.1.2. THYMOPOIETINS

The closely related thymopoietin I and II (TP I and II) were extracted from bovine thymus by Goldstein (1968; see Chapter 7).

When injected parenterally into the guinea pig, the peptides cause myositis, while their prolonged administration leads to a myasthenia-like neuromuscular block. In vitro, they decrease the muscle tension in an indirectly stimulated neuromuscular preparations and this effect could be suspended with d-tubocurarine and neostigmine (Goldstein 1968). In doses between 4 and 32 ng/mouse, both TP I and II inhibit neuromuscular transmission postsynaptically (Goldstein and Manganaro 1971), though they do not combine directly with the acetylcholine receptor (ACh-R), since an acute block or acute depolarization does not occur. In vivo, an 18 hour latency period is

needed for the development of the effect, which then persists for several days. The primary role of thymus hormones, including thymopoietins, is to promote the differentiation of lymphopoietic cells, their action on neuromuscular transmission being only secondary. Lymphopoietic stem cells contain cholinergic receptors similar to those of the skeletal muscles and the TPs may act through these receptors (Goldstein 1974), however, the receptor recognition sequence remains unknown for the time being.

TPs, by eliciting a myasthenia-like neuromuscular block, were suggested to have a role in the pathogenesis of myasthenia gravis, a neuromuscular disorder characterized by muscular fatiguability and weakness (reviewed by Drachman 1978). Although the exact pathogenetic role of the thymus in myasthenia gravis is unclear at present, thymectomy proved to be effective in 50-80% of the patients (Wijermans et al. 1980). The thymus produces several hormones, but only serum thymic factor shows increased serum levels in myasthenia gravis which, on the other hand, is inactive in the electromyographic assay (Bach et al. 1972). Although the TPs have an established neuromuscular point of attack (Kelemen et al. 1979), their involvement in myasthenia gravis appears to be of secondary importance at best, since ACh-R antibodies persisting in the circulation proved to have a primary role in the impairment of neuromuscular transmission characteristic of this disease.

11.2. PEPTIDES AFFECTING THE RESPONSIVENESS OF SKELETAL MUSLCE TO NEURAL (ELECTRIC) STIMULI

(1) Insulin is probably the most important factor in the regulation of musle protein turnover (its well-known effects on glucose metabolism and amino acid transport were excellently summarized in a review by Goldberg et al. 1980). Insulin causes a dose-dependent depression of the contraction of slow twitch muscles, while it has no effect on the activity of fast twitch muscles. Its contraction-depressing concentrations cor-

respond to those of terbutaline, a β_2-adrenoceptor agonist. However, the action of insulin cannot be blocked with propranolol. Following KCl depression in vitro, insulin, partly at least, restores the contractile tension in both types of muscles (Holmberg and Waldeck 1980). This effect of the hormone is probably due to its ability to stimulate Na^+ and K^+ transport across the cell membrane, causing depolarisation (Zierler 1966).

(2) Opiates. Evidence has been presented demonstrating the ability of opiate drugs to suppress action potentials in frog skeletal muscle (Frank 1975a,b Frank and Morwaha 1979). In electrophysiological model experiments, meperidine was found to cause an initial depression of both sodium and potassium conductance mechanisms, followed by a secondary depression of sodium current only. The latter is supposed to be a specific action at an opiate receptor because only the secondary depressant action of meperidine could be antagonized by the opiate antagonist naloxone in a low organ-bath concentration (10^{-8} mol/l). In high bath concentration (10^{-4} mol/l), nalorphine alone produces opiate agonist action as was described for meperidine previously (Ary and Frank 1983). These data point to the skeletal muscle as a potential target tissue for opiate actions. The physiological significance of this phenomenon, however, remains to be clarified.

11.3. DIPEPTIDES AND THE MUSCLE CONTRACTILE MECHANISM

(1) Lysyl-tryptophan. The dipeptide was isolated from the hydrolysate of Fur Seal muscle by Tamiya and Matsumoto (1979). The dipeptide elicits a two-fold increase in ATPase activity in carp myosin B (actomyosin + regulatory proteins) in vitro. The mechanism by which ATPase activity is affected by the dipeptide is unknown at present.

(2) Carnosine (β-alanyl-histidine) and anserine (β-alanyl-methylhistidine). These dipeptides occur in highest concentrations in the skeletal muscle. They are also present in the brain, heart, and the liver, though in much lower concentrations. Carnosine is synthesized by the enzyme carnosine synthetase (β-alanine, L-histidine ligase: EC 6.3.2.11). This enzyme has the highest activity in skeletal muscle (0.95 nmol/h/mg protein), but is also present in the brain (0.05 nmol/h/mg protein) and heart (0.26 nmol/h/mg protein) (Seely and Marshall 1982). Anserine is probably produced by methylation of carnosine. The physiological elimination of carnosine is catalysed by β-alanyl-histidine dipeptidase (EC 3.4.13.5, carnosinase), and γ-homoalanyl-histidine dipeptidase (EC 3.4.13.6, homocarnosinase). The biological half-life of these dipeptides is 3 weeks in rat, and 4 weeks in chicken muscle (Harms and Winnick 1954). The level of carnosine in skeletal muscle is 180-250 mg/100 g muscle wet weight, depending primarily on the distance of the sample from nerve endings (Severin et al. 1963). The carnosine level is higher around the nerve terminals than in other areas. In contrast to the high concentration of carnosine in skeletal muscle, its level in myocardium is only 43.9 mg/kg wet weight.

These imidazole dipeptides play an active role in muscle contractions by stimulating the ATPase activity of actomyosin (Gergely et al. 1959, Severin et al. 1963, Yun and Parker 1965). Contractions of the neurally stimulated muscle immersed in a carnosine-containing Tyrode bath continue, in spite of its lowered ATP and creatine phosphate content, as compared to the control muscle, which loses its reactivity to indirect nerve stimulation. The exact physiological roles of these dipeptides are unclear at present.

11.4. PEPTIDE HORMONES AFFECTING DEVELOPMENT AND PROTEIN METABOLISM IN THE SKELETAL MUSCLE

(1) Somatomedins are growth hormone-dependent single chain peptides with growth promoting and insulin-like activities (see Chapter 8). In skeletal muscle cell cultures, and in experiments on isolated muscles (Florini et al. 1977, Ewton and Florini 1980, 1981a,b) somatomedin-A, somatomedin-C and multiplication-stimulating activity acted as stimulators of the early developmental stage of the muscle (myoblast and myotube stadium), enhancing amino acid incorporation (Judson et al. 1974), increasing the levels of muscle-specific proteins such as creatine kinase (CK), a marker for muscle cell differentiation (Ewton and Florini 1981a). Somatomedins bind specific receptors which undergo phosphorylation on the cell surface and are internalized before being degraded (Jacobs et al. 1983). The differentiation-promoting activities of polyamines are also closely associated with the action of somatomedins, the former ones being insufficient to carry out the differentiation effect alone (Kuehn and Atmar 1983).

(2) Somatostatin (growth hormone release-inhibiting hormone: GH-RIH) is a cyclic tetradecapeptide, which was first isolated from ovine hypothalami (reviewed by Schally et al. 1978; see also Chapters 3 and 5). Although the occurrence of somatostatin in skeletal muscle has not yet been documented, a soluble somatostatin-binding protein was found (Ogawa et al. 1977), suggesting that skeletal muscle is a potential target tissue for somatostatin. It is highly probable that, similarly to substance P (Furness et al. 1982) and vasoactive intestinal peptide (Jarhult et al. 1980), somatostatin occurs in special neuron terminals in the vascular bed of the skeletal muscle. The biological half-life of somatostatin is extremely short in the serum (less than 4 min), and the peptide is split by cytosolic and membrane-bound sulfhydryl-dependent endopeptidases and aminopeptidases (Conlon et al. 1982).

In a concentration of $2x10^{-8}$ mol/l, somatostatin reduces the extent of alanine release, and increases glutamine release in rat skeletal muscle preparations (Magno-Sumbilla et al. 1980). However, it has no effect on the incorporation of alanine, glutamine, glutamic acid, leucine, isoleucine and valine into muscle proteins. The alanine release-inhibitory, and the glutamine release-enhancing effects of somatostatin occur at concentrations observed in other somatostatin-reactive target tissues. The exact mechanism by which somatostatin acts in muscle tissue is unknown at present. Available information supports a mode of action similar to that in pancreatic β-cells, where somatostatin acts distally to cAMP to elicit insulin release.

(3) Insulin and growth hormone (GH). We only mention, but do not discuss here these two peptide hormones. Within the frame of their widespread effects on intermediary metabolism, insulin and GH are also renowned for their anabolic effects through which they profoundly affect, especially the protein metabolism of the skeletal musculature.

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CHAPTER 12

POSSIBLE INVOLVEMENT OF ENDOGENOUS PEPTIDES IN SOME DISEASES

J. GROF

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12.1. ENDOGENOUS PEPTIDES IN UREMIC INTOXICATION

In 1972, Babb et al. extended the already long list of putative uremic toxins by adding to the list a new group of serum components, referred to as middle molecules (MMs). In their middle molecular hypothesis (MMH) it was postulated, purely on a theoretical basis, that molecules with M_rs ranging between 300 and 5,000 might play an etiological role in the development and/or the maintenance of some of the symptoms characteristic of uremic intoxication.

12.1.1. CHEMISTRY OF MMs

The existence of MMs, and their potential role in the molecular etiology of uremic intoxication were initially deduced exclusively from clinical observations made on patients subjected to different dialysis strategies. Attempts to elucidate the chemical characteristics of MMs from different body fluids have only started in recent years. The introduction of up-to-date separation techniques and chemical analytical methods has made it finally possible to undertake a more detailed analysis, in terms of quantity as well as composition, of serum MMs.

Since the pioneering observation by Christol and Monnier (1938), circumstantial evidence on the peptidic character of uremic MMs has continuously been presented in the literature. The initial observations which indicated the peptidic nature of, at least some uremic MMs can be summarized as follows: partially purified MM fractions (a) ranged between 300-5,000 D in their molecular mass; (b) reacted positively with chemical reagents used for the detection of free amino groups and peptide bonds; (c) yielded free amino acids upon acid or alkalic hydrolyses. Only recently, however, has the peptidic nature of uremic MMs been experimentally documented, partly by establishing the qualitative amino acid composition of some highly purified MM preparations (Peters et al. 1974, Bergstrom 1975, Migone et al. 1975, Funck-Brentano et al. 1976, Lutz 1976, Bovermann et al. 1980, Buzio et al. 1980, Grof and Menyhart 1981, Menyhart and Grof 1981, Shaykh et al. 1984), and partly by isolating, characterizing as well as sequencing some of the uremic peptides, as summarized below.

A. Uremic peptides with known amino acid sequences

Lote et al. (1976): Lys-His-Gly

Abiko et al. (1974, 1978a,b, 1979a,b):

1. Asp-Gly
2. His-Gly-Lys (positions 58-60 in the α-,β-,γ- and δ-chains of hemoglobin)
3. Gln-Asp-Gly (positions 397-399 in the α- and β-chains of fibrinogen)
4. Asp-Leu-Trp-Glu-Lys (positions 123-127 in the β-chain of fibrinogen)
5. His-Pro-Ala-Glu-Asn-Gly-Lys (positions 13-19 in β_2-microglobulin)

Bovermann et al. (1982): Ala-Phe-Phe-Gly-Gly-Gln

B. Uremic peptides with known number of constituent amino acids (the number of constituent amino acids is indicated in parentheses)

Lutz (1975, 1976):

1. Glu(1),Asp(1),Leu(2),Ser(1),Arg(3),Thr(1) (also contains a polyamine and two carbohydrate moieties).
2. Ala(1),Gly(2),Glu(3),Ser(1 or 2),Thr(3)
3. Ala(2),Asp(1),Gly-(2),Lys(2),Arg(3),Phe(1)
4. Asp(1),Leu(3),Val(2),Arg(3),Thr(1),Phe(1),His(3)
5. Glu(1),Leu(3),Gly(2),Lys(2),Ser(2),Thr(2),Phe(1),His(3)

Boverman et al. (1980): Ala(1),Glu(1),Asn(1),Gly(2),Phe(2)

Buzio et al. (1980):

1. Ala(1),Glu(2),Gly(4-5)
2. Ala(1),Glu(2),Asp(1),Gly(3),Ser(1)
3. Ala(1),Glu(3-4),Asn(1),Leu(1),Gly(2),Ser(1)
4. Ala(1-2),Glu(2),Asp(2-3),Gly(5-6),Lys(4),Ser(2-3),Val(1)
5. Ala(2),Glu(3),Asn(2),Gly(6),Lys(2),Ser(3),Val(1)
6. Ala(2),Glu(4),Asn(2-3),Gly(5),Lys(1-2),Ser(4), Val(1),Arg(1),Thr(1),Ile(1),His(1)

C. Qualitative amino acid composition of highly purified uremic peptides (residues in parentheses indicate alternatives; Uir=unidentified residue; m=methylated residue; Orn=ornithine, Pse=phosphoserine, Cit=citruline; Tau=taurine)

Furst et al. (1980): Gly+Uir

Ota et al. (1980): Glu,Gln,Asp,Cys

Menyhart and Grof (1981):

1. Gly,Lys
2. Gly(Ser),Lys
3. Asp,Gly
4. Ala,Gln,Asn,Lys
5. Gln(Thr),Gly,Ser,Tyr,Cys,Uir
6. Asn,Leu,Thr,Tyr,Cys
7. Ala,Glu,Gln,Ser,Arg,Uir
8. Glu,Gln(Ser),Gly,Tyr,Cys
9. Asn,Gly,Thr,Tyr,Cys,Uir
10. Gly,Ser,Arg,Thr,Pro,Cys,Uir

11. Gly,Ser,Val,Arg,Thr,Cys,Uir
12. Glu,Asn,Gly,Ser,Thr,Cys
13. Ala,Gln(Thr),Asn,Ser,Met,Phe,Cys,Uir
14. Gln,Asn,Gly,Lys(Arg),Ser,Phe,Tyr,His,Uir
15. Gln,Asn,Lys,Ser,Arg,Thr,Cys,Uir
16. Glu,Gln,Leu,Lys,Thr,Met,Phe
17. Ala,Gln,Asn,Lys,Ser,Met,Cys,His,Uir
18. Asn,Leu,Gly,Lys,Arg,Thr,Met,Phe,Uir
19. Ala,Glu,Asp,Leu,Lys,Ser,Arg,Phe
20. Lys,Ser,Arg,Thr,Met,Phe,Tyr,His,Uir
21. Gln,Asn,Gly,Lys,Ser,Thr,Phe,Tyr,Cys,Uir
22. Ala,Asp,Asn,Leu,Lys,Ser,Val,Arg,Thr,Phe,Uir

Shaykh et al. (1984):

1. Ala,Glu,Asp,Gly,Lys,Ser,Thr,Ile,Phe,Tyr,His,Orn,Pse
2. Ala,Glu,Asp,Gly,Lys,Ser,Thr,Ile,Met,Phe,Tyr,His,Orn,Pse
3. Ala,Asp,Gly,His,Ser,Val,Thr,Pro,Ile,Phe,Tyr,His,Orn,Pse
4. Ala,Glu,Asp,Gly,Lys,Ser,Arg,Thr,Ile,Met,Phe,Tyr,His, Orn,Pse
5. Ala,Glu,Asp,Gly,Lys,Ser,Val,Thr,Pro,Ile,Phe,Tyr,His, Orn,Pse
6. Ala,Glu,Asp,Gly,Lys,Ser,Val,Arg,Thr,Pro,Ile,Met,Phe, Tyr,His,mHis,3mHis,Cit,Orn,Pse,Tau

The total synthesis of peptides isolated by Abiko's group as well as the determination some of their biological activities has also been undertaken (Abiko et al. 1978a, 1979a,b, 1980).

The presence of sugar-containing moieties, like glucuronic acid conjugates, oligosaccharides (Cueille et al. 1980a,b, Furst et al. 1980, LeMoel et al. 1980a,b) or polyamines (Lutz 1976) in certain MM fractions, indicated that besides simple peptides nonpeptide substances such as oligosaccharides, poliols are also present in these fractions (Man et al. 1978), and that MM peptides might participate in various interactions with other molecules, thereby producing molecular complexes of a particular character (Lutz 1975, Russo et al. 1980).

The unique amino acid composition of the uremic MM peptides identified so far strongly supports the contention that uremic MM components might represent a distinct group of peptides characteristic of the uremic state.

12.1.2. TOXICITY OF UREMIC MMs

Today, there is no decisive evidence which would justify the attribution of a causal role to MMs in the clinical features of uremic toxemia, or in the molecular etiology of uremic intoxication. When emphasizing this fact, however, it must be noted that a relevant number of available information strongly suggests the toxic nature of uremic MMs or of their individual components, rendering MMs likely candidates for uremic toxins. This information has been collected from clinical observations, as well as from data obtained from in vivo and in vitro experiments.

Clinical observations indicating toxicity of MMs

Clinical observations have provided circumstantial evidence on the toxicity of MMs. The main points can be summarized as follows: (1) the incidence of neuropathy in uremic patients treated with peritoneal dialysis (which, unlike hemodialysis, removes organic and inorganic serum components of small molecular mass less effectively than MM sized ones) is significantly lower than in patients treated with hemodialysis (Scribner 1965, Babb et al. 1972); (2) an increase in the frequency, or in the duration of hemodialyses, or in the surface area of dialysing membranes, all result in a more complete removal of MM components from the serum, and simultaneously arrest uremic neuropathies or prevent their progression (Man et al. 1973, Manji et al. 1974, Mirahmadi et al. 1974, Funck-Brentano et al. 1976); (3) uremic patients benefited more from hemodialyses if membranes preferentially penetrable for larger molecules were used for their treatment (Man et al. 1973,

Funck-Brentano et al. 1975); (4) favourable experiences have also been collected with both hemoultrafiltration (HUF) (Jorsted et al. 1980) and hemoperfusion (Chang and Michelsen 1973, Chang et al. 1974, Asaba et al. 1979), methods eliminating MM components more effectively than conventional dialysers; (5) while practically identical urea and creatinine levels can be detected in symptomless patients and in patients with toxemia, some MM fractions were shown to be much higher in patients with severe uremic symptoms (Asaba et al.1980); (6) MM concentrations in the serum of severely uremic patients who developed pericarditis, neuropathy, and malabsorbtion-induced starvation were high, whereas low MM concentrations were detected in patients devoid of such symptoms, due to adequate dialysis or effective conservative treatment (Furst et al. 1974, Bergstrom 1975); (7) a positive correlation has been established between the serum levels of MMs, the decrease in conduction velocity of the motor nerves, and the clinical severity of neuropathies (Man et al. 1978, Bortella et al. 1980); (8) following kidney transplantation, the elevated serum concentrations of MMs decreased to normal values and this was accompanied by the alleviation of the clinical symptoms (Grof et al. 1974, Asaba et al. 1977. Grof and Menyhart 1979, Gal and Grof 1983).

In vivo evidence on the toxicity of MMs

(1) Some partially purified MM fractions have been reported to induce symptoms typical for neurotropic agents in mice, and killed the animals within 1-3 minutes following intraperitoneal injection. A fraction with similar activity could not be separated from sera of healthy volunteers (Grof et al. 1974, Grof and Menyhart 1977, Menyhart and Grof 1977, Grof and Menyhart 1979); (2) Long-term treatment of mice with MM components partially purified from uremic serum, resulted in a rise of MM serum concentrations to a level comparable with that found in uremia. Such artificial elevation of serum MM concentration caused an inhibition in the incorporation of

^{59}Fe into red blood cells, and reduced the hemoglobin concentration as well. Iron uptake of immature erythroblasts remained unchanged at the same time (Scigalla et al. 1980a,b,c); (3) Injection of allogeneous spleen lymphocytes into lethally irradiated mice preincubated in the presence of MMs, caused a marked depression in the "graft versus host" (GVH) reaction. When a MM-containing solution was continuously infused into rats, a significant delay was observed in rejection of the skin allograft (Navarro et al. 1980); (4) MMs isolated from uremic sera inhibited ^{3}HTdR incorporation into the bone marrow cells of mice (Scigalla et al. 1980a);(5) Uremic MM components with M_r ranging between 500 and 1,000 inhibited tubular sodium reabsorbtion in rats; (6) A tripeptide had inhibitory effects on some neurons in the dorsal horn of the spinal cord (Lote et al. 1976).

In vitro evidence on the toxicity of MMs

MM substances partially or completely purified from various body fluids of uremic patients were shown to inhibit a whole array of biological processes in vitro. These include: (1) proliferation of hemopoietic cells, 3T3 MLV2 cells (Delaporte 1980, Navarro et al. 1980), and lymphocytes (Touraine et al. 1975, Navarro et al. 1979, 1980, Bovermann et al. 1982); (2) blastic transformation and rosette formation of lymphocytes (Korz et al. 1977, Hanicki et al. 1976, Abiko et al. 1979a, Navarro et al. 1980) and sheep erythrocyte rosette formation (Abiko et al. 1979a, 1980); (3) ^{3}HTdR incorporation into fibroblast, medullary-, HeLa- and rat bone marrow-cells; (4) phagocytosis of leukocytes (Ringoir et al. 1980, Jorsted et al. 1980, Wideroe et al. 1980); (5) in vitro synthesis of hemoglobin (Goubeaud et al. 1977, Leber et al. 1978); (6) first phase of the ADP-induced platelet aggregation (Crevat et al. 1980); (7) glucose utilization in different tissues (Dzurik et al. 1972, 1973); (8) membrane potential of neuroblastoma cells (Crevat et al. 1980); (9) amplitude of the action potentials in electrically stimulated isolated frog

nerves (Funck-Brentano et al. 1976, Boudet et al. 1980, Man et al. 1980); (10) respiration of isolated mitochondria (Rinaudo et al. 1979, Crevat et al. 1980); (11) activities of the adenylcyclase, pyruvate-kinase, glucokinase (Cloix et al. 1976), insulin inducible lipoprotein-lipase enzymes (Lutz 1975, 1976) and the activity of LDH (Abiko et al. 1980). In addition, MM substances were shown to be cytotoxic for a number of cell lines, and to increase the hemolysis of red blood cells (Man et al. 1973, Navarro et al. 1979, Delaporte 1980, Ota et al. 1980).

Based on these observations MM components can be suspected to mediate, at least some of the toxic symptoms characteristically accompanying uremic intoxication, such as neuropathy, immunodeficiency and the increased sensitivity towards infections.

12.1.3. ORIGIN OF THE MM SUBSTANCES

As to the site of origin and the mechanism of the production of MM substances, information is sporadic and uncertain. According to our own observations (Gal and Grof 1983), the quantity of individual MM fractions isolated by ion exchange chromatography increased in the sera of conserved blood samples at 4°C, depending on the duration of storage. This observation suggests that the production of MM components may be a membrane-linked process in the blood cells, or that various MM components might be metabolized or transformed into one another as a result of some membrane-linked mechanism. This is also supported by earlier observations that membraneous structures of hepatic origin apparently produced MM substances identical with those found in uremic sera (Klein et al. 1978).

It is generally known that, by enzymatic or chemical degradation of serum proteins, toxic polypeptides of MM character may be generated (Rinderknecht and Niemand 1959). Increased protein catabolism is a well-known feature of chronic uremia. One can speculate that due to an increased protein catabolism, the degradation of proteins may be incomplete or blocked at

the level of proteases that normally carry out a complete decomposition of toxic protein intermediates. This is also supported by the observation of Abiko's group (1978a, 1979), demonstrating the structural identity of some of the sequenced uremic peptides with fragments of normal serum proteins.

According to the clinical observations of Malkki et al. (1977) a considerable amount of MMs must be of dietary origin, and consequently the blood level of these substances could be substantially reduced by hydrolyzing food proteins with exogenous proteases in the gut before absorbtion.

12.2. ENDOGENOUS PEPTIDES IN NEUROPSYCHIATRIC DISORDERS

Major aspects of this topic have been discussed in Chapter 5. However, the emerging significance of prion proteins in some peculiar neurologic diseases remained unmentioned in previous discussions.

12.2.1. PRION PROTEIN-ASSOCIATED NEUROLOGIC DISEASES

Creutzfeldt-Jakob disease (CJD), Gertsmann-Straussler syndrome (GSS), and kuru in man and scrapie in animals represent CNS-related pathological conditions with a number of common characteristics: they are transmissible diseases with common histological features in the CNS; there are no signs of a viral etiology in any of these diseases; there is no inflammatory or immune response during their course. Highly purified preparations of the scrapie agent are, by all indications, devoid of nucleic acids (viruses), and they consist primarily of a sialoglycoprotein of M_r 27,000-30,000 which proved inseparable from infectivity. To distinguish between the infectious agents causing CJD, GSS, kuru and scrapie, and the viruses, the term "prion" was coined by Prusiner (1982) to designate the putative pathogenic agent. Presently, the only known macromolecules among prions are prion proteins (PrP). Of these cDNA-derived amino acid sequences of hamster (Oesch et al.

1985), human (Kretzschmar et al. 1986) and murine (partial sequence only) origin (Chesebro et al. 1985) have been hitherto reported. The human and hamster proteins consist of 253 and 254 amino acid residues, respectively. Conservation of PrP amino acid sequence between hamster and human is about 90%, reflecting similar structural features and shared antigenicity of the two proteins. PrPs likely are membrane-bound structures having two possible glycosylation sites. The conservation between the hamster and human proteins suggests that they may have important (conserved) functions in cellular metabolism and may simultaneously explain the similarities between scrapie and the corresponding human diseases. Although the etiological role of PrPs in the mentioned diseases are widely accepted, Manuelidis and associates (1987) have recently questioned the causal relationship between PrP and CJD (for more details and references on PrPs see Carlson et al. 1986, Prusiner 1986, Sparkes et al. 1986, Bannister and McCormick 1987).

12.3. ENDOGENOUS PEPTIDES IN MISCELLANEOUS DISEASES

Pathological conditions causally associated with one or the other endogenous peptides have been sporadically mentioned in the course of previous discussion. However, known or unknown peptides have also been postulated as etiologic factors in a number of other disease states such as the toxemia of pregnancy (e.g., Vorne et al. 1974) neoplastic diseases (see e.g., the whole volume of the journal: Drugs under Experimental and Clinical Research, Vol. 13, suppl. 1, 1987), psoriasis (e.g., Tagami et al. 1982, Weber et al. 1982) and the post-burning condition (e.g., Gay et al. 1980). Since, however, truly convincing evidence on the causal involvement of peptides in the molecular etiology of these diseases is lacking at present, no attempt is made for their further discussion.

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CHAPTER 13

NONMAMMALIAN MESSENGER PEPTIDES

J. MENYHART

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INTRODUCTION

Until recently, in the notion of scientists there has been a close association of the messenger molecules with particular anatomical structures in mammals, e.g., with the endocrine and nervous systems, respectively. First, since glandular structures arose at the level of the vertebrates, it was assumed that hormone molecules were restricted to the vertebrates. Second, because nerves did not appear evolutionarily until the level of the early metazoa, it was postulated that the neurotransmitter molecules did not arise phylogenetically until that level. It is now clear that peptides with important biological functions may be found in many species at strikingly distant stages of phylogenetic development,indicating an ubiquitous occurrence of these molecules along the whole spectrum of the evolutionary scale. Apart from mammals, biologically active peptides have been found in aves, reptiles, octopods, amphibians, insects, and also in unicellular organisms, to mention only the main sources (for review see LeRoith and Roth 1984, O'Shea and Schaffer 1985). In this context, it is worth stressing that peptides found in completely different animal species may be very closely related, both chemically and pharmacologically, and conversely, closely related species may contain completely different peptides. From taxonomical and evolutionary points of view, it may be of interest that restricted groups of species, e.g.,amphibians living in far distant geographic areas, may have closely related or occasionally identical peptide messengers. Most, if not all, of the nonmammalian peptides have their counterpart(s) in mammals. Therefore, the discovery of new peptides in nonmammalian or-

ganisms may be a useful impetus in the search for the same or similar peptides in mammalian organisms.

13.1. PEPTIDES IN NONMAMMALIAN VERTEBRATE SPECIES

As to the nonmammalian vertebrate peptides, those present in amphibians were studied most thoroughly. Interestingly, amphibian skin has been recognized as a real factory and storehouse of a multitude of bioactive peptides. Based on structural homologies, amphibian peptides are divided into four main categories: (1) bradykinin-like peptides, or bradykinins; (2) physalaemin-like peptides or tachykinins; (3) caerulein-like peptides; (4) bombesin-like peptides.

13.1.1 BRADYKININ-LIKE PEPTIDES (BK-LP)

Amongst the nonmammalian peptides hitherto recognized, bradykinin (BK) and its sister peptides (Table 13.1) are the most widely distributed inside and outside the amphibian kingdom. The name bradykinin refers to the slowly developing smooth muscle contracting activity of the peptide (bradus=slow, Greek), as opposed to the rapidly acting tachykinins (tachus=fast, Greek). BK-LPs beside those listed in Table 13.1 include: bufokinin, a structurally uncharacterized peptide (M_r 1,000) with a BK-like activity that was isolated from the toad B u f o m a r i n u s p a r a c n e m i s; pachykinins are synthetic BK analogs having permeability factor-like activities rather than kinin activities; ornithokinin, one of the avian plasma kinins with an unknown structure is ineffective in mammals but hypotensive in birds (for information on the kinin systems in the blood of lower vertebrates and aves see Erdos et al. 1967, Seki et al. 1973); BK-potentiating factors (BK-PF) are structurally related peptides discovered in venoms of some poisonous snakes that, besides being smooth muscle

Table 13.1. Bradykinin-like peptides

N°	Name	Positions+ 1 2 3 4 5 6 7 8 9
1.	Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg=BS
2.	Thr^6-bradykinin	BS(Thr^6)
3.	Val^1, Thr^6-bradykinin	BS(Val^1, Thr^6)
4.	Lys-bradykinin (kallidin)	Lys-BS
5.	Met-Lys-bradykinin (Met-kallidin)	Met-Lys-BS
6.	Ala-Arg, Thr^6-bradykinin (polisteskinin-R)	Ala-Arg-BS(Thr^6)
7.	Polisteskinin	pGlu-Thr-Asn-Lys-Lys-Lys-Leu-Arg-Gly-BS
8.	Vespulakinin	Thr-Ala-Thr-Thr-Arg-Arg-Arg-Gly-BS
9.	Vespakinin X	CH CH Ala-BS-Ile-Val
10.	Vespakinin M	Gly-BS(His^3)-Ile-Asp-SO_3H
11.	Phyllokinin	BS-Ile-Tyr
12.	Ranakinin-N	BS-Val-Ala-Pro-Ala-Ser
13.	Ranakinin-R	(Thr^6)BS-Ile-Ala-Pro-Glu-Ile-Val
14.	Bombinakinin-O	BS-Gly-Lys-Phe-His

+Note: BS= basic structure, which is the struture of authentic bradykinin. Substitutions within the BS are indicated by residues in parentheses. Extensions of the BS are indicated by amino acids or amino acid sequences preceding (N-terminal extension) or following (C-terminal extension) the symbole BS. CH indicates carbohydrate moieties.

contracting agents on their own, potentiate smooth muscle actions of BK-LPs. There are indications that BK-LPs act on different receptor types (Camargo and Ferreira 1971).

The activity spectra of natural BK-LPs are qualitatively, but not quantitatively, similar. The typical action of intravenously administered BK-LPs is a general vasodilatation with a consecutive hypotension resulting from a direct action on the vascular smooth muscles. Being, however, potent catecholamine releasers, BK-LPs may secondarily elicit a vasoconstriction and blood pressure elevation. Systematically administered BK markedly affects the intracranial extracerebral circulation and enhances vascular permeability. BK is a potent stimulant of various extravascular smooth muscles acting directly or through releasing prostaglandins. Centrally administered BK has marked behavioral effects, inhibits neurotransmission in the spinal cord and potentiates pentobarbital sleeping time (Okada et al. 1977). BK itself, and its sister

peptides with N-terminal extensions are potent algogenic agents. BK was shown to inhibit tumor growth in vivo (Koppelmann et al. 1975) and to stimulate the growth of various cells in culture (reviewed by Stewart and Channabasavaih 1979).

BK and related peptides occur in a broad spectrum of living organisms and are especially widely distributed in defense secretions (venoms) of various species such as bees, wasps, snakes and in amphibian skin glands. The toxic actions of venoms released at bite of some poisonous snakes are enhanced by a simultaneous release of potent BK-PFs into the venom. Furthermore, venoms of some wasps contain serotonin, histamine and polisteskinin which act synergistically in producing pain and inflammation (for a review on BK see Bertaccini 1976).

13.1.2. TACHYKININS (TK)

The group of tachykinins, also termed physalaemin-like peptides, display characteristic structural resemblances (Table 13.2). Uperolein resembles physalaemin most closely and substance P (SP) less closely. The functionally important part of SP has a close analogy to Met-enkephalin: although SP does not bind to opiate receptors, the two peptides have an intricate relationship in the neuronal network subserving pain perception and pain control in mammals (Basbaum and Fields 1984; see also Chapter 5).

Although the majority of TKs is of nonmammalian origin, SP represents a mammalian peptide within the group (see Chapters 5, 6 and 9).

TKs are rather powerful hypotensive and ileum-contracting agents that cause vasodilatation also in the brain and the coronaries. The effect of TKs on capillary permeability is more marked than that of BKs or even histamine (Rocha e Silva 1972). TKs, and especially SP were shown to be potent releasers of histamine from rat mast cells in vitro (Erdos and Johnson 1973, Johnson and Erdos 1973).

The generally contracting effects of TKs on extravascular smooth muscles display species- and organ-dependent quantita-

Table 13.2. Tachykinins

N°	Name	Positions[x] 12	11	10	9	8	7	6	5	4	3	2	1
1.	Physalaemin	pGlu-	Ala-	Asp-	Pro-	Asn-	Lys-	Phe-	Tyr-	Gly-	Leu-	MetNH$_2$	
2.	Phyllomedusin	pGlu-	Asn-	Pro-	Asn-	Arg-	Phe-	Ile-	Gly-	Leu-	MetNH$_2$		
3.	Uperolein	pGlu-	Pro-	Asp-	Pro-	Asn-	Ala-	Phe-	Tyr-	Gly-	Leu-	MetNH$_2$	
4.	Eledoisin	pGlu-	Pro-	Ser-	Lys-	Asp-	Ala-	Phe-	Ile-	Gly-	Leu-	MetNH$_2$	
5.	Kassinin	Asp-	Val-	Pro-	Lys-	Ser-	Asp-	Gln-	Phe-	Val-	Gly-	Leu-	MetNH$_2$
6.	Substance P (mammalian)	Arg-	Pro-	Lys-	Pro-	Gln-	Gln-	Phe-	Phe-	Gly-	Leu-	MetNH$_2$	

[x]Note: Numbering of constituting amino acids starts at the C-terminal ends of the molecules.

tive differences. TKs are potent stimulants of the salivary and lacrimal glands in many species (Bertaccini and de Caro 1965, Emmelin and Leninger 1967). Prolonged administration of physalaemin, but not eledoisin, caused an obvious hypertrophy in the main salivary glands of rats (Cantalamessa et al. 1975). Physalaemin, and eledoisin stimulate the exocrine pancreas and cause a depolarization in spinal motoneurons by direct actions. Centrally administered physalaemin and eledoisin display qualitatively different behavioral effects in rats.

13.1.3. CAERULEIN-LIKE PEPTIDES (C-LP)

Of this group of peptides shown in Table 13.3, the first three are of nonmammalian and the remaining two of mammalian origin that share many structural and biological properties. The nonmammalian molecules were found in the skin of various frogs and other amphibians. The major structural determinants of gastrin- and CCK-like activities are the appropriately positioned sulfated tyrosyl residues (see Table 13.3). Therefore, C-LPs possess CCK-like activity, but little gastrin-like activity. Caerulein, its C-terminal octapeptide and CCK-8 are more potent than is the parent CCK-33 molecule in eliciting a CCK-like activity.

The C-terminal heptapeptide sequences of C-LPs are of crucial importance in respect of their biological activities,

Table 13.3. Caerulein-like peptides

N°	Name	Positions[x] 10 9 8 7 6 5 4 3 2 1
1.	Caerulein	pGlu-Gln-Asp-Tyr(+)-Thr-Gly-Trp-Met-Asp-PheNH$_2$
2.	Peptide from Hylambates maculatus	pGlu-Asn-Asp-Tyr(+)-Leu-Gly-Trp-Met-Asp-PheNH$_2$
3.	Phyllocaerulein	pGlu-Glu-Tyr(+)-Thr-Gly-Trp-Met-Asp-PheNH$_2$
4.	CCK 8	Asp-Tyr(+)-Met-Gly-Trp-Met-Asp-PheNH$_2$
5.	Hexagastrin	Tyr(+)-Gly-Trp-Met-Asp-PheNH$_2$

[x]Note: Numbering of constituting amino acids starts at the C-terminal ends of the molecules. Cross marks indicate sulfated Tyr residues.

whereas the N-terminal portions seem to be devoid of any particular importance. Some activities are still retained in the C-terminal tri- and dipeptide sequences.

Caerulein and its sister peptides are only moderately hypotensive even in much higher doses than those required for their major pharmacological actions.

As to their main pharmacological effects, the activity spectra of C-LPs largely resemble those of gastrin and CCK. However, in contrast to all other polypeptides discussed here, caerulein has no bronchoconstrictor activity in anaesthetized guinea pigs.

13.1.4. BOMBESIN-LIKE PEPTIDES (B-LP)

This is the most recently discovered, and therefore the least thoroughly investigated group of the hitherto discussed nonmammalian peptides (Table 13.4). The nonmammalian members of the group were isolated from the skin of amphibians, mostly frogs, whereas neuromedin B, the only shown mammalian representative of the group, was isolated from the spinal cord of some mammals (for additional mammalian B-LPs see Chapters 5 and 6).

Table 13.4. Bombesin-like peptides

N°	Name	Positions[x] 17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
1.	Bombesin							pGlu-	Gln-	Arg-	Leu-	Gly-	Asn-	Gln-	Trp-	Ala-	Val-	Gly-His-Leu-MetNH$_2$
2.	Alytesin							pGlu-	Gly-	Arg-	Leu-	Gly-	Thr-	Gln-	Trp-	Ala-	Val-	Gly-His-Leu-MetNH$_2$
3.	Litorin										pGlu-	Gln-	Trp-	Ala-	Val-	Glu-	His-	Phe-MetNH$_2$
4.	Phyllolitorin										pGlu-	Leu-	Trp-	Ala-	Val-	Gly-	Ser-	Phe-MetNH$_2$
5.	Leu phyllolitorin										pGlu-	Leu-	Trp-	Ala-	Val-	Gly-	Ser-	Leu-MetNH$_2$
6.	Rhodei litorin										pGlu-	Leu-	Trp-	Ala-	Thr-	Gly-	His-	Phe-MetNH$_2$
7.	Ranatensin									pGlu-	Val-	Pro-	Gln-	Trp-	Ala-	Val-	Gly-	His-Phe-MetNH$_2$
8.	Ranatensin C									Glx-	Thr-	Pro-	Gln-	Trp-	Ala-	Val-	Gly-	His-Phe-MetNH$_2$
9.	Ranatensin R	Ser-	Asp-	Ala-	Thr-	Leu-	Arg-	Arg-	Tyr-	Asn-	Gln-	Trp-	Ala-	Thr-	Gly-	His-	Phe-	MetNH$_2$
10.	Neuromedin B (mammalian)									Gly-	Asn-	Leu-	Trp-	Ala-	Thr-	Gly-	His-	Phe-MetNH$_2$

[x]Note: Numbering of amino acid residues starts at the C-terminal ends of the individual molecules.

The C-terminal octapeptide sequences are identical or closely related in all B-LPs, and the structural similarities are associated with similarities in their pharmacological activities.

In general, B-LPs have modest hypertensive effects in large doses that appear to be independent of β-receptors and of norepinephrine release. The cross-tachyphylaxis between ranatensin and the structurally unrelated angiotensin suggests that the two peptides occupy closely associated receptors. Other important effects such as gastrin-dependent stimulation of gastric acid secretion and stimulation of CCK-release (Bertaccini et al. 1973, Impiccatore et al. 1973) could be elicited with much lower doses of bombesin than could with caerulein. Bombesin was shown to stimulate pancreatic juice secretion and gallbladder emtying probably through CCK release (Linari and Baldieri-Linari 1975). Bombesin affects gut motility in a rather unpredictable manner, the quality of the response being species-dependent. Bombesin reduces VIP secretion in the cat and man (Melchiorri et al. 1975) and B-LPs elicit contractions in many extravascular smooth muscles. B-LPs, especially rhodei litorin have a substantial affinity for rat urinary bladder receptors (Barra et al. 1985).

13.2. PEPTIDES IN LOWER VERTEBRATE AND INVERTEBRATE SPECIES

There are additional nonmammalian peptides not listed in the previously described groups which possess interesting or unique biological activities, or chemical properties that form the bases of occasionally intriguing physiological functions in some lower vertebrate and invertebrate species. There was a general consensus amongst the authors of this volume that leaving these peptides entirely unmentioned would be rather unfortunate. Therefore, it was decided that a limited number of admittedly arbitrarily selected peptides operating in lower vertebrates and invertebrates be included and briefly discussed, without aiming towards completeness.

With a few exceptions (Dockray and Dimaline 1985) data on avian peptides are rather scanty. The best studied ones are discussed in Chapters 5, 6 and 8 no attempts were made to include further avian peptides in the following discussion.

13.2.1. PEPTIDES IN LOWER VERTEBRATE SPECIES

13.2.1.1. UROTENSIN

The term urotensin (UT) refers to the four peptides (UT I-IV) hitherto purified from the urophysis, the caudal neurosecretory system of teleostean fishes, that all have potent actions on the smooth muscles. Of the UTs, UT-I and UT-II have been sequenced so far. The amino acid sequence of UT-I (M 4,864) isolated from the teleost C a t a s t o m u s c o m m e r s o n i is as follows:
Asn-Asp-Asp-Pro-Pro-Ile-Ser-Ile-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Asn-Met-Ile-Glu-Met-Ala-Arg-Ile-Glu-Asn-Glu-Arg-Glu-Gln-Ala-Gly-Leu-Asn-Arg-Lys-Tyr-Leu-Asp-Glu-ValNH_2 (Lederis et al. 1982). UT-I exhibits a mild vasopressin-like activity in fishes and other cold-blooded vertebrates, a potent hypotensive activity in mammals and birds and a CRF-like activity in both fishes and mammals (Fryer et al. 1983, Lederis et al. 1985, Ishida et al. 1986, Iwanaga et al. 1986).

The primary structure of UT-II (M_r 1,360) isolated from the teleost *Gillichthys mirabilis* is the following: Ala-Gly-Thr-Ala-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val. (Pearson et al. 1980). This sequence is homologous to that of somatostatin in positions 1-2 and 7-10. UT-II exerts hypertensive, smooth muscle-contracting and osmoregulatory effects as its main biological activities.

13.2.1.2. MELANIN-CONCENTRATING HORMONE

A putative melanin granule (melanosome)-concentrating hormone (MCH) is synthesized in the teleost hypothalamus and stored in, and released from, the neurohypophysis (Baker and Rance 1983). The primary structure of MCH is as follows: Asp-Thr-Met-Arg-Cys-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val, (Kawauchi et al. 1983, Wilkes et al. 1984) in which Cys(5) and Cys(14) are disulfide bonded.

MCH stimulates melanosome concentration (centripetal aggregation) within melanophores of all teleostean species, but stimulates melanosome dispersion (centrifugal movement) within the melanophores of some frogs and lizards indicating that MCH may exhibit both melanosome-concentrating and -dispersing activities depending on the species studied, and that such a hormone might play a role in chromatic responses (color changes) of these species. MCH-dependent melanosome aggregation can be reversed by equimolar concentration of α-melanocyte-stimulating hormone.

13.2.1.3. SAUVAGINE

Sauvagine isolated from the skin of the frog *Phyllomedusa sauvagei* was identified as a 40 amino acid peptide with the following primary structure: pGlu-Gly-Pro-Pro-Ile-Ser-Ile-Asp-Leu-Ser-Leu-Glu-Leu-Leu-Arg-Lys-Met-Ile-Glu-Ile-Glu-Lys-Gln-Glu-Lys-Glu-Lys-Gln-Gln-Ala-Ala-Asn-Asn-Arg-Leu-Leu-Leu-Asp-Thr-IleNH_2 (Montecucchi and

Henschen 1982). A considerable proportion of the sequence of sauvagine is homologous to those of corticotropin-releasing factor (CRF) and UT-I, idicating that these peptides may be in an ancestral relationship (Vallai et al. 1983). In harmony with their structural homologies, and with similarities in their surface properties (Lau et al. 1983), the three peptides share many biological activities, of which the CRF-like effect manifested in the release of ACTH and β-endorphin should be especially stressed (Negri et al. 1985).

13.2.1.4. BOMBININ

Bombinin isolated from the frog *Bombina variega*, the same frog from which bombesin was isolated, is a 24 amino acid peptide:
Gly-Ile-Gly-Ala-Leu-Ser-Ala-Lys-Gly-Ala-Leu-Lys-Gly-Leu-Ala-Lys-Gly-Leu-Ala-Glu-His-Phe-Ala-Asn (for references see Bertaccini 1976). From the same amphibian three smaller peptides (one hexa- and two nonapeptides) related to bombinin have also been isolated. A potent hemolytic action can be regarded as the most characteristic biological activity of bombinin (Csordas and Michl 1970).

13.2.1.5. XENOPSIN (XP)

The C-terminal region of the octapeptide XP pGlu-Gly-Lys-Arg-Pro-Trp-Ile-Leu (Araki et al. 1973) isolated from the skin of the frog *Xenopus laevis* has striking structural and biological similarities to neurotensin, a mammalian neuropeptide (see Chapters 5 and 6). The generation of XP-related peptides has also been observed in mammalian gastric tissues (Carraway and Feurle 1985).XP exhibits a hypotensive effect in vivo and contracting effects on isolated rat stomach and guinea pig ileum preparations. XP also has a remarkable ability to induce cyanosis and hyperglycemia. The peptide is markedly tachyphylactic.

13.2.1.6. THYROTROPIN-RELEASING HORMONE AND CYCLO(His-Pro)

Tyrotropin-releasing hormone (TRH), a hypothalamic tripeptide in mammals (see Chapters 3 and 5) was also isolated from frog skin (Yasuhara and Nakajima 1975). Cyclo(His-Pro) [c(HP)] or histidyl-proline diketopiperazine, a cyclic dipeptide is a metabolite of TRH that is generated by a limited enzymatic proteolysis from TRH (Prasad and Peterkovsky 1976). Subsequently, c(HP) was also demonstrated in the brain and gastrointestinal tract of mammals, in the human blood (for details and references see Prasad et al. 1984), as well as in the skin and brain of frogs of different genera (Prasad et al. 1982, 1984). The concentrations of c(HP) in the brain and gastrointestinal tract were found to be much higher than those of TRH. c(HP) elicits a rather wide spectrum of predominantly endocrine and CNS activities in mammalian species (for details and refrences see Prasad et al. 1984).

13.2.1.7. TRYPTOPHYLLINS

A whole set of tryptophane-containing peptides, termed tryptophyllins (TPHs), has been isolated from the skin of *Phyllomedusa rhodei*, a South American frog (Montecucchi et al. 1984).

The primary structures of the presently known TPHs are shown in Fig.13.1 (Montecucchi et al. 1984, 1986, Gozzini et al. 1985; for a review see Perseo et al. 1985).

TPH-like peptides including cyclo(Trp-Lys), a diketopiperazine structure, have also been demonstrated in the skin of other Phyllomedusa species and the brain of turkeys and cows (Swann et al. 1980). Various TPHs were shown to be homologous to caerulein-like peptides and xenopsin (Gozzini et al. 1985) as well as to corresponding segments of some mammalian and viral proteins (Montecucchi et al. 1986).

TPHs-4:

```
                4
pGlu-Pro-Trp-ValNH2
pGlu-Pro-Trp-MetNH2
pGlu-Pro-Trp-Met
pGlu-Ala-Trp-Met
 Phe-Pro-Pro-Trp
```

TPHs-5:

```
Phe-Pro-Pro-Trp-ValNH2
 -   -   -   - MetNH2
 -   -   -   - LeuNH2
 -   -   -   - Leu
```

TPH-7:

Val-Pro-Pro-Leu-Gly-Trp-Met

TPH-13:

```
                                        10
pGlu-Glu-Lys-Pro-Tyr-Trp-Pro-Pro-Pro-Ile-Tyr-Pro-Met
```

Fig. 13.1. Primary structures of tryptophyllins (TPH) of various length. Dashes indicate residues identical to those in the first row of that group

Preliminary biological studies on TPH-7 and TPH-13 failed to reveal any activities on mammalian smooth muscles in vitro and in vivo, but TPH-7 was shown to induce sedation and sleep in the pigeon, and TPH-13 to exert endocrine and behavioral effects in rats following a central administration. Repeated subcutaneous administration of TPH-7 and a synthetic analog increased liver protein synthesis and the body weight in the rat (for references see Gozzini et al. 1985, Perseo et al. 1985, Montecucchi et al. 1986).

13.2.1.8. ANGIOTENSIN S AND F

Decapeptides with angiotensin (AT)-like structures and activities have been isolated from both snake (AT-S; Nakayama et al. 1974) and fowl sources (AT-F; Nakayama et al. 1973). In the structure Asp-Arg-Val-Tyr-Val-His-Pro-Phe-Ser/Tyr-Leu, the Ser/Tyr substitutions at position 9 indicate the only difference between AT-S (Ser) and AT-F (Tyr).

13.2.1.9. SNAKE NEUROTOXINS

Venoms of elapid and hydrophid snakes contain a large number of basic, curaremimetic polypeptides known as postsynaptic neurotoxins (M_rs 6,000-8,000) of which approximately 90 individual toxins have been sequenced so far (for references see Grant and Chiappinelli 1985). Cloning and sequencing of a snake neurotoxin precursor cDNAs have also been reported (Tamiya et al. 1985).

The most invariant structural features of snake venom neurotoxins are the presence of four completely conserved disulfide bonds. The long (66-77 residues) and short neurotoxins (60-62 residues) characteristically block the transmission at vertebrate neuromuscular junctions and in electric tissues of electric fishes by high affinity binding to nicotinic acetylcholine receptors. In general, the blockade caused by short neurotoxins, while relatively potent, is more readily reversed than that produced by long neurotoxins with κ-bungarotoxin as the most irreversibly acting long neurotoxin. The so-called short toxins (57-61 residues) generally display very little toxicity on their own. Finally, the so-called cytotoxins (other names: cardiotoxins, direct lytic factors, cobramines and membrane-active polypeptides) affect a great variety of cells, eliciting a persistent depolarization of the plasma membranes and thereby causing functional and structural impairments of the affected cells.

Although α-bungarotoxin shows a considerable structural homology to other postsynaptic neurotoxins, its several regions are unique if compared to other postsynaptic neurotoxins providing it with unusual physiological properties. Our present knowledge concerning the structure and function of the nicotinic receptors in the vertebrate muscles and the electric organs of some marine species is largely based on the use of neurotoxins as probes for these receptors. By comparison, little is known of the neuronal nicotinic receptors present on ganglionic neurons (for further data on snake venom neurotoxins see Dufton and Hider 1983, Grant and Chiappinelli 1985, Politi et al. 1985, Aird et al. 1986).

13.2.1.10. MISCELLANEOUS PEPTIDES RESEMBLING MAMMALIAN PEPTIDES IN VARIOUS NONMAMMALIAN SPECIES

Peptides resembling known mammalian regulatory peptides such as neuropeptide Y (Osborne et al. 1985, Kimmel et al. 1986), peptide YY (Bottcher et al. 1985), various neuropeptides (Gaudino et al. 1985, Holmgren et al. 1985), peptides from the APUD system (Magistris et al. 1985) glucagon, somatostatin, substance P and vasoactive intestinal polypeptide (Conlon et al. 1985), heparin-binding growth factors (Lagente et al. 1986), glial growth factors (Brockes and Kintner 1986), nerve growth factor (Siigur et al. 1985, 1986), growth hormone and prolactin (Kawauchi et al. 1986, Yasuda et al. 1986), insulin (Cutfield et al. 1986) platelet-activating glycoprotein (Marlas 1985) and many more have been detected in various nonmammalian vertebrate species.

13.2.2. PEPTIDES IN INVERTEBRATE SPECIES

13.2.2.1. COAGULOGENS

Coagulogens isolated from the hemocytes of various horseshoe crab species proved to be structurally related peptides with 175-176 residues and a M_r of about 19,700. The amino acid sequences of coagulogens derived from three different biological sources are shown in Fig.13.2. The gelation process (see below) involves a limited proteolysis that liberates the 28 amino acid connecting (C) peptides from the inner regions of the molecules leaving behind the 18 amino acid A chains and the 129 or 130 amino acid B chains, respectively. The functionally important A and B chain regions of various coagulogen species show extremely high sequence homologies, whereas the functionally less important C-peptide regions are relatively variable. This situation is very similar to that found in mammalian fibrinogen from which fibrinopeptides A and B are released by α-thrombin upon coagulation.

```
                                        10                                  20
L.p.: Gly-Asp-Pro-Asn-Val-Pro-Thr-Cys-Leu-Cys-Glu-Glu-Pro-Thr-Leu-Leu-Gly-Arg-Lys-Val-
T.t.: Ala - Thr - Ala - Ile - - - Asp - - Gly-Val - - - Thr-Gln
T.g.: Asp - Thr - Ala - Leu - - - Asp - - Gly-Ile - - - - Glu
                                        30                                  40
L.p.: Ile-Val-Ser-Gln-Glu-Thr-Lys-Asp-Lys-Ile-Glu-Glu-Ala-Val-Gln-Ala-Ile-Thr-Asp-Lys-
T.t.: - - Thr-Thr - Ile - - - - - - Lys - - Glu - Val-Ala-Gln-Glu-
T.g.: Phe - - Asp-Ala - - Thr-Ile - - Lys - - Glu-Glu-Val-Ala-Lys-Glu
                                        50                                  60
L.p.: Asp-Glu-Ile-Ser-Gly-Arg-Gly-Phe-Ser-Ile-Phe-Gly-Gly-His-Pro-Ala-Phe-Lys-Glu-Cys-
T.t.: Ser-Gly-Val - - - - - - - - Ser-His - - Val - Arg - -
T.g.: Gly-Gly-Val - - - - - - - Leu - Ser-His - - Val - Arg - -
                                        70                                  80
L.p.: Gly-Lys-Tyr-Glu-Cys-Arg-Thr-Val-Thr-Ser-Glu-Asp-Ser-Arg-Cys-Tyr-Asn-Phe-Phe-Pro-
T.t.: - - - - - - - - - Arg-Pro - His - - - - - - Pro -
T.g.: - - - - - - - - - Arg-Pro - His - - - - - - Pro -
                                        90                                 100
L.p.: Phe-Ser-His-Phe-His-Pro-Glu-Cys-Pro-Val-Ser-Val-Ser-Ala-Cys-Glu-Pro-Thr-Phe-Gly-
T.t.: - Thr - - Arg-Leu - - - - - Thr-Arg-Asp - - - Val - -
T.g.: - Ile - - Arg-Ser - - - - - Thr-Arg-Asp - - - Val - -
                                       110                                 120
L.p.: Tyr-Thr-Thr-Ser-Asn-Glu-Leu-Arg-Ile-Ile-Val-Gln-Ala-Pro-Lys-Ala-Gly-Phe-Arg-Gln-
T.t.: - - Val-Ala-Gly - Phe - Val - - - - - - Arg - - - - -
T.g.: - - Ala-Ala-Gly - Phe - Val - - - - - - Arg - - - - -
                                       130                                 140
L.p.: Cys-Val-Trp-Gln-His-Lys-Cys-Arg-Ala-Tyr-Gly-Ser-Asn-Phe-Cys-Gln-Arg-Thr-Gly-Arg-
T.t.: - - - - - - - - - - Phe - - - Ser - Gly-Tyr-Asn - -
T.g. - - - - - - - - - - - - - - Asn - Gly-Phe-Asn - -
                                       150                                 160
L.p.: Cys-Thr-Gln-Gln-Arg-Ser-Val-Val-Arg-Leu-Val-Thr-Tyr-Asp-Leu-Glu-Lys-Gly-Val-Phe-
T.t.: - - - - - - - - - - - - - - - Asn - - - Asn-Gly -
T.g.: - - - - - - - - - - - - - - Phe-Asn - - - Asn-Gly -
                                       170                 175
L.p.: Phe-Cys-Glu-Asn-Val-Arg-Thr-Cys-Cys-Gly-Cys-Pro-Cys-Arg-Ser
T.t.: Leu - - Ser-Phe - - - - - - - - - - - - Phe(176)
T.g.: Leu - - Thr-Phe - - - - - - - - - - - - Phe(176)
```

Fig. 13.2. Amino acid sequences of coagulogens isolated from the horseshoes crabs, Limulus polyphemus (L.p.), Tachypleus tridentatus (T.t.) and Tachypleus gigas (T.g.). Dashes indicate residues identical to those in the L.p. sequence. Deletions in T.t. and T.g. sequences at position 129 were introduced for maximizing homology

The coagulogens present in the hemocytes circulating in the hemolymph of horseshoe crabs are, in fact, the invertebrate counterparts of vertebrate fibrinogens. When the hemocytes are exposed to endotoxins, the coagulogens undergo gelation: a clotting enzyme converts soluble coagulogens to insoluble gels, named coagulin. The gelation reaction is responsible both for hemostasis and defense against invading microorganisms (for data on a novel coagulogen see Srimal et al. 1985).

13.2.2.2. PIGMENT-DISPERSING HORMONES

13.2.2.2.1. BLANCHING HORMONE

Blanching hormone, also termed crustacean color change hormone, or red pigment-concentrating hormone isolated from the eyestalk of the prawn *Pandalus borealis* has the following primary structure: pGlu-Leu-Asn-Phe-Ser-Pro-Gly-TrpNH_2 . (Fernlund and Josefsson 1972). Acting as a true hormone, this peptide regulates pigment dispersal in the cuticle of prawns and shrimps causing their blanching. A white pigment-concentrating hormone acting on leucophores in the midgut and hindgut of the fresh water shrimp, *Caridina denticulata* has also been described (Miyawaki and Tsuruda 1985; see also Riehm et al.1985).

13.2.2.2.2. MELANIZATION AND REDDISH COLORIZATION HORMONE

Melanization and reddish colorization hormone (MRCH) an insect neuropeptide exists in three molecular forms (MRCH I, II, and III) with M_rs ranging between 6,400 and 8,000. MRCHs were isolated from the head regions of common armyworms and silkworms (Matsumoto et al. 1985). Partially determined N-terminal amino acid sequences of MRCHs and the C-terminal end of mammalian insulin-like growth factor II show about 50% sequence homology. In insects undergoing color polymorphism MRCHs are responsible for the formation of melanin in the cuticle and ommochrome in the epidermis.

13.2.2.3. SCORPION NEUROTOXINS

Scorpion neurotoxins are generally grouped into two main categories, α - and β-toxins, on the basis of their binding properties to rat brain synaptosomes and the mechanism of their action on the sodium channels of excitable membranes. α-toxins are present in the venoms of African and Asian scor-

```
a: Lys-Glu-Gly-Tyr-Leu-Met-Asp-His-   Glu-Gly-Cys-Lys-Leu-Ser-Cys-Phe-   Ile-Arg-
b:  -  Asp  -  -  -  Val-Glu-Lys     Thr  -  -  -  Lys-Thr  -  Tyr-Lys-Leu-Gly-
c:  -   -   -  -  -  Val-Lys-Lys-Ser-Asp  -  -  -  Lys-Asp  -  -  Trp-Leu-Gly-
d:  -   -   -  -  -  Val-Asn-Lys-Ser-Thr  -  -  -  Tyr-Gly  -  Leu-Lys-Leu-Gly-
e:  -   -   -  -  -  Val-Lys-Lys-Ser-Asp  -  -  -  Tyr-Gly  -  Leu-Lys-Leu-Gly-
f:  -   -   -  -  -  Val-Ser-Lys-Ser-Thr  -  -  -  Tyr-Glu  -  Leu-Lys-Leu-Gly-

a: Pro-Ser-Gly-Tyr-Cys-Gly-Arg-Glu-Cys-Gly-Ile-Lys-Lys-Gly-   Ser-Ser-Gly-   Tyr-
b: Glu-Asn-Asp-Phe  -  Asn  -  -  -  Lys-Trp  -  His-Ile-Gly-Gly  -  Tyr-Gly  -
c: Lys-Asn-Glu-His  -  Asn-Thr  -  -  Lys-Ala  -  Asn-Gln-Gly-Gly  -  Tyr-Gly  -
d: Glu-Asn-Glu-Gly  -  Asn-Lys  -  -  Lys-Ala  -  Asn-Gln-Gly-Gly  -  Tyr-Gly  -
e: Glu-Asn-Glu-Gly  -  Asp-Thr  -  -  Lys-Ala  -  Asn-Gln-Cis-Gly  -  Tyr-Gly  -
f: Asp-Asn-Asp  -  -  Leu  -  -  -  Lys-Gln-Gln-Tyr  -  Lys  -  -  -  Gly  -

a: Cys-   Ala-Trp-Pro-Ala-Cys-Tyr-Cys-Tyr-   Gly-Leu-Pro-Asn-Trp-Val-Lys-Val-Trp-
b:  -  Tyr-Gly-Phe     Gly  -  -  -     Glu  -  -  -  Asp-Ser-Thr-Gln-Thr  -
c:  -  Tyr  -  Phe      -   -  Trp  -     Glu  -  -  -  Glu-Ser-Thr-Pro-Thr-Tyr-
d:  -  Tyr  -  Phe      -   -  Trp  -     Glu  -  -  -  Glu-Ser-Thr-Pro-Thr-Tyr-
e:  -  Tyr  -  Phe      -   -  Trp  -     Glu  -  -  -  Glu-Ser-Thr-Pro-Thr-Tyr-
f:  -  Tyr  -  Phe      -   -  Trp  -     Thr-His  -  Tyr-Glu-Gln-Ala-Trp  -  -

a: Asp-Arg     Ala-Thr-Asn-Lys-Cys(61)
b: Pro-Leu-Pro-Asn-Lys          -  Thr(63)
c: Pro-Leu-Pro-Asn-Lys          -  Ser(63)
d: Pro-Leu-Pro-Asn-Lys          -  Ser-Ser(64)
e: Pro-Leu-Pro-Asn-Lys     Ser  -  (63)
f: Pro-Leu-Pro-Asn-Lys-Thr      -  Asn(64)
```

Fig. 13.3. Amino acid sequence of β-type toxin VII of the South American scorpion, T y t i u s s e r r u l a t u s (a), and those of various β-type toxins designated CsEI (b), CsEv1 (c), CsEv2 (d), CsEv3 (e), and CssII (f) of the North and Central American subfamily of scorpions, C e n t r u r o i d e s s c u l p t u r a t u s (Cs). The sequences are aligned with respect to the half cystine residues. Deletions (empty spaces) were introduced for maximal homology. Hyphens indicate residues identical to those of sequence "a". The number of residues is in parenthesis

pions (B u t h i n a e subfamily), β-toxins in that of North and Central American scorpions (C e n t r u r i n a e subfamily), whilst both α- and β-toxins can be found in the venom of South American scorpions (T i t y n a e subfamily). Several β-type toxins of the C e n t r u r i n a e subfamily have been sequenced earlier. The amino acid sequence of toxin VII, the most active and most abundant β-type toxin of the South American scorpion T y t i u s s e r r u l a t u s and those of several β-type toxins from the C e n t r u r i n a e subfamily are comparatively shown in Fig.13.3 (for details see Bechis et al. 1984 and the references therein).

The T y t i u s s e r r u l a t u s toxin designated toxin-γ or toxin II-11 by Possani et al. (1977) and the Tytius toxin VII reported by Bechis et al. (1984) proved to be identical. Two novel toxins from the venom of the scorpion

Leiurus quin questriatus that block distinct classes of calcium-activated potassium channels have been identified by Castle and Strong (1986; for structural informations on 18 additional scorpion toxins see Possani et al. 1985).

13.2.2.4. PROCTOLIN

Proctolin isolated from the hindgut of the cockroach Periplaneta americana as a transmitter candidate (Brown 1975) is a pentapeptide Arg-Tyr-Leu-Pro-Thr (Starrat and Brown 1975). The peptide is a potent myotropic substance stimulating the hindgut (proctodeal muscles) and the heart of cockroaches via receptors located on the membranes of muscle fibers. A proctolin-like peptide was also isolated from the lobster as a possible neurohormone (Schwartz et al. 1984).

13.2.2.5. NEUROHORMONE D

Neurohormone D (NH-D) was isolated from corpora cardiaca of the cockroach Periplaneta americana (Fig.13.4; Baumann and Penzlin 1984).

Subsequently two myotropic substances, myotropic peptides 1 and 2 (M-1 and M-2), also termed corpora cardiaca peptides 1 and 2 (CC-1 and CC-2) have been isolated and sequenced from cockroach corpora cardiaca (O'Shea et al. 1984, Scarborough et al. 1984, Witten et al. 1984). NH-D and M-1/CC-1 proved to be structurally identical (see Fig.13.4). These peptides show remarkable homologies to locust adipokinetic and hyperglycemic hormones (see below) and to the red pigment-concentrating hormone of prawns (see section 13.2.2.2.). Both M-1 and M-2 are potent stimulants of visceral muscle activities (hindgut and heart).

a: pGlu-Val-Asn-Phe-Ser-Pro-Asn-TrpNH_2
b: - Leu-Thr - Thr - - -
8

Fig. 13.4. Primary structures of neurohormone D (a) and myotropic peptide-2 (b). Dashes indicate residues identical to those in sequence "a"

13.2.2.6. LEUCOKININ AND LEUCOSULFAKININ

A four member group of structurally related insect neuropeptides, termed leucokinins (LK) I, II, III and IV has been identified in head extracts of the cockroach, L e u c o p h a e a m a d e r a e (Fig.13.5; Holman et al. 1986a,b).

	8
I:	Asp-Pro-Ala-Phe-Asn-Ser-Trp-GlyNH_2
II:	- - Gly - Ser - - -
III:	- Gln Gly - - - - -
IV:	- Ala Ser - His - - -

Fig. 13.5. Amino sequences of leucokinins I, II, III and IV. Hyphens indicate residues identical to those in sequence I

From this same source an additional myotropic neuropeptide, called leucosulfakinin (LSK) has also been isolated and shown to have the following primary structure:
Glu-Gln-Phe-Glu-Asp-Tyr-Gly-His-Met-Arg-PheNH_2 in which the Tyr(6) residue is sulfated (Nachman et al. 1986). LSK, human gastrin II (the sulfated form of gastrin-17) and cholecystokinin exhibit the highest sequence homologies (55%) ever reported between insect and vertebrate neuropeptides. The hindgut-, heart- and hemolymph-stimulating activities of LKs and LSK in cockroaches are analogous to the gastrin-induced increase in motility and blood circulation in the mammalian intestine (for details and references see Nachman et al. 1986).

13.2.2.7. ADIPOKINETIC AND HYPERGLYCEMIC HORMONES

Adipokinetic hormone (AKH) and hyperglycemic hormone (HGH), two additional corpora cardiaca peptides, play an important role in locusts and other insects as regulators of the energy metabolism during flight or fleeing. Both peptides exist in multiple molecular forms (Fig.13.6). These peptides, especially the decapeptide AKH I, are closely related to blanching hormone, or red pigment-concentrating hormone demonstrated in prawns (see section 13.2.2.2., and also Stone et al. 1976, Gade et al. 1984).

```
                                                        10
AKH I-SL:  pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH2
                                                    8
AKH II-S:     -   -   -   -   Ser-Thr-Gly-TrpNH2
                                                    8
AKH II-L:     -   -   -   -   Ser-Ala-Gly-TrpNH2
                                                    9
AKH-H  :      -   -  Thr  -   -  Ser-Ser  -  GlyNH2
```

Fig. 13.6. Primary structures of hitherto sequenced adipokinetic hormones (AKH). Dashes indicate residues identical to those of AKH I-SL. The capital letters separated by dashes refer to the species from which the peptides were isolated (see text).

The octapeptide AKH II (Carlsen et al. 1979) exhibiting AKH activities in locusts and blanching hormone activities in shrimps (for references see Gade et al. 1984) exists in two molecular forms (see Fig.13.6): AKH II-S and AKH II-L where the suffixes S and L refer to the two insects, Schistocercus gregarius and Locustus migratorius, respectively, that served as sources of their isolation. Similarly the term AKH-H refers to an additional AKH peptide that was isolated from the ear worm moth, Heliothis Zea (see Fig.13.6 and also Jaffe et al. 1986).

As to the HGH peptides, their two known forms, termed HGH I and HGH II have been alternately named myotropic peptides-1 and 2 (M-1 and M-2) or corpora cardiaca peptides 1 and 2 (CC-1 and CC-2) depending on their biological activities tested (myotropic or hyperglycemic activities) and on the sources of their isolation (corpora cardiaca). Consequently the HGH peptides are structurally identical with peptides shown in Fig.13.4 (for additional HGHs see Primley 1984, Siegert et al. 1986).

As to the biological significance of AKHs and HGHs, they play important roles, partly in supplying the flight muscles of the insect with energy-carrying metabolites through mobilizing specific diacyglycerols from the fat body of the locust during flight, and partly in stimulating the oxidative processes in the flight muscles (for references see Gade et al. 1984).

13.2.2.8. SARCOTOXIN I

Sarcotoxin I (ST I), an antibacterial peptide present in the hemolymph of the larvae of the flesh fly *Sarcophagus peregrinus* was identified as a mixture

```
                +       +  +          +  +  10  -  +                    +        +  -  20
ST IA: Gly-Trp-Leu-Lys-Lys-Ile-Gly-Lys-Lys-Ile-Glu-Arg-Val-Gly-Gln-His-Thr-Arg-Asp-Ala-
ST IB:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
ST IC:  -   -   - Arg  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

                                          30                                      39
ST IA: Thr-Ile-Gln-Gly-Leu-Gly-Ile-Ala-Gln-Gln-Ala-Ala-Asn-Val-Ala-Ala-Thr-Ala-Arg
ST IB:  -   -   - Val-Ile  - Val  -   -   -   -   -   -   -   -   -   -   -   -
ST IC:  -   -   - Val  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
```

Fig. 13.7. Primary structures of sarcotoxin I (ST I) peptides (A,B and C). Dashes indicate residues identical to those of ST IA. Charges are indicated

of three structurally related peptides (Fig.13.7; Okada and Natori 1985). The suggested mechanism of the antibacterial activities of these peptides is discussed by Okada and Natori (1985).

13.2.2.9. MELITTIN AND APAMIN

Both melittin and apamin are constituents of the venom of the honey bee, *Apis melifera*. Besides the 25 amino acid melittin: Gly-Ile-Gly-Ala-Val-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln (Pisano 1968, Habermann 1972) honey bee venom contains another 27 amino acid melittin-like peptide which differs from melittin in having a seryl residue at position 21 and the sequence Lys-Arg-Gln-Gln at positions 24 through 27. Although the blood pressure effect of the two peptides is unimpressive, both are potent in lowering the surface tension of aqueous solutions and both have hemolytic, mastolytic and antibacterial activities and cause a depression of the electrocardiogram in cats (Ishay et al. 1975, Soderhall 1985). Moreover melittin stimulates insulin secretion (Morgan et al. 1985), and enter into

a specific interaction with calmodulin (Caday and Steiner 1986; see also the next section).

The 18 amino acid apamin: Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu-Cys-Ala-Arg-Arg-Cys-Gln-Gln-His, also a bee venom constituent, has a long-lasting excitatory effect on the CNS (for more details on bee venom peptides see Pisano 1968, Haberman 1972).

13.2.2.10. MASTOPARANS

The mastoparans (MPs) were discovered in wasp venoms. The presently known members of the group (Fig.13.8; for references see Argiolas and Pisano 1984) include MP, the parent molecule, MP-C (where C refers to the isolation of the peptide from the venom of the European hornet, *Vespa crabro*), MP-M (isolated from *Vespa mandarinia*), MP-X (isolated from *Vespa xanthoptera*) and Polistes mastoparan, or P-MP (isolated from *Polistes jadwigae*).

MPs have the same potency as melittin has in facilitating phospholipase A_2, but melittin is more active than are MPs in lysing erythrocytes. The amylase-releasing effect of MPs from isolated pancreatic acini is also due to a lytic action on the acinar cells. MPs and melittin bind to lysosomes and their common amphiphilicity promotes their binding to natural phospholipids. MPs also bind to calmodulin with the highest activity ever observed for a peptide (for references see Argiolas and Pisano 1984, McDowell et al. 1985). Both MPs and melittin facilitate phospholipase A_2 -catalyzed release of arachidonic acid from egg yolk lecithins and from a number of cells and

```
                                                10              14
MP  : Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-LeuNH2
MP-C: Leu - - - - - Leu - Val - - - - -
MP-M: - - - - - Ile - - - - - - - -
MP-X: - - Trp - Gly-Ile - - Met - - - Leu -
MP-P: Val-Asp-Trp - Lys-Ile-Gly-Gln-His-Ile-Leu-Ser-Val -
```

Fig. 13.8. Amino acid sequences of various mastoparans (MP). The capital letters separated by dashes refer to the species from which the peptide was isolated (see text). Hyphens indicate residues identical to those in MP sequence

tissues in vitro, suggesting that at least one of the functions of MPs (and melittin) may be to facilitate phospholipases both in the venoms and the victims (for a review see Nakajima et al. 1986).

13.2.2.11. CRABROLIN

Crabrolin, a 13 amino acid peptide (Phe-Leu-Pro-Leu-Ile-Leu-Arg-Lys-Ile-Val-Thr-Ala-LeuNH_2) isolated from the venom of the European hornet V e s p a c r a b r o releases histamine from rat peritoneal mast cells (Argiolas and Pisano 1984). Although crabrolin exhibits weak mastoparan-like activities, its unique structure predicts as yet undiscovered activities as its main functions. Hornetin, a lethal protein in the venom of the hornet V e s p a f l a v i t a r s u s has recently been characterized by Ho and Ko 1986.

13.2.2.12. HISTAMINE-RELEASING PEPTIDES

Two histamine-releasing peptides, HR-I and HR-II (Fig.13.9; for references see Argiolas and Pisano 1984) have been isolated from the venom of the hornet V e s p a o r i e n t a l i s where its concentration is much lower than are those of mastoparan C (MP-C) and crabrolin.

Both HRP peptides release histamine from rat peritoneal mast cells and lyse erythrocytes with potencies similar to those of MP-C and crabrolin. However, HR-II is four times as potent as is HR-I in lysing erythrocytes, whereas crabrolin, which resembles HR-II (69% homology), is only one fourth as potent as is MP-C in the same lysing test (for more details and references see Argiolas and Pisano 1984).

```
                                        10                  14
HR-I : Ile-Asn-Leu-Lys-Ala-Ile-Ala-Ala-Leu-Val-Lys-Lys-Val-LeuNH2
HR-II: Phe-Leu-Pro-Leu-Ile-Leu-Gly-Lys -  -  -  Gly-Leu - NH2
```

Fig. 13.9. Primary structures of histamine-releasing (HR) peptides

13.2.2.13. PROTHORACICOTROPIC HORMONE AND OTHER PEPTIDES INVOLVED IN INSECT DEVELOPMENT

The metamorphosis of insects involves a series of hormonally regulated cellular and tissue changes that are initiated by ecdysone, a steroid hormone, and come to a completion with the emergence of the adult from the old pupal cuticle, a process named eclosion. The signal for the latter events is provided by the "eclosion hormone" exerting pronounced behavioral and metamorphosis-related somatic effects following its release from brain neurosecretory cells.

In the silkworm B o m b y x m o r i prothoracicotropic hormone (PTTH) produced by neurosecretory cells in the pars intercerebralis of the insect is released into the hemolymph at an appropriate stage of the developmental cycle to activate the prothoracic gland, where it initiates the synthesis of ecdysone. The hormonal activity of PTTH has been recognized since 1922, but its purification and characterization have only recently been achieved (for references see Nishitsutsuji-Uwo and Nishimura 1984, Matsumoto et al. 1985, Nagasawa et al. 1986). In the head of the adult silkworm, two forms of PTTHs with distinct biological activities can be found: 4K-PTTH and 22K-PTTH with M_rs 4,400 and 22,000, respectively. 4K-PTTH exists in at least three molecular forms: 4K-PTTH-I, -II, and -III, whose N-terminal portions show remarkable sequence homologies with vertebrate insulin and related peptides such as insulin-like growth factors and relaxin(Matsumoto et al. 1985). Presently, a complete amino acid sequence is available only for 4K-PTTH-II (Nagasawa et al. 1986). Similarly to vertebrate insulin, 4K-PTTH-II consist of two nonidentical peptide chains: the A and the B chain, respectively (Fig.13.10). The A chain consists of 20 amino acid residues while there are four microheterogenous forms of the B chain with the following structures: Ala(5)B28 (meaning a 28 residue peptide with alanine at position 5 of the B chain), Ala(3)B26, Gly(5)B28 and Gly(3)B26. The 26 amino acid peptides lack the first two N-terminal residues of the 28 amino acid peptides, but keep pGlu residues as their N termini.

A chain: Gly-Ile-Val-Asp-Glu-Cys-Cys-Leu-Arg-Pro-Cys-Ser-Val-Asp-Val-Leu-Leu-Ser-Tyr-Cys
10 20

B chain: pGlu-Gln-Pro-Gln-Ala-Val-His-Thr-Tyr-Cys-Gly-Arg-His-Leu-Ala-Arg-Thr-Leu-Ala-Asp-Leu-Cys-Trp-Glu-Ala-Gly-Val-Asp
10 20 28

Fig. 13.10. Amino acid sequences of the A and B chains of 4K-PTTH-II, one of the prothoracicotropic hormones

Purification of, and a partial sequence for, the eclosion hormone of the silkworm has also been reported (Nagasawa et al. 1985). An acidic eclosion hormone of M_r~40,000 was described in the tobacco hawkmoth *Manduca sexta*. Three other peptides, cardioacceleratory peptide-1 (M 1,000), cardioacceleratory peptide-2 (M_r~500) and bursicon, the tanning hormone, (M_r~20,000) localized in the perivisceral organ of the adult insect also contribute to the completion of eclosion behavior (for references see Tublitz et al. 1986). Interestingly, the release of these agents appears to be under a steroid hormone control. (For other developmental hormones contributing to the completion of the eclosion behavior see Tublitz et al. 1986).

13.2.2.14. CECROPINS AND ATTACINS

Because insects have neither lymphocytes nor immunoglobulins, they must possess an immune system entirely different from that functioning in higher vertebrates. Studies on the diapausing pupae of *Cecropia* have revealed that the humoral immune response of these arthropods includes a lysozyme-dependent enzymatic mechanism which is, however, insufficient alone, and needs to be completed with a nonenzymatic mechanism to provide the insects with an efficient defense against bacteria. The latter operates through two newly recognized classes of bactericidal polypeptides, termed cecropins and attacins that proved to be effective against a remarkable number of Gram-positive and Gram-negative bacteria.

In a period of two years, seven distinct but structurally related cecropins (Ce) species: CeA,B,C,D,E,F and factor G

```
                                                  10                  20
Hc A: Lys-Trp-Lys-Leu-Phe-Lys-Lys-Ile-Glu-Lys-Val-Gly-Gln-Asn-Ile-Arg-Asp-Gly-Ile-Ile

Hc B:  -   -   -  Val  -   -   -   -   -   -  Met  -  Arg  -   -   -  Asn  -   -  Val

Ap B:  -   -   -  Ile  -   -   -   -   -   -   -   -  Arg  -   -   -  Asn  -   -   -

Hc C:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
                1
Hc D:      -  Asn-Pro  -   -  Glu-Leu  -   -   -   -   -  Arg-Val  -   -  Ala-Val  -
                1
Ap D:      -  Asn-Pro  -   -  Glu-Leu  -  Arg-Ala  -   -  Arg-Val  -   -  Ala  -   -
                1
Hc E:      -  Asn-Pro  -   -  Glu-Leu  -   -   -   -   -  Arg-Val  -   -  Ala-Val  -
                1
Hc F:      -  Asn-Pro  -   -  Glu-Leu  -   -   -   -   -  Arg-Val  -  Asn-Ala-Val  -


                                                  30                      37
Hc A: Lys-Ala-Gly-Pro-Ala-Val-Ala-Val-Val-Gly-Gln-Ala-Thr-Gln-Ile-Ala-Lys-R
                                                                  35
Hc B:  -   -   -   -   -  Ile  -   -  Leu  -  Glu  -  Lys-Ala-LeuNH2

Ap B:  -   -   -   -   -   -   -   -  Leu  -   -   -  unknown C-terminal

Hc C:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

Hc D: Ser  -   -   -   -   -   -  Thr  -  Ala  -   -   -  Ala-Leu  -   -  -R

Ap D: Ser  -   -   -   -   -   -  Thr  -  Ala  -   -   -  Ala-Leu  -   -  -R

Hc E: Ser  -   -   -   -   -   -  Thr  -  +++  -  +++  -  +++ +++ +++ +++

Hc F: Ser  -   -   -   -   -   -  Thr  -  +++  -  +++  -  +++ +++ +++ +++
```

Fig. 13.11. Amino acid sequences of various cecropins (A,B,C,D,E,F) derived from the moth Hyalophora cecropia (Hc) and the Chinese oak silk moth Antheraea pernyi (Ap), respectively. Dashes indicate residues identical to those in the Hc A sequence. Crossmark triplets and C-terminal "R"s represent unidentified residues and unidentified blocking groups, probably amide groups, respectively.

have been identified in pupae of the Cecropia moth Hyalophora cecropia (Hc). Of these, HC CeA,B,C and D have been fully sequenced, whilst only partial sequences are available for Hc CeE and F, and factor G remaines unsequenced at present (for sequence data see Steiner et al. 1981, Hultmark et al. 1982). CeB and D have also been identified in pupae of the Chinese oak silk moth Antheraea pernyi (Ap). Ap CeD has been fully but Ap CeB only partially sequenced (Qu et al. 1982). The known sequences of Hc and Ap cecropins are shown in Fig.13.11. Although the primary structure of Hc factor G presently is unknown, its amino acid composition was found to be different from those of the other cecropins. The sequence homologies observed between cecropins from the two insects suggest that they originate from a single gene.

All major cecropins were found to be efficient against several Gram-negative bacterial strains. Molecular cloning and sequencing of a cDNA encoding Hc CeB (Hofsten et al. 1985) and the chemical synthesis of the natural peptide have led to a correct sequence for Hc CeB (Hc B in Fig.13.11) that proved to be different from that previously reported (Steiner et al. 1981).

Besides lysozyme and cecropins, a third class of closely related antibacterial proteins consisting of six members, attacins A,B,C,D,E and F (ATs A-F) have been isolated from the hemolymph of the immunized pupae of the cecropia moth *Hyalophora cecropia*. (The term attacin refers to attacini, a saturniid species to which *Hyalophora cecropia*, the biological source of attacins, belongs). The different ATs, whose M_rs scatter between 20,000 and 23,000 were distinguished according to their pIs (Hultmark et al. 1983) and thus the six ATs could be grouped into two main categories: ATs A-D constituting a basic group (bATs) and ATs E-F an acidic group (aATs), respectively. The intragroup N-terminal sequences proved to be identical, whilst the intergroup N-terminal sequences differed slightly (Hultmark et al. 1983). Subsequently, aAT F was fully sequenced (Engstrom et al. 1984). Moreover, two cloned cDNAs corresponding to an aAT and bAT, respectively, were also isolated and sequenced (Kockum et al. 1984). The amino acid sequences of aAT and bAT are shown in Fig.13.12. It is noteworthy that sequence data obtained for AT F by direct sequencing and those predicted from cloned aAT cDNA proved to be identical. Previous results obtained with AT and immune protein P4 cDNA clones (Lee et al. 1983) were also consistent with those obtained by Kockum et al. (1984).

The results presented above imply the existence of only two different genes for attacins. Mature aAT and bAT showed a high degree of sequence homology (76% at the nucleotide level and 79% at the amino acid level). However, while coding information for the C-terminal tetrapeptide sequence Ser-Lys-Tyr-Phe could be detected in both cDNA clones, this sequence was missing from the chemically determined sequence of AT F. It is therefore possible that this C-terminal sequence is removed by

```
                                        10                                  20
bAT: Arg-Asn-Ala-Gly-Ala-Leu-Thr-Ile-Asn-Ser-Asp-Gly-Thr-Ser-Gly-Ala-Val-Val-Lys-Val-
aAT: Asp-Ala-His -  -  -  -  Leu -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
                                        30                                  40
bAT: Pro-Ile-Thr-Gly-Asn-Glu-Asn-His-Lys-Phe-Ser-Ala-Leu-Gly-Ser-Val-Asp-Leu-Thr-Asn-
aAT:  -  Phe-Ala -  -  Asp-Lys-Asn-Ile-Val -  -  Ile -  -  -  -  -  -  -  -  -  -  Asp-
                                        50                                  60
bAT: Gln-Met-Lys-Leu-Gly-Ala-Ala-Thr-Ala-Gly-Leu-Ala-Tyr-Asp-Asn-Val-Asn-Gly-His-Gly-
aAT: Arg-Asn -  -  -  -  -  -  -  -  -  -  Val -  Leu -  -  Ile -  -  -  -  -  -  -  -
                                        70                                  80
bAT: Ala-Thr-Leu-Thr-Lys-Thr-His-Ile-Pro-Gly-Phe-Gly-Asp-Lys-Met-Thr-Ala-Ala-Gly-Lys-
aAT: Leu-Ser -  -  Asp -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
                                        90                                  100
bAT: Val-Asn-Leu-Phe-His-Asn-Asp-Asn-His-Asp-Phe-Ser-Ala-Lys-Ala-Phe-Ala-Thr-Lys-Asn-
aAT:  -  -  Val -  -  -  -  -  -  -  -  Ile-Thr -  -  -  -  -  -  -  Arg -
                                        110                                 120
bAT: Met-Pro-Asn-Ile-Pro-Gln-Val-Pro-Asn-Phe-Asn-Thr-Val-Gly-Ala-Gly-Val-Asp-Tyr-Met-
aAT:  -  -  Asp -  Ala-Asn -  -  -  -  -  -  -  -  -  Gly -  Ile -  -  -
                                        130                                 140
bAT: Phe-Lys-Asp-Lys-Ile-Gly-Ala-Ser-Ala-Asn-Ala-Ala-His-Thr-Asp-Phe-Ile-Asn-Arg-Asn-
aAT:  -  -  -  -  -  -  -  -  -  -  Ser -  -  -  -  -  -  -  -  -  -  -  -  -
                                        150                                 160
bAT: Asp-Tyr-Ser-Leu-Gly-Gly-Lys-Leu-Asn-Leu-Phe-Lys-Thr-Pro-Thr-Thr-Ser-Leu-Asp-Phe-
aAT:  -  -  -  -  Asp -  -  -  -  -  -  -  -  -  -  Asp -  -  Ile -  -
                                        170                                 180
bAT: Asn-Ala-Gly-Trp-Lys-Lys-Phe-Asp-Thr-Pro-Phe-Phe-Lys-Ser-Ser-Trp-Glu-Pro-Ser-Thr-
aAT:  -  -  -  Phe -  -  -  -  -  -  -  -  Met -  -  -  -  -  -  -  Asn-Phe-
                                    188
bAT: Ser-Phe-Ser-Phe-Ser-Lys-Tyr-Phe
aAT: Gly -  -  Leu -  -  -  -
```

Fig. 13.12. Amino acid sequences of basic (b) and acidic (a) attacins (AT). Dashes indicate identities

proteolysis from some mature proteins. This, and the suggested N-terminal blocking of some bATs by pyroglutamate groups (Engstrom et al. 1984) would result in a removal of a distinct number of positive charges from the molecules, that could explain all the six forms of ATs mentioned above.

In contrast to cecropins that proved to be efficiently antibacterial against a variety of Gram-positive and Gram-negative bacteria, ATs proved to be effective only against a few Gram-negative bacteria, a phenomenon that may be due to established differences in the mechanisms of their actions (Steiner et al. 1981). An antibacterial and antineoplastic glycoprotein, termed aplysianin A, has been recently identified in the albumen gland of A p l y s i a k u r o d a i, a molluscan species (Kamiya et al. 1986).

13.2.2.15. CARDIOREGULATORY PEPTIDES

Throughout the sixties, a whole array of cardioactivities unassociated with usual neurotransmitters were detected in extracts of molluscan ganglia, a number of which turned out to be peptides (Frontali et al. 1967). Since then, several cardioregulatory peptides with M_rs ranging between 700 and 1,500 have been described in various molluscs (Greenberg and Price 1980). Of these, the tetrapeptide FMRFamide (the name refers to the one letter symbols of the constituting amino acids Phe-Met-Arg-PheNH_2) was first isolated and sequenced from ganglia of the mollusc *Macrocallista nimbosa* (Price and Greenberg 1977). Subsequently, related peptides have been isolated and sequenced from several other molluscan species (for more details and references see Morris et al. 1982, Taussig and Scheller 1986). These substances have a wide spectrum of biological activities (Cotrell et al. 1983, Belardetti et al. 1987) including excitatory effects on the heart in many molluscan species, a blood pressure-elevating effect observed in rats following intravenous administration. The heptapeptide pQDPFLRFamide (where pQ refers to pyroglutamine) isolated from the ganglia of *Helix aspersa* proved to be about a hundred times more potent than FMRFamide on isolated Helix hearts (Price et al. 1985). The recent finding that Aplysia neurons express a gene encoding multiple FMRFamide-like neuropeptides that display, among others, cardiomodulatory activities deserves a special attention (Schaefer et al. 1985).

Antisera specific for the C-terminal end of FMRFamide react with several substances in the CNS of many vertebrate species (Chronwall et al. 1984, O'Donohue et al. 1984). One of these immunoreactive materials has recently been isolated from chicken brain and identified as LPLRFamide. This peptide was demonstrated to increase the blood pressure in anesthetized rats (Barnard and Dockray 1984). FMRFamide-like peptides were shown to be present also in the nervous system of the horseshoe crab, *Limulus polyphemus* (Watson et al. 1984), birds (Dockray and Dimaline 1985), and in Aplysia

(Brown et al. 1985). It is worth mentioning that the Phe-Met-Arg-Phe sequence occurs at the C-terminal end of Met-enkephalin-Arg^6-Phe^7, a mammalian opioid heptapeptide, (Akil et al. 1984).

The well-established atrial cardiac hormones are now recognized to play an important role in the regulation of sodium and water balance and in vasoregulation in many vertebrate species, including man. The hormones characterized so far have been variously termed cardiodilatin, cardionatrin, atrial natriuretic factor, atrial natriuretic peptide, and atriopeptin by different authors (see Chapter 9). The first report on the occurrence of a similar peptide(s) in invertebrates has been published by Nehls and associates (1985), who have presented evidence on the existence of a cardiodilatin-like substance in the neurocardiac axis of the adult summer snail, H e l i x p o m a t i a (for additional cardioregulatory peptides see section 13.2.2.16.5).

13.2.2.16. BEHAVIORAL PEPTIDES

Aplysia, a marine molluscan species, proved to be a real factory of neuropeptides performing a wide range of functions including the organization of complex fixed behavioral patterns.

Recently collected information give rise to the notion that there exist a so-called egg laying hormone (ELH) gene family consisting of five members per haploid genome in Aplysia. The identified five members of the ELH gene family encodes the following products: (1) α, β, γ and δ bag cell peptides (BCPs) are expressed in the bag cells a special group of neurons in the abdominal ganglion; (2) atrial gland peptides A and B (AGP_A and AGP_B are expressed in the atrial gland which is a part of the reproductive system in Aplysia; (3) ELH is partially or fully expressed, both in the bag cells and the atrial gland; (4) acidic protein (AP) is expressed in the bag cells; (5) the small cardioactive peptides A and B (SCP_A and SCP_B) are expressed in neurons of the buccal ganglion. A

number of these peptides have been sequenced by the classical methods, whereas amino acid sequences of others have been predicted from appropriate cDNA clones, or by both.

(1). Bag cell peptides

This group of peptides includes α, β, γ and δ BCPs. Their genomic representation has a flanking order β-γ-δ whilst α BCP follows them at a considerable distance in the genome. α BCP is a nonapeptide: Ala-Pro-Arg-Leu-Arg-Phe-Tyr-Ser-Leu. On the other hand β and γ BCPs are pentamers that differ only in their C termini (His in the β, and Asp in the γ peptide), while the remaining four amino acids common to both BCPs (Arg-Leu-Arg-Phe) are also found in the C-terminal region of atrial gland peptides and also in β BCP. Finally, δ BCP is a heptapeptide: Asp-Gln-Asp-Glu-Gly-Asn-Phe. BCPs, and especially α BCP, were shown to have neurotropic effect inhibiting the firing in selected ganglionic neurons. In contrast to AGPs which may act at a considerable distance from the site of their release. α BCP will only act over a short distance due to its instability and susceptibility to protease attacks associated with its unamidated C terminus.

(2) Atrial gland peptides

Atrial gland extracts induce egg laying in Aplysia. This activity is partly mediated by two peptides, termed peptide A and peptide B (AGP_A and AGP_B), respectively (Fig.13.13). Both peptides are related to, but are different from the egg-laying hormone (see below; Heller et al. 1979, Strumwasser et al. 1980). It is suggested that copulation-related mechanical or hormonal stimulation of the atrial gland results in a release of peptides that trigger bag cell bursts and results in egg laying. AGPs may act as pheromones mediating communication between individuals of the species.

```
                    10                                  20
A: Ala-Val-Lys-Leu-Ser-Ser-Asp-Gly-Asn-Tyr-Pro-Phe-Asp-Leu-Ser-Lys-Glu-Asp-Gly-Ala-
B: -   -   -   Ser -   -   Tyr-Glu-Lys -   -   -   -   -   -   -   -   -   -   -
                    30                          36
A: Gln-Pro-Tyr-Phe-Met-Thr-Pro-Arg-Leu-Arg-Leu-Arg-Phe-Tyr-Pro-IleNH2
B: -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
```

Fig. 13.13. Amino acid sequences of atrial gland peptides A and B. Dashes indicate residues identical to those in the A sequence

(3) Egg-laying and egg-releasing hormones

Egg-laying hormone (ELH) is expressed in the bag cells, but a somewhat different ELH and related peptides are also expressed in the atrial gland of Aplysia (for details see Nagle et al. 1986). ELH expressed in the bag cells is a 36 amino acid peptide with the following primary structure: Ile-Ser-Ile-Asn-Gln-Asp-Leu-Lys-Ala-Ile-Thr-Asp-Met-Leu-Leu-Thr-Glu-Gln-Ile-Arg-Glu-Arg-Gln-Arg-Tyr-Leu-Ala-Asp-Leu-Arg-Gln-Arg-Leu-Leu-Glu-LysNH_2.

Egg-releasing hormone (ERH) is an atrial gland peptide in Aplysia californica that displays ELH-like activities (Chiu et al. 1979, Schlesinger et al. 1981). The 34 amino acid peptide has the following primary structure: Ile-Ser/Val-Ile-Val-Ser-Leu-Phe-Lys-Ala-Ile-Thr-Asp-Met-Leu-Leu-Thr-Glu-Gln-Ile-Tyr-Ala-Asn-Tyr-Phe-Ser-Thr-Pro-Arg-Leu-Arg-Phe-Tyr-Pro-Ile (the double residue at position 2 indicates an uncertainty). Acting on the CNS and the ovotestis, ELH elicits profound behavioral effects associated with egg-laying and it also has a direct effect on the cardiac output (for details and references see Strumwasser et al. 1980, Scheller et al. 1984, Mark and Scheller 1986).

(4) Acidic protein

Acidic protein (AP), a 27 amino acid peptide [Ser(P)-Ser-Gly-Val-Ser-Leu-Leu-Thr-Ser-Asn-Lys-Asp-Glu-Glu-Gln-Arg-Glu-Leu-Leu-Lys-Ala-Ile-Ser-Asn-Leu-Leu-Asp] where Ser(P) is a phosphorylated seryl residue] is also a product of the ELH gene family. AP is expressed in the bag cells and its gene flanks the ELH gene within the genome. It is assumed that AP may be concerned with organizing behavioral patterns associated with egg-laying, but no specific physiological role has been attributed to this peptide so far (Scheller et al. 1983).

(5) Small cardioactive peptides

The known small cardioactive (SCPs) expressed in the neurons of the buccal ganglion in Aplysia are SCP_A and SCP_B. Their cDNA-derived amino acid sequences are shown in Fig. 13.14 (Mahon et al. 1985). Within the predicted sequence

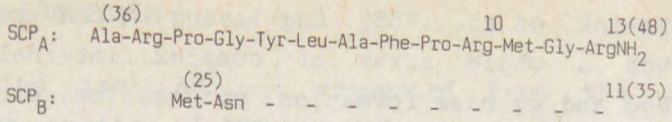


Fig. 13.14. cDNA-derived amino acid sequences of small cardioactive peptides (SCP). Dashes indicate identical residues. Numbers in parentheses refer to the positions of the N- and C-terminal residues of SCP_A and SCP_B within the precursor molecule.

of the 136 amino acid precursor, SCP_B is represented by residues at position 25 through 35 whereas, SCP_A by those at positions 36 through 48, respectively (see Fig.13.14). SCP_A and SCP_B that lack the two C-terminal residues of the peptides shown in Fig.13.14 but keep their C-terminal methionyl residues amidated have also been isolated from Aplysia (Morris et al. 1982, Lloyd et al. 1987).

SCPs, especially SCP_B stimulate contractile activity in the gut, increase the amplitude of the heart beats and enhance the contractile response of the feeding-related radula closer muscle, also called radula protractor muscle in Aplysia. The widespread distribution of these peptides in various tissues of Aplysia suggests, however, that they may have other functions as well (for more details and references on neuropeptide-dependent regulation of Aplysia behavior see the reviews by Scheller et al. 1984, Lloyd 1986, Lloyd et al. 1986, Tublitz et al. 1986).

13.2.2.17. OVULATION HORMONE/CAUDODORSAL CELL HORMONE

Ovulation hormone or caudodorsal cell hormone (CDCH), a 36 amino acid peptide (Leu-Ser-Ile-Thr-Asn-Asp-Leu-Arg-Ala-Ile-Ala-Asp-Ser-Tyr-Leu-Tyr-Asp-Gln-His-Trp-Leu-Arg-Glu-Arg-Gln-Glu-Glu-Asn-Leu-Arg-Arg-Arg-Phe-Leu-Glu-$LeuNH_2$) has been isolated from the neurosecretory caudodorsal cells of the fresh water pulmonate snail L y m n a e a s t a g n a l i s. CDCH shows a somewhat less than 50% homology with ELH and related

peptides (Ebberink et al. 1985). Egg-laying in the mentioned snail involves a whole array of complex internal events (ovulation, egg and gg-mass formation, oviposition, etc.) that all are initiated and coordinated by the neuroendocrine caudodorsal cells. CDCH also stimulates the synthesis of secretory products in the female accessory sex glands and affects identified neurons in the neuronal feeding network (for more details see Ebbernik et al. 1985).

13.2.2.18. EGG-LYSIN

Although the involvement of "acrosins", proteins secreted from the sperm acrosome, in the penetration of spermatozoa into the eggs in various species have been suggested, the molecular mechanism of sperm penetration remains largely unknow.

Egg-lysin isolated and sequenced from spermatozoa of the abalone H e l i o t i s r u f e s c e n s, a molluscan species, (Fridberger et al. 1985) is a 134 amino acid protein (Fig.13.15) containing 36 charged and many hydrophobic residues in a peculiar structural arrangement that is consistent with the biological activities characteristic of this protein. Egg-lysin was shown to interact with egg membrane components resulting in a dissolution of egg vitelline layers and a fusion of the egg and sperm membranes, thereby facilitating sperm penetration into the eggs (for more details see Fridberger et al. 1985).

```
                                                  10                  20
Arg-Ser-Trp-His-Tyr-Val-Glu-Pro-Lys-Phe-Leu-Asn-Lys-Ala-Phe-Glu-Val-Ala-Leu-Lys-
                                                  30                  40
Lys-Gln-Phe-Ile-Ala-Gly-Phe-Asp-Arg-Gly-Leu-Val-Lys-Trp-Leu-Arg-Val-His-Gly-Arg-
                                                  50                  60
Trp-Leu-Ser-Trp-Val-Gln-Lys-Lys-Ala-Leu-Tyr-Phe-Val-Asn-Arg-Arg-Tyr-Met-Gln-Thr-
                                                  70                  80
His-Trp-Ala-Asn-Tyr-Met-Ile-Trp-Ile-Asn-Lys-Lys-Leu-Asp-Ala-Leu-Gly-Arg-Thr-Pro-
                                                  90                  100
Val-Val-Gly-Asp-Tyr-Thr-Arg-Leu-Gly-Ala-Asp-Ile-Gly-Arg-Arg-Ile-Asp-Met-Ala-Tyr-
                                                  110                 120
Phe-Tyr-Asp-Phe-Leu-Lys-Asp-Lys-Asn-Met-Ile-Pro-Lys-Tyr-Leu-Pro-Tyr-Met-Gln-Glu-
                                                  130         134
Ile-Asn-Arg-Met-Arg-Pro-Ala-Asp-Val-Pro-Val-Lys-Tyr-Met
```

Fig. 13.15. Amino acid sequence of molluscan egg-lysin

13.2.2.19. CONOTOXIN

Conotoxins isolated and sequenced from the venoms of fish-eating snails of the genus C o n u s (Gray et al. 1981, McIntosh et al. 1982, Olivera et al. 1985) are toxic peptides utilized for studying fundamental aspects of impulse transduction at the neuromuscular junctions. The amino acid sequences of the representatives of the functionally grouped conotoxins are demonstrated in Fig.13.16. α-toxins, the first conotoxins for which chemical structures were determined act postsynaptically on acetylcholine receptors at the neuromuscular junctions, while μ-toxins specifically block postsynaptic sodium channels in the muscles. Because the postsynaptically acting α- and μ-toxins cause death by directly paralyzing the neuromuscular system, they are also named paralytic toxins. The "shaker" peptides, whose names refer to their capability of inducing tremor in mice following their central administration, belong to the group of ω-conotoxins. The latters can be divided into two subgroups: one which presynaptically blocks the voltage-sensitive Ca^{2+}-channels and thus acetylcholine release at amphibian synapses ("blockers"), and another which does not ("nonblockers").

A recently discovered peptide toxin from C o n u s m a g u s is similar in size to ω-conotoxins. Since its effect is directed both on the pre- and the postsynaptic potassium channels, it was named K-conotoxin. No sequence data is available for this peptide at present. Amongst the nonparalytic toxins present in snail venoms the sleeper peptides deserve mentioning. One of them has been sequenced (see Fig.13.16) and shown to produce a prolonged sleep-like state in mice. This peptide is extremely acidic due to the presence of five γ-carboxyglutamate residues. The "convulsant toxin", another acidic nonparalytic peptide is an about 100 amino acid peptide presently with an unknown structure that induces death-causing convulsions in mice, without any apparent effects on fish. The nonapeptide conopressin, a vasopressin homolog of basic character is also a nonparalytic snail venom peptide (for references see Olivera et al. 1985). When mice

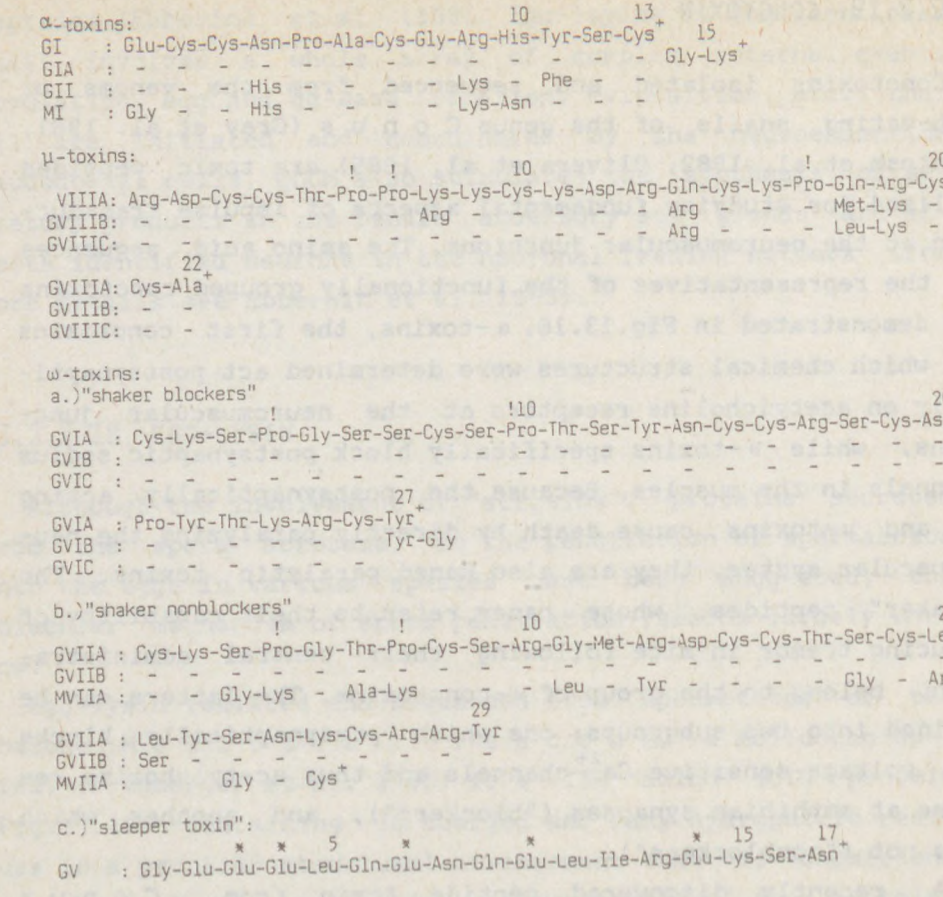


Fig. 13.16. Amino acid sequences of the specific groups of conotoxins. Glu residues marked with asterisks indicate γ-carboxyglutamate residues. Dashes indicate residues identical to those in the first row of each group. Pro-s marked with exclamation marks indicate trans-4-hydroxyprolines. Crossmarked C termini indicate their amidation, while lack of the crossmarks at the C termini indicate that no assignment has yet been made. Deletions (empty spaces) were introduced for maximizing homology (Olivera et al. 1985)

are injected intracerebrally with conopressin, they scratch themselves, why it is also called the "scratcher peptide". Vasopressin has a similar effect in mice.

All conotoxins are highly cross-linked with disulfide bonds. The fish-eating cones use them to paralyze their prey for nutritional purposes. Conotoxins, however, may be dangerous also for humans. Because the mammalian nervous system

is clearly affected by conotoxins, they are of potential significance in studying molecular mechanism of the impulse transmission at the neuromuscular junction in mammalian species (for more information on conotoxins and related peptides see Gray et al. 1984, Hider 1985, Kosuge et al. 1985, Olivera et al. 1985).

13.2.2.20. INSULIN- AND GLUCAGON-LIKE PEPTIDES

Glucose and glycogen probably are present in all forms of living organisms, as are many of the protein and lipid pathways known to be influenced by insulin and glucagon in invertebrate species. By all indications, insulin- and glucagon-like peptides function also in molluscs and insects, though the exact molecular structure of these peptides remain to be elucidated. Readers interested in insulin- and glucagon-like peptides in insects and molluscs are referred to the review by Thorpe and Duve (1984).

13.3. NEUROPEPTIDES IN HYDRA, UNICELLULAR ORGANISMS AND IN HIGHER PLANTS

A neuropeptide (M_r 1,142) isolated and sequenced from the fresh water coelenterate *Hydra attenuata* was named head activator because it controls head-specific growth and differentiation processes. It is a undecapeptide having the following amino acid sequence: pGlu-Pro-Pro-Gly-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe (Bodenmuller 1984). In hydra, the first organism in the animal kingdom to possess a nervous system, the head activator is produced by nerve cells and stored there in neurosecretory granules. The role of the neuropeptide head activator in nerve cell function and development was discussed in a recent paper by Schaller and Bodenmuller (1985).

Peptides structurally identical with the head activator were also isolated from human and bovine hypothalami and from rat intestine (Bodenmuller and Schaller 1984). Since the func-

tion of head activator is unknown in mammals, one can only speculate that, in analogy to hydra, the peptide might have some growth regulatory functions in the developing embryos. Whether it has additional functions in adults as a transmitter or a modulator remains to be elucidated (for a review see Bodenmuller and Roberge 1985).

Accumulated data indicate that substances similar to known hormonal peptides and neurotransmitter molecules of the vertebrates are present in unicellular organisms and also in the plants (for a reviews see LeRoith and Roth 1984, Krieger 1985, Lukas et al. 1985). Studies published to date suggest that these peptides originated in the unicellular organisms and have been maintained in higher organisms during the course of evolution. In unicellulars, these molecules serve as primitive elements of intercellular communication. The alternative, though far less likely possibility, namely that the genes for these peptides arose in vertebrates and were introduced by plasmids into the unicellular organisms, cannot be excluded completely until the genes are demonstrated and analyzed.

Several peptides closely resembling the classic vertebrate hormones and neuropeptides such as insulin (LeRoith et al. 1981, LeRoith and Roth 1984), epidermal growth factor (Allen et al. 1985, Oka et al. 1985), ACTH, β-endorphin (LeRoith et al. 1982), proopiomelanocortin, somatostatin, relaxin (LeRoith et al. 1985), vasotocin, calcitonin, cholecystokinin, (personal communications, referred to by LeRoith and Roth 1984), TSH (Macchia et al. 1967, Weiss et al. 1983), to mention only a few, were found in unicellular organisms, though their exact chemical identity has not been established as yet.

Some sex pheromones present in eukaryotic microbes are also peptides (Miyake 1968, Kitamura and Hiwatashi 1978, Kochert 1978). Of these, the yeast peptides, termed mating factors, deserve a special attention. They inform yeast cells of one mating type of the presence of the opposite mating type within the vicinity, and induce mating-specific processes in the target cell. The mating factors from the yeast *Saccharomyces cerevisiae* are known as α-factor, a product of the cells of α-mating type, and "a"-factor a product of the cells of "a" mating type (Ciejek

et al. 1977, Masui et al. 1977, for a review see Sprague 1983). The α-factor is a 13 amino acid peptide (Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Glu-Pro-Met-Tyr) while the "a" factor consists of 11 amino acid residues (Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Ala-Asx-Pro).

The α-type mating factor also exists in a Trp(1)-depleted form. On the other hand, "a"-factor probably is a mixture of two peptides: one, termed a_1-factor, is identical with the aforementioned "a"- factor and another, called a_2-factor in which the Val(6) residue is substituted for a Leu(6) residue (for details see Sprague 1983). Each mating factor acts to arrest the cells of the opposite mating type at a point known as start in the cell cycle. Although "a"-factor and α-factor have no sequence similarities, recent results demonstrate close parallels between responses of "a" cells to α-factor and α cells to "a"-factor (Rine 1986). An N-terminal portion of α-factor is structurally similar to LH-RH, a mammalian sex hormone (Hunt and Dayhoff 1979) and the yeast factor has a specific though rather weak LH-releasing effect in cultured rat pituitary cells (Loumaye et al. 1982).

Several bacteria contain substances that are similar, both immunologically and biologically, to human chorionic gonadotropin (hCG) (Maruo et al. 1979) and they also have specific binding sites for this human hormone (Richert and Ryan 1977). The "sex-pheromones" produced by some bacteria are also reproduction-related peptides (Dunny et al. 1979). Gramicidin, a peptide produced in certain bacilli stimulates conversion from the vegetative form to spore (Mukherjee and Paulus 1977, Paulus et al. 1979).

*

Data accumulated recently clearly indicate that peptides with distinct primary structures and biological activities are probably more widely present in nature than were formerly thought and might represent universal information carrying molecules in a wide spectrum of living organisms.

It is only natural that a majority of research is channelled into studies on mammalian species in that they most closely resemble the human condition. On the other hand, non-mammalian species, and especially the lower vertebrates and invertebrates have much to offer in terms of potential availability, large number, relative ease of experimentation and, at least superficially, a simpler basic design of the exploration of fundamental physiological and biochemical problems. From this point of view, the aforementioned species deserve greater attention in respect to biologically active peptides than they have hitherto received.

The study of nonmammalian peptides appears to be intriguing, not only because they elicit a strikingly wide spectrum of biological activities also in mammals, but because of the often astonishing structural similarities between peptides of mammalian and nonmammalian origin. Almost all groups of non-mammalian peptides hitherto recognized have one or more counterparts in mammalian species, including human. This suggests that studying active peptides in lower organisms may occasionally provide valuable clues leading to the discovery of new peptides functioning in mammalian organisms.

Further research on nonmammalian peptides will certainly produce striking discoveries in the future, and also new peptide molecules with interesting biological activities, and will contribute to our more complete understanding of the general biological significance of peptides throughout the whole spectrum of the phylogenetic scale (for reviews see Arlot-Bonnemains et al. 1986, Habermehl and Krebs 1986, Haunderland and Bowers 1986, Tyrer 1986).

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MAGYAR TUDOMÁNYOS AKADÉMIA KÖNYVTÁRA

Relying on the latest data the present work offers an overall survey of biologically active endogenous peptides. Emphasis is laid on mammalian systems but some nonmammalian regulatory processes are also treated.

The selection of endogenous regulatory peptides discussed in the book is based on the biological regulatory function of the peptides rather than their size. Some polypeptides dealt with here consist of more than hundred amino acids and would qualify as proteins. It was only such a selection principle that rendered it possible to bring all relevant regulatory endogenous peptide molecules under the same heading. Enzyme proteins are not included although their regulatory function is paramount, for the obvious reason that enzymes constitute a distinct class.

As the book is of interdisciplinary character it can be a helpful guide to workers in all biological sciences, as well as to organic chemists engaged in related fields.

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Chemistry, biology and medical significance

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