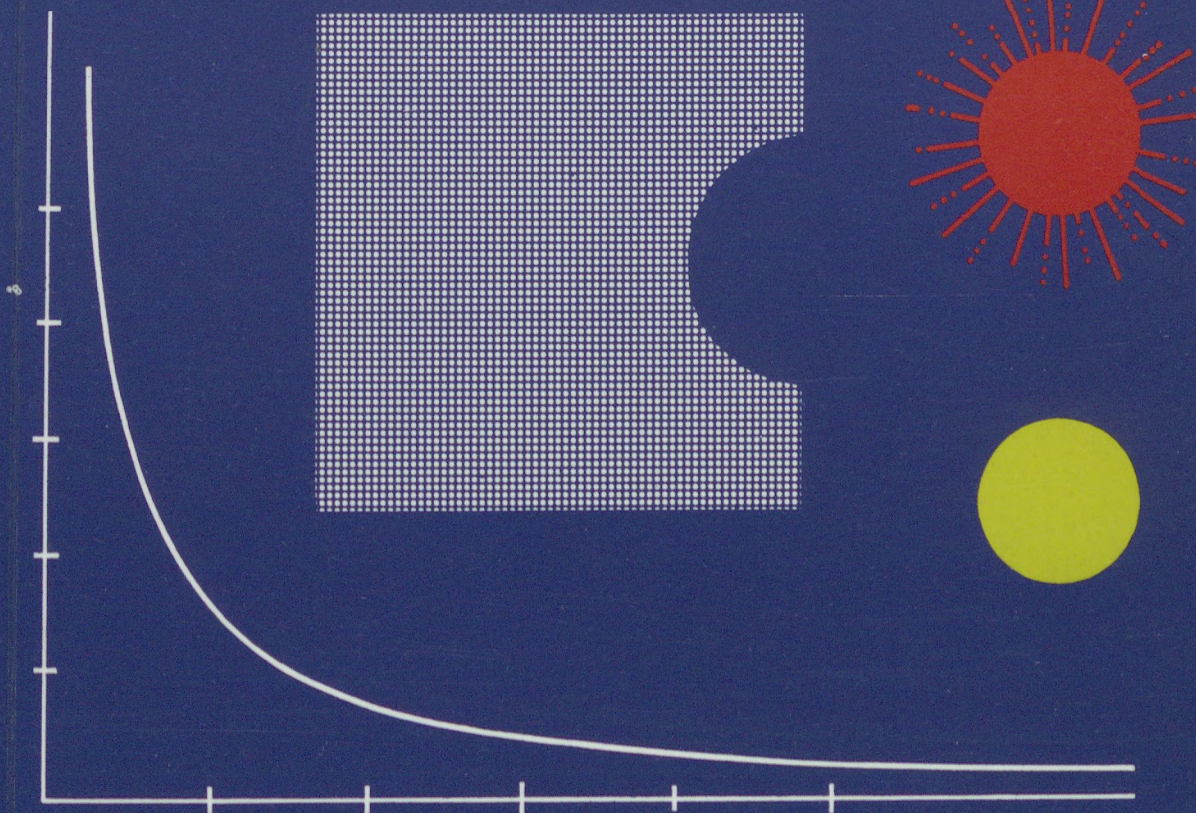


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CLINICAL APPLICATION OF RADIOIMMUNOASSAY



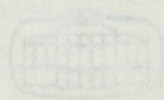
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and

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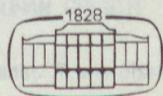
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By
FERENC A. LÁSZLÓ
and
TAMÁS JANÁKY



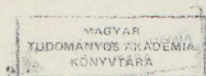
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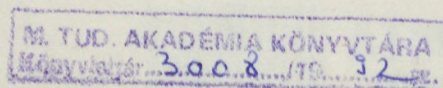
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INTRODUCTION

The emergence of radioimmunoassay (RIA) and similar methods based on protein binding opened up new dimensions in studies on the functioning of the living organism. In comparison with the previous biological and physico-chemical examination procedures, these methods permit the detection of amounts of material lower by several orders of magnitude.

The basic principles of radioimmunoassay were described by Yalow and Berson in 1960 (in 1977, Rosalyn Yalow was awarded the Nobel Prize for achievements in medical physiology). They applied their earlier results on the metabolism of ^{131}I -insulin and on antibody formation to devise a procedure for the determination of human insulin; the method was based on the competition of labelled and non-labelled insulin for the binding sites of the antibody.

Similarly in 1960, Ekins reported the method of competitive protein binding assay (CPBA), which he developed for the determination of serum thyroxine; in this, natural thyroxine binding globulin (TBG) present in the living organism was used as binding protein. Although this was not an immunological examination, the fundamental principle of the method was the same as that of RIA.

The introduction of RIA and CPBA led to revolutionary changes in endocrinology, and subsequently in haematology, gastroenterology, pharmacology and cancer research. Simple kits containing all the reagents necessary for the determination became available commercially, and this greatly accelerated application of the methods in routine clinical laboratories and in biochemical and medical research. Several hundred different kits are now on the market for the determination of various steroid, peptide and protein hormones, drugs, proteins, vitamins, viruses, tumour markers, enzymes, etc.

RIA has the advantage over the previous methods that the substance to be measured can be determined accurately and reproducibly, with high sensitivity (10^{-6} - 10^{-12} g/ml) and specificity (only one substance is measured), on a small quantity of sample (5-100 μl).

The nomenclature of the methods based on protein binding is not uniform in the literature. Orientation is hampered by the use of different definitions, frequently meaningless technical terms, and synonymous con-

Table 1. Analytical methods based on protein binding

Binding agent	Labelling substances	Name of the method
Natural binding protein	Radioactive isotope	Competitive protein-binding assay
	Radioactive isotope	Radioreceptor assay
Tissue receptor	Enzyme	Enzymereceptor assay
		Labelled antigen immunoassay
	Radioactive isotope	Labelled antibody immunoassay
	Enzyme	Enzymeimmunoassay
	Fluorescent compound	Fluoroimmunoassay
Antibody	Chemiluminescent compound	Chemiluminescent or bioluminescent immunoassay
	Bacteriophage	Viroimmunoassay
	Metal	Metalloimmunoassay

cepts. The basis of the method is the binding reaction between a ligand and a binder. The various binding proteins (polyclonal antibody, monoclonal antibody, specific serum proteins, cellular receptors) and the use of different reaction conditions have led to the following expressions: radioimmunoassay, radioligand assay, radioimmunoanalysis, equilibrium assay, saturation analysis, displacement analysis, competitive protein-binding analysis, radioreceptor assay, non-equilibrium assay, etc. The terms often overlap one another, and their interpretation depends on the aspect from which the process is being studied.

Of the various analytical methods based on protein binding (Table 1), in this book we discuss only radioimmunoanalysis, as the most frequently used procedure in the endocrine laboratory.

Some of the biologically active substances to be found in the blood are known to exhibit a definite diurnal fluctuation. The time of day when a blood sample is taken is not irrelevant. The results may further be influenced by dietary factors and by pharmacons.

Certain substances (e.g. the middle-sized polypeptides) circulate "freely" (i.e. in a state not bound to protein) in the blood, and disappear rapidly from the plasma. In such cases, the examination of a single sample may be misleading, for it does not represent a longer time period. Longer observation would necessitate an indwelling cannula, but use of this is inconvenient. The cannula may be avoided by the performance of provocative tests in clinical practice. If the suspicion of hypofunction arises, stimulation tests may be applied to measure the functional reserve capacity of the tissue producing the substance in question. Suppression tests are carried out if a hyperfunction is suspected. Knowledge of the basal level and the results of stimulation or suppression tests provide good information for an assessment of the functioning of the organs.

Reliable results can be obtained only if there is good co-operation between the laboratory and the ward. In our experience, it is of great help in this respect if the physicians are fully familiar with the laboratory methods. The results may be made completely uncertain if, for example, the blood is not sampled in the prescribed way. The determination is generally not performed on whole blood. The plasma or the serum should be separated from the blood elements as quickly as possible. To inhibit the rapid decomposition of the substance to be measured, enzyme inhibitors (e.g. Trasylol) are added or the material is frozen. Care must be taken that the heparin added to inhibit coagulation should not be in great excess, for it may disturb the determination.

In this book, an account will be given of the more important clinical applications of RIA. No effort can be made to achieve completeness: the number of methods described is well in excess of two hundred. The main aspect that we have borne in mind during the selection was whether the procedure has been introduced widely in clinical practice. Only the more important data are presented in the descriptions of the various methods; a detailed account of the course of the assay is not given, for two reasons: this would increase the length of the book considerably, and there are often a number of accepted methods for the determination of certain substances, and we could not undertake to decide in all cases which procedure is the best. The individual determinations will be discussed in accordance with the following scheme:

Chemical structure. Besides a description of the structure of the substance, the immune properties and stability factors will be presented.

Physiological conditions. In addition to the normal physiological function, the more essential regulatory mechanisms will be outlined.

Method. Brief accounts will be given of the antigen preparation, the more important cross-reactions, the extraction procedures, the preparation of the radioactive substance, the sensitivity of the method, and the separation methods.

Clinical applications. Attention will be paid to the more frequent hyper- and hypofunctional diseases, special tests, the difficulties of sample taking, the normal and the pathological values, and question of differential diagnostics.

The individual chapters have been written on the basis of the available literature, with utilization of our own experience. Our RIA laboratory operates within the Endocrine Unit and Research Laboratory at Szent-Györgyi Albert University Medical School in Szeged, and performs hormone determinations according to the given profile, the majority of them with the use of kits. We ourselves have developed some RIA procedures (progesterone, oestradiol, testosterone, aldosterone, vasopressin, oxytocin); in collaboration with the Department of Medical Chemistry in Szeged and with the Agricultural Combine in Mezöhegyes, we have produced a large number of specific antisera and have also determined the necessary parameters. A small proportion of the observations we have made in this way are similarly to be found in the compilation.

The book has primarily been written for specialists who come into contact with RIA results in the course of their work. It may be used effectively by those working in RIA laboratories who want to know more about the diagnostic value of their work. Clinicians and practising physicians daily receive data obtained by means of RIA. Without a knowledge of the principles of the RIA procedure, it will scarcely be possible for them to evaluate these results correctly. We deal with data relating to healthy subjects, together with the more important pathological states and diseases in which deviations from the normal values are to be expected. In this way the book will also assist the physician to reach the correct diagnosis.

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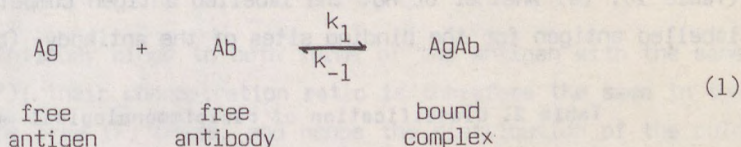
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Chapter 1

FUNDAMENTAL PRINCIPLES OF IMMUNOANALYSIS

ANTIGEN-ANTIBODY BINDING REACTION

Radioimmunoanalysis is based on a reversible immunochemical reaction between an antigen (ligand) and an antibody (binder), in which a water-soluble antigen-antibody complex is formed. The complex is held together by weak, short-range forces (hydrogen-bond, ionic bond, hydrophobic interaction, van der Waals bond); its stability therefore depends to an extremely large extent on the conformations and the three-dimensional structures of the two components during the reaction. The reaction continues until equilibrium is attained, when a proportion of the antigen and of the antibody are present in bound form (AgAb), the remainder existing in free form (Ag and Ab):



The rate constant of the complex formation reaction (association) is k_1 , while that of the opposite reaction (dissociation) is k_{-1} . The absolute rate depends on the concentrations of individual components. Initially, the rate of formation of AgAb is high and little dissociation occurs. As the reaction progresses, the quantities of free Ag and Ab, and hence the rate of association, decrease. At the same time, the concentration of the complex increases, this being accompanied by an increase in the dissociation. Equilibrium is attained when the same amount of AgAb is formed in unit time as the amount that decomposes, i.e. there is no longer any change in the concentrations of the components. This equilibrium state can be described by means of the law of mass action:

$$K = \frac{[\text{AgAb}]}{[\text{Ag}][\text{Ab}]} \quad (2)$$

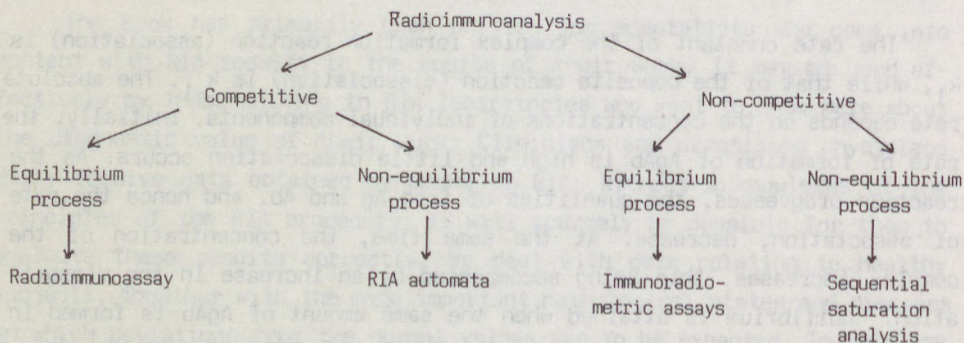
where $[Ag]$, $[Ab]$ and $[AgAb]$ are the concentrations of free antigen, free antibody and bound complex, respectively, and K is the equilibrium constant of the reaction (the affinity constant).

Since the final concentrations are governed by the rate constants, the equilibrium constant is the quotient of the rate constants of the two opposite reactions: $K = k_1/k_{-1}$. With a large value of K , the reaction is shifted in the direction of association: the antibody is almost completely saturated, and the amount of free antibody is slight. It may be stated on the basis of the law of mass action that, after equilibrium has been attained in the presence of a constant amount of antibody with given affinity constant, **the distribution of the antigen between the bound and free fractions is controlled by the total amount of antigen present.**

CLASSIFICATION OF RADIOIMMUNOANALYTICAL PROCEDURES

The radioimmunoanalytical methods are classified from two aspects (Table 2): (a) whether or not the labelled antigen competes with the non-labelled antigen for the binding sites of the antibody; (b) whether or not

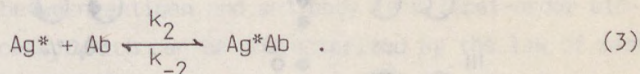
Table 2. Classification of radioimmunological methods



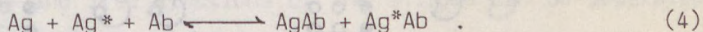
the reaction between the antigen and the antibody attains equilibrium.

LABELLED ANTIGEN ASSAYS

Radioimmunoassay (equilibrium, competitive method). For the determination of the distribution of the antigen between the bound and free fractions in RIA, an antigen labelled with a radioactive isotope is used. The labelled antigen (Ag^*), binds to the antibody, similarly to the non-labelled antigen and the complex Ag^*Ab being formed:



In the RIA system, the simultaneously present labelled and non-labelled antigen compete for the limited binding sites of the antibody, and the reaction continues until equilibrium is attained:



In theory, the antibody binds to both forms of the antigen with the same affinity ($K = K^*$); their concentration ratio is therefore the same in the bound (B) and the free (F) phase, and hence the distribution of the cold antigen (B/F) may be characterized by the distribution of the radioactive antigen (B^*/F^*).

It may be seen from Eq. (4) that, if constant amounts of antibody and labelled antigen are used, the ratio of the bound and free fractions is governed by the amount of non-labelled antigen. With the increase of the concentration of the non-labelled antigen, the radioactivity of the bound fraction decreases (that of the free fraction increases), i.e. the amount of bound labelled antigen is inversely proportional to the amount of non-labelled antigen. A special case of RIA is illustrated in Fig. 1: the antibody is totally saturated at every antigen concentration (conditions: $[Ag^*] > [Ab]$ and K is infinitely large). As the amount of cold antigen is increased, the labelled antigen is diluted; this is a model of substoichiometric isotope dilution analysis.

In the vast majority of RIAs, however, the affinity constant is not sufficiently large for the antibody to be completely saturated at any antigen concentration: the degree of saturation is a function of the amount of antigen, the two increasing together.

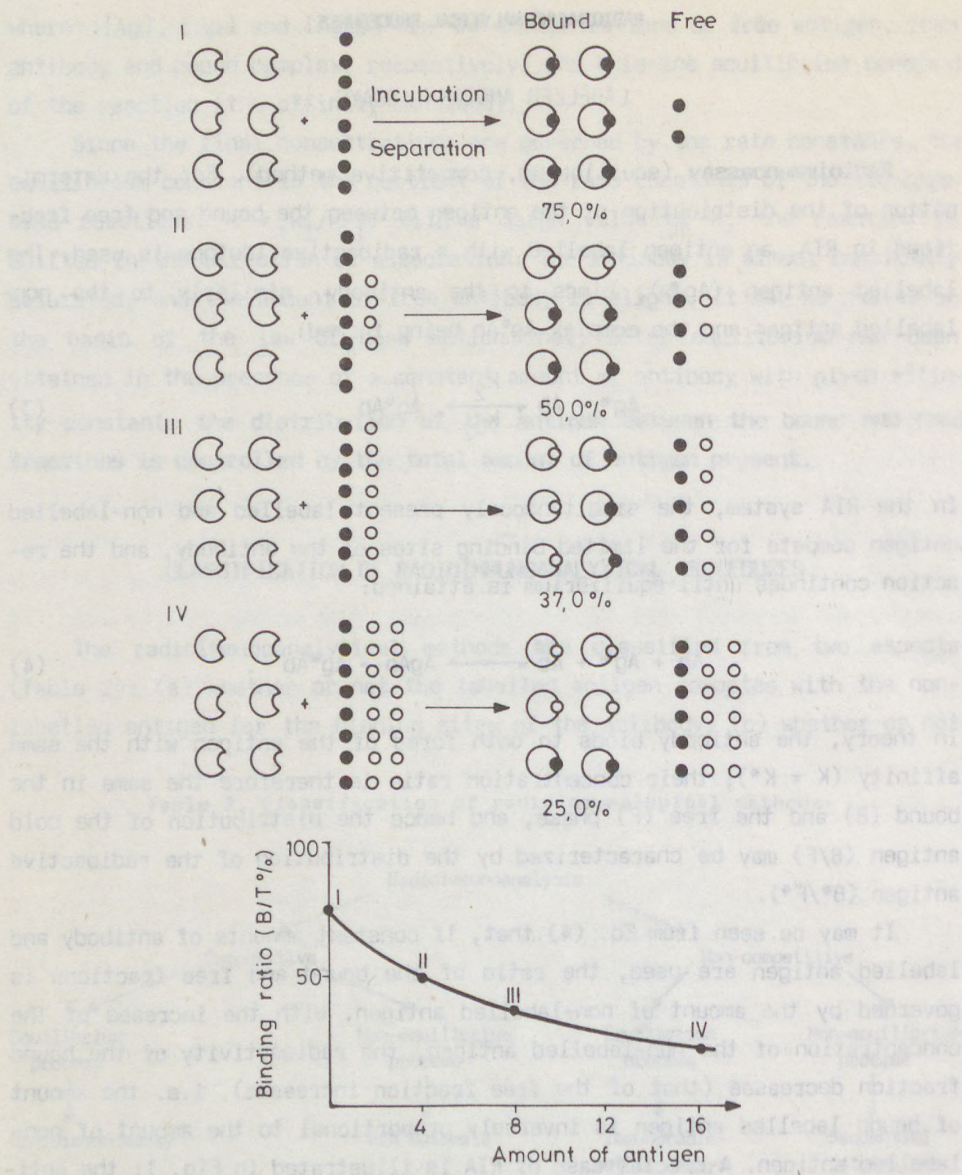


Fig. 1. Principle of radioimmunoassay (RIA). The radioactive (●) and cold antigen (o) present together in the system compete for the binding sites (C) of the antibody. The calibration curve depicts the ratio of the radioactivity bound to the antibody to the total radioactivity (B/T) as a function of the amount (concentration) of cold antigen

The model of RIA described so far involves a simple reaction between an antigen and an antibody, but in fact the situation is much more complicated. Some basic assumptions may be made in regard to the components in the reaction:

- (i) the antigen and antibody consist of homogeneous, chemically uniform material;
- (ii) both the antigen and the antibody are monovalent, i.e. one antigen molecule reacts with only one antibody molecule, other combinations not being possible;
- (iii) the reaction between antigen and antibody is a first-order biomolecular process, which can be characterized by the law of mass action;
- (iv) the non-labelled and the radioactively labelled antigens behave completely identically from the aspect of binding to antibody;
- (v) the reaction between the antigen and the antibody continues until equilibrium is attained;
- (vi) the bound and free fractions of the antigen can be separated without disturbance of the equilibrium.

These are the conditions for ideal RIA, but for a number of reasons they are never satisfied simultaneously in practice.

Non-equilibrium, competitive method. In a situation calling for rapid determinations, e.g. drug measurements, assays can be shortened to the extent that the sensitivity and precision may be compromised somewhat. In these methods, the reaction is stopped (by separation of the bound and free fractions) in a state when equilibrium has not yet been reached. In all such cases, the chosen incubation period must be adhered to extremely strictly, for even a slight variation will cause appreciable inaccuracy in the determination. A constant incubation time and temperature can readily be achieved with RIA automata, the majority of these instruments operating with non-equilibrium methods.

Sequential saturation analysis (non-equilibrium, non-competitive method) (Fig. 2). In this assay, Ag is first mixed with an amount of antibody whose binding capacity exceeds the total amount of antigen added, so that essentially all of the antigen becomes bound during this incubation period. In the second step, labelled antigen (Ag^*) is added to react with the binding sites remaining unoccupied by the non-labelled antigen during the first incubation step. It is desirable that the non-labelled antigen-antibody complex ($AgAb$) formed first should remain essentially unassociated during the second step.

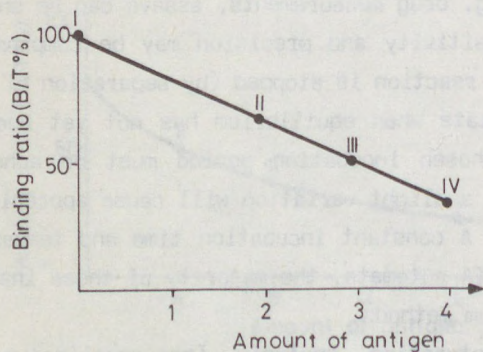
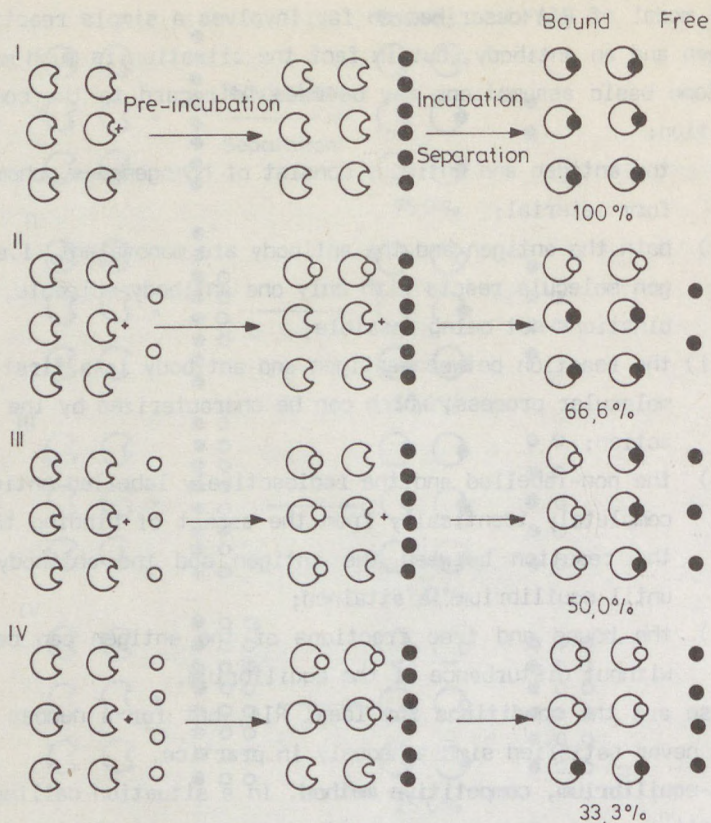


Fig. 2. Principle of sequential saturation analysis. After the binding of the cold antigen (o), the radioactive antigen (•) occupies the remaining antibody binding sites (C). The calibration curve depicts the proportion of the bound fraction (B/T) as a function of the amount of cold antigen

In the majority of cases the affinity of the antibody is not infinitely large; accordingly, dissociation of the complex AgAb cannot be neglected. Through appropriate choice of the length of the second incubation period, it can be achieved that the labelled antigen does not displace bound non-labelled antigen, i.e. there is no competition.

Since more non-labelled antigen is bound to the antibody in sequential saturation analysis than in equilibrium RIA, a small increase in the quantity of material causes a larger $[Ag*Ab]$ decrease, and hence the slope of the dose-response curve is higher. This leads to an increase in the sensitivity of the method in many cases.

Sequential saturation analysis has the great advantage that it can also be applied well in systems in which the labelled and non-labelled antigens bind with considerably different affinities to the antibody.

LABELLED ANTIBODY ASSAYS

Immunoradiometric analysis (equilibrium, non-competitive method). There are fundamental differences between labelled antibody assays and labelled antigen assays. In these methods, ^{125}I -labelled antibody is used to bind the antigen, and the bound and unreacted antibodies should be separated from one another.

Two variants of immunoradiometric analysis have become widespread:

1. **Immunoradiometric assay (IRMA)**. The procedure involves two steps: first, the antigen reacts with the radioactive antibody applied in excess, and the process continues until equilibrium is reached. In the second step, the unreacted labelled antibody is removed from the system by the addition of a large amount of antigen coupled to a solid phase (immunoabsorbent), and the radioactivity of the antigen-labelled antibody complex remaining in solution can be measured. Figure 3 reveals that the quantity of soluble antigen-labelled antibody complex increases in direct proportion to the increase in the amount of antigen.

2. **Sandwich immunoradiometric assay (two-site IRMA)**. In this type of radioimmunoanalytical procedure (Fig. 3), the reaction between the antigen and the labelled antibody is preceded by an immune-extraction step, in which the whole of the antigen of interest in the sample is extracted by a large amount of specific antibody coupled to the solid phase. This step is therefore a purification and concentration process; the antigen is bound to

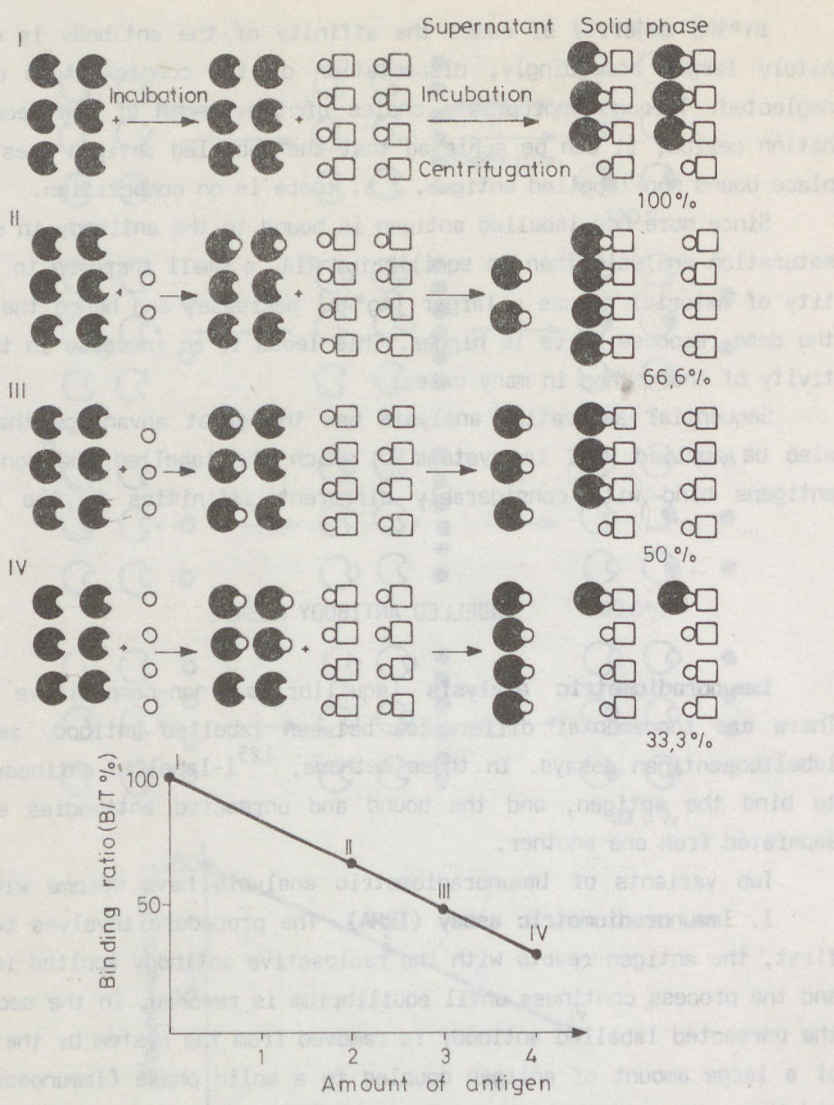


Fig. 3. Principle of immunoradiometric analysis (IRMA). Those radioactive antibodies (●) which do not bind cold antigen (o) during the incubation are reacted with antigen coupled to a solid phase (□). The proportion of labelled antibody bound to the solid phase decreases as the quantity of cold antigen increases

the solid-phase antibody, and substances possibly disturbing the analytical reaction can be removed. The bound antigen is then reacted with a known excess of labelled antibody; this binds to the antigen, assuming that it possesses at least two determinant sites, and the antisera are specific for these different binding sites. The fact that the antigen must be bound by two antibodies at two different sites at the same time means that this procedure is extremely specific. The amount of bound labelled antibody is directly proportional to the quantity of antigen extracted, so that a higher antigen concentration in the sample results in a higher labelled antibody binding (see Fig. 3).

The name of the method indicates that the antigen is held in a sandwich-like manner by the solid-phase antibody and the labelled antibody.

The immunoradiometric methods have the following advantages:

(a) The problems in the labelling of the antigen can be avoided.
(b) The antibodies are iodinated on an immunoabsorbent, and thus the binding site is protected.

(c) The labelled antibodies have a large affinity constant (these are isolated with the immunoabsorbent, and hence the sensitivity of the method is higher than that of a simple RIA).

(d) Sandwich IRMA has a high specificity.

Their drawbacks:

(a) Preparation of the labelled antibody is difficult and requires considerable practice.

(b) A large amount of antigen is need for preparation of the immunoabsorbent, and a large amount of antibody is needed for binding of the antibody to the solid phase.

(c) The methods are suitable only for the determination of large molecules possessing at least two antigenic determinants.

(d) The extent of non-specific binding is high in certain cases.

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GLOSSARY OF RADIOIMMUNOANALYTICAL PROCEDURES

Accuracy: a measure of the extent to which an assay can approximate the true value.

Active site: that portion(s) of a protein that must be maintained in specific shape and amino acid content to be functional (in antibody, the part that binds antigen; in hormone, the portion that recognizes the cell receptor).

Adjuvant: non-specific immune-stimulator which enhances the immune response to the immunogen during antibody production (immunization). Inorganic substances, such as gels of aluminium hydroxide, phosphate and sulphate, and charcoal can adsorb antigens; they are good adjuvants. Freund's adjuvant contains mineral oil and emulsifier.

Affinity: the strength of the interaction between a monovalent antigen and an antibody; it can be characterized by the affinity constant.

Affinity chromatography: a technique for the separation of molecules by their affinity to bind to ligands (or binders, like antibodies) attached to an insoluble matrix (e.g. Sepharose). The bound molecules can subsequently be eluted in a pure state.

Affinity constant: (K_a , unit l/mol) the equilibrium constant of the antigen-antibody reaction. It characterizes the stability of the antigen-antibody complex. The sensitivity of a method is influenced by the magnitude of the affinity constant: a high affinity usually means a sensitive method.

Adsorption chromatography: a technique for separating molecules by their different polarities on a layer or in a column filled with the sorbent (e.g. silica gel). It is used to purify synthetic antigens and radioactive tracers (e.g. tritiated steroids).

Adsorption method: (a) an isolation and concentration procedure to obtain the analyte in appropriate form (see extraction methods); (b) a technique for separation of antibody-bound and free antigen in a binding system. The adsorbent (activated charcoal, cellulose, glass powder, talc, etc.) binds the free antigen, which is separated from the bound fraction by centrifugation.

Analyte: substance to be measured by an analytical method.

Antibody: an immunoglobulin produced by lymphoid cells (plasma cells) in response to foreign substances antigens and capable of coupling specifically with its homologous antigen (the one that stimulates the immune response) or with substances that are chemically very similar to that same antigen.

Antigen: a foreign molecule that is capable of inducing an immune response in vertebrates and that reacts specifically with the antibodies in an assay. Those substances (molecular weight > 5000) which stimulate the production of antibodies are complete antigens or immunogens. A complex antigenic molecule may carry several antigenically distinct sites (determinants). Those which are too small to stimulate antibody synthesis itself are incomplete antigens or haptens.

Antigenic determinant: a small part of an antigen which determines the specificity of an antigen-antibody reaction and which is recognized by the binding sites of immunoglobulins.

Antiserum: serum from an immunized animal (or *in vitro* cell culture) containing antibodies specific for the antigen that stimulates antibody production.

Association constant: (K_1 , unit l/mol) the rate constant of the antigen-antibody complex-forming reaction.

Automatization: the possibility of carrying out a binding assay with an instrument. It substitutes the human labour (partly or completely) in the successive steps of an assay: identification and dispensing of samples, addition of reagents, incubation, separation of bound and free fractions, radioactivity counting, calculation of results and evaluation.

Avidity: the total combining power (energy) of an antibody with an antigen. It involves both the affinity of each binding site and the number of binding sites per antibody and antigen molecule.

Bacteriostatic agents: additives in the assay buffer of a binding system, they prevent bacterium growth (sodium azide, Merthiolate, etc.).

B cells: are parts of the immune system in vertebrates. In response to an antigen stimuli, they are transformed to plasma cells (during successive cell division), which produce their own, specific antibodies.

Becquerel: (Bq) the SI unit of radioactivity. 1 Bq = 1 dps (radioactive nucleus disintegration per second). (1 kBq = 27 nCi).

Beta-ray: a beam of negatively charged particles (electrons) emitted by an atomic nucleus undergoing radioactive decay (e.g. ^3H , ^{14}C). It can be measured (counted) by liquid scintillation counting, for example.

Binder or binding agent: a protein molecule (antibody, receptor, natural protein) which binds specifically to another molecule (ligand).

Binding percentage (B/T) bound fraction of labelled ligand (B) related to the whole amount of tracer (T) added to an assay tube. It is usually corrected by the non-specific binding (B-NSB/T-NSB).

Bioassay: *in vitro* or *in vivo* method for determination of the relative potency or effectiveness of a substance (e.g. a hormone or a drug) by comparing its biological effect on test animals, tissues or cells with the use of appropriate controls (e.g. quantitation of vasopressin by its antidiuretic action on rats). The result of a bioassay sometimes differs from that given by other methods.

Blank value: gives information on the labelled antigen quality and its non-specific binding (NSB) (on tube walls, precipitates, carrier proteins). It is usually determined by the incubation of tracer with assay buffer instead of antiserum. After separation, it should be less than 5% in a well-designed and performed method.

Bolton-Hunter reagent: (N-succinimidyl-3-(4-hydroxyphenyl)propionate) an appropriate molecule for conjugation labelling (see there). It may be iodinated either before or after coupling to the compound to be labelled.

"Bound" fraction: (B) that part of the ligand (labelled and non-labelled) which is attached to antibody and separated from the free fraction.

Bovine serum albumin: (BSA) a carrier protein which is coupled to haptens to make them immunogenic. It is a usual additive to assay buffers: it prevents adsorption losses onto the surface of tubes and stabilizes proteins and peptides.

^{14}C : a radioactive isotope of normal carbon (^{12}C) emitting weak beta particles (0.156 MeV). The half-life of ^{14}C is 5730 years. It can be counted with a liquid-scintillation spectrometer.

Carbodiimide coupling method: a technique used to conjugate molecules of low molecular weight (haptens) to protein carrier. The reagent (e.g. water-soluble 1-ethyl-3-[3-dimethylamino]propyl carbodiimide) promotes the reaction between free amino and carboxyl groups on the haptens and proteins.

Carrier protein: an immunogenic molecule to which a hapten is coupled, thereby making the hapten capable of inducing an immune response. They are proteins of large molecular weight (e.g. serum albumins from different species, thyroglobulin, keyhole-limpet haemocyanin, or synthetic homogeneous polypeptides (e.g. poly-L-lysine)).

Charcoal: one of the best adsorbents for the adsorption of substances of small molecular weight. It separates free and antibody-bound antigens on the basis of their size and charge. Covering with dextran (dextran-coated charcoal) helps to prevent the adsorption of bound fraction via its molecular-sieving effect. The charcoal should be activated by heat before use.

Chemical precipitation: a usual method to terminate a ligand-binder reaction. Inorganic salts (e.g. ammonium sulphate) and organic solvents (e.g. ethanol, dioxane, acetone, polyethylene glycol) precipitate the immunoglobulins usually by dehydrating their hydrate sheath. The pellet (bound fraction of ligand) formed is separated by centrifugation.

Chloramine-T: (N-monochloro-p-toluenesulphonamide) is a strong oxidizing agent applied for iodine labelling. The procedure is simple, requiring only mixing of a solution of the substance to be labelled, Na^{125}I , and chloramine-T; the reaction is stopped by addition of a reducing substance (sodium metabisulphite) or albumin. The iodinated product should be purified (chromatography, dialysis, electrophoresis).

Chromatography: a technique used for separating and identifying the components from mixtures of molecules having similar chemical and physical properties. The dissolved compounds are applied onto a porous stationary phase and then washed with liquid mobile phase. Since the molecules migrate at slightly different rates, they are eventually separated.

Coated tubes: a convenient tool for the separation of antibody-bound and free fractions of antigen. The antibodies are bound to the inner walls of a plastic tube (by adsorption or by covalent bonds), and after the immune reaction has taken place, the free antigen is poured out of the tube. Both fractions can be easily counted.

Column chromatography: any form of chromatography that uses a column or tube to hold the stationary phase (adsorbent, ion-exchange resin, gel, etc.).

Competitive binding: the basic principle of most radioligand assays. The labelled and non-labelled antigens compete for the limited number of binding sites on the binding agent. The reaction is governed by the Law of Mass Action.

Competitive protein binding assay: (CPBA) a term used to characterize those radioligand assays in which the binding reagent is a naturally-occurring protein (e.g. thyroxine binding globulin).

Conjugation labelling: a iodinated tracer production method for compounds which do not contain a iodine residue or are not accessible for steric reasons, or which are sensitive to oxidation. In this procedure, phenol (tyrosine methyl ester, tyramine, Bolton-Hunter reagent) or an imidazole group (histamine) capable of iodination is coupled to the substance to be labelled.

Cross-reacting material: any substance (antigen) that reacts with antibodies raised against another antigen (e.g. FSH, LH and TSH react with anti-hCG serum because all four antigens share a common antigenic determinant (alpha-chain)).

Cross-reaction: union of an antibody with an antigen other than the one used to stimulate the formation of that antibody; such cross-reactions usually involve antigens that are chemically similar or those that share antigenic determinants. It is done by giving the extent of non-specific binding with a substance the structure of which is related to that of the antigen to be determined. For its evaluation, one should compare the quantities of the two substances that inhibit binding of the labelled antigen to the antibody to the same extent (50%).

Curie: unit of radioactivity. One curie is equal to the quantity of any radioactive isotope undergoing 3.7×10^{10} disintegrations per second (dps) or 2.22×10^{12} disintegrations per minute (dpm).

Dalton: unit of mass equal to 1/12 the mass of the carbon atom with mass number 12.

Damage: in a fraction of labelled antigen or antibody which cannot react with the other components (physico-chemical damage). It can occur during labelling ("iodination damage"), storage ("storage damage") or incubation ("incubation damage").

Dextran-coated charcoal: separation reagent (see charcoal).

Dilution curve: (or titration curve) is a means of evaluating the titre characteristic of an antiserum. Titration is performed by assaying the

ability of a fixed amount of labelled antigen to bind antiserum at various dilutions. It is a sigmoid curve if the binding percentage is presented versus antiserum dilution.

Disequilibrium assay: (or non-equilibrium assay) in which the bound and free fractions are separated before the system attains thermodynamic equilibrium, or in which the labelled and non-labelled antigen are not added to the antibody simultaneously (see sequential saturation).

Dissociation constant: (K_{-1} or K_d) the rate constant of the antigen-antibody complex decomposition reaction.

Dose-response curve: (calibration or standard curve) is the base for quantitation of an assay. In RIA it involves the incubation of a fixed amount of antibody and labelled antigen with known amounts of non-labelled (or "cold") antigen (standard or calibrator). Increase of the quantity of "cold" antigen inhibits binding of the labelled antigen to the antibody. In the curve, the response (relative binding: B/T , B/B_0 , etc.) is plotted versus dose. The antigen concentration to be measured is determined from the calibration curve by extrapolating the extent of inhibition it gives rise to.

Double antibody separation method: is a universal technique to differentiate the antibody-bound and free antigen. The separation is based on a second immune reaction, in which the primary antibody-antigen complex forms a precipitate with a **second antibody**. Because of the low immunoglobulin concentration employed in the ligand assay, even this new complex (second antibody-first antibody-antigen) does not always precipitate from solution. For a centrifugable precipitate to form, it is necessary to add a high concentration of second antibody (dilution 1:10-1:100) and accompanying gamma-globulin to the incubation system.

Electrophoresis: a separation technique based on the different mobilities of substances on a matrix (paper, starch gel, polyacrylamide gel, etc.) between two poles.

Enzyme inhibitors: are applied to prevent ligands (e.g. peptides) from effecting enzymatic degradation during sample preparation (extraction) and incubation. Ethylenediaminetetraacetic acid (EDTA), dimercaprol, phenylmethylsulphonyl fluoride (PMSF) and trasylol are well-known inhibitors.

Equilibrium: is the state of a reversible reaction (antigen-antibody) in

which the rates of complex formation (association) and decomposition (dissociation) are equal. The situation can be described by the Law of Mass Action.

Equilibrium assay: a type of radioimmunoanalytical method in which the reaction components are allowed to come into thermodynamic equilibrium and are then separated.

Equilibrium constant (K) is the ratio of the concentration of the antigen-antibody complex and the product of the concentrations of the free antigen and free antibody at equilibrium, or the ratio of the association and dissociation rates. The unit of K is litre/mole (l/mol).

Extraction methods: techniques applied to isolate the analytes from biological materials. They are used to concentrate the analyte and to eliminate interfering factors. Extraction with organic solvents (e.g. dichloromethane, acetone, etc.) or adsorption on a solid phase (glass powder, silica gel, reversed-phase silica gel, immunosorbents, etc.) are widely-used methods.

F_{ab} fragment: that fragment of a papain-digested immunoglobulin molecule which bears a single antigen-binding site and contains one intact light chain and a portion of one heavy chain.

F_c fragment: that crystallizable fragment of a papain-digested immunoglobulin molecule which contains only portions of two heavy chains and no antibody binding sites. This fragment does, however, bind complement and is responsible for the binding of immunoglobulin to various types of cell in a non-antigen-specific manner.

"Free" fraction: (F) that part of a ligand (labelled or non-labelled) which is not attached to antibody and is separated from the bound fraction. It can be referred to as the unbound fraction.

Freeze-drying: (lyophilization) a method of dehydrating a solution by rapid freezing and subliming the ice in the frozen state under vacuum. Peptides and proteins (e.g. antibodies) are usually more stable in a lyophilized form than in solution.

Freund's adjuvant: a widely-used adjuvant. It may contain killed, dried Mycobacterium tuberculosis (complete Freund's adjuvant) suspended in the oil phase of a water-in-oil emulsion, which further enhances the effectiveness of the adjuvant through its general and local inflammatory effect.

Gamma-counter: an instrument for the measurement of gamma-ray emitting radioactive isotopes (e.g. ^{125}I , ^{131}I). The quantitation is based on detecting light photons emitted by a scintillator (Tl-activated sodium iodide crystal), when hit by electromagnetic radiation. The flash detected by the photo-cathode of a photomultiplier tube is converted into an electronic pulse, which is amplified, screened and counted in the scaler. Gamma-counting is much simpler and cheaper than liquid-scintillation counting. When the counter is connected to a computer, results can be easily calculated.

Gamma-ray: electromagnetic radiation of short wavelength emitted by an atomic nucleus undergoing radioactive decay (isomeric transition or internal conversion). The activity of a gamma-ray radiating isotope (e.g. ^{125}I , ^{131}I) is measured in a gamma-counter.

Gel filtration: a chromatographic separation method in which substances are separated on soft gels (Sephadex, Biogel) on the basis of their size in solution. Useful in purification of biopolymers.

Half-life: (a) biological: the time required for the body to eliminate half of a dose of a given substance, (b) radioactive: the time lapse during which a radioactive mass loses half its radioactivity. Each radio-nuclide has a unique half-life.

HPLC (high-performance liquid chromatography): a chromatographic method in which the column is filled with different stationary phases (silica gel, reversed-phase silica gel (C_{18} , C_8 , CN, NH_2), ion-exchange resin, polymer resin) of very small particle size (3-10 μm) and the mobile phase is pumped through the column under high pressure. HPLC usually has high resolving power and it is much faster than conventional separation methods.

Hormone: an organic compound produced in one part of an organism and transported to other parts where it exerts a profound effect.

Hybridoma: a cell resulting from the fusion of an antibody-producing cell (a B lymphocyte) and a myeloma (a bone marrow cancer) cell. Such a hybrid cell produces a clone that can be maintained in tissue culture or as an animal tumour, and the clone may secrete only a single kind of antibody (see monoclonal antibody).

^{125}I : a radioactive isotope (with a half-life of 60.14 days) of iodine, emitting gamma-rays (35 keV) and X-rays (27-32 keV). Its radioactivity can be measured with a gamma-counter.

Immune response: the physiological response(s) stemming from activation of the immune system by antigens. The cells mainly involved in an immune response are T and B lymphocytes and macrophages. B cells mature to produce immunoglobulins or antibodies that react with antigens. The first contact with an antigen "sensitizes" the animal and results in a primary immune response. Subsequent contact of the sensitized animal with the same antigen (booster injection) results in a more rapid and elevated reaction, called the secondary immune response, which is most easily demonstrated by monitoring the level of circulating antibodies in the serum.

Immunization: administration of an immunogen to animals for the purpose of stimulating an immune response to it. The results are influenced by several factors, e.g. quality and quantity of immunogen, use of adjuvant, animal species, route and schedule of immunization, etc.

Immunogen: a substance which causes an immune response. The chemical structure and molecular size of a compound are important in determining its immunogenicity. Substances with a molecular weight above 5000 dalton are generally good immunogens, whereas smaller ones are much weaker stimulants (see haptens).

Immunoglobulin: (antibody) a glycoprotein secreted by mature lymphoid B cells. The basic structure of all immunoglobulins is similar to a pair of large polypeptide chains (heavy- or H-chain) associated with two smaller chains (light- or L-chain), forming a Y-shape. On the basis of the structural and physico-chemical properties of the H-chain, the immunoglobulins are classified into five main groups: IgG, IgM, IgA, IgD and IgE. Both the L- and the H-chain contain one constant and one variable region. The IgG molecule (the main immunoglobulin) possesses two antigen binding sites in its hypervariable regions, which are located at the outer end of each H-L-chain pair.

Immunoprecipitation: precipitation of an antigen-antibody complex (bound fraction) by a second antibody, an antigamma-globulin (see double-antibody separation method).

Immunoradiometric assay: (IRMA) non-competitive radioimmunoanalytical method. An excess amount of ^{125}I -labelled antibody is reacted with the antigen; after equilibrium is reached, the unbound labelled antibody is separated. Two types of immunoradiometric assay are used: classical one-site IRMA and two-site or "sandwich" IRMA (see there).

Immunosorbent: the antibody is coupled to an insoluble solid phase or

matrix (cellulose, Sephadex, polystyrol, polypropylene, acrylamide) via adsorption or covalent bonding. This is a convenient tool for the separation of bound and free antigen: at the end of the incubation, the solid-phase antibody-bound ligand is centrifugable. It is also used to extract antigen from biological samples for IRMA.

Incubation: refers to the conditions under which the antigen-antibody reaction takes place, such as time, temperature, medium composition, pH, etc.

Iodination: (iodine labelling) a tracer preparation method in which ^{125}I or ^{131}I is introduced into a compound containing a iodine-accepting group (tyrosine, histidine, tyramine, etc.). The iodide anion is converted into a iodonium cation by an oxidizing agent (chloramine-T, lactoperoxidase, iodogen) and this cation reacts with an aromatic side-chain of the substance. The iodinated product must be purified before use in an assay (adsorption, chromatography, electrophoresis, etc.).

Iodogen: (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril) is a mild oxidizing agent used to iodinate sensitive peptides and proteins.

Ion-exchange chromatography: a mode of chromatography in which substances are separated on a cationic or anionic resin by virtue of their ionic character.

Isoelectric focusing: a separation method in which the environment of electrophoretic separation is altered to create zones of pH differences. Proteins migrate to the zone of their isoelectric point and there cease to migrate.

Isotopes: one of the several forms of a chemical element. The different isotopes of a given element have the same number of protons and electrons, but differ in the number of neutrons contained in the atomic nucleus. Hence, they have identical chemical properties, but differ in atomic weight.

Isotope dilution analysis: a method of chemical analysis for a component of a mixture. The method is based on the addition of a known amount of labelled component with known specific activity, followed by isolation of a quantity of the component and measurement of the specific activity of the sample.

Kits: commercial preparations containing all of the reagents (antibody, standards, assay buffer, quality control sample, separation reagent) necessary for the determination of a substance, together with a detailed description of the method.

- Label:** (radioactive) a radioactive atom introduced into a molecule to facilitate the study of its distribution.
- Labelling:** a procedure where a label is introduced into a molecule.
- Law of Mass Action:** a mathematical description of a reversible reaction that attains equilibrium. The rate of reaction is proportional to the concentration of each substance taking part in the reaction.
- Ligand:** a molecule that will bind to a complementary site on a given structure (binder) (e.g. an antigen is a ligand which binds to an antibody). In radioimmunoanalysis, the term refers to the substances being measured as opposed to the binding agent.
- Ligand assay:** a general term for a type of assay in which the quantitation is based on a reaction between a ligand and a binding agent (natural binding protein, antibody, tissue receptor).
- Ligand-free serum:** a medium for construction of the dose-response curve, which does not contain analyte. For correct measurement, it is sometimes indispensable to compensate the effects of other components in the sample.
- Liquid-scintillation counter:** an electronic instrument for measuring beta-ray emitting radioisotopes (e.g. ^3H , ^{14}C , ^{32}S). The sample to be measured is dissolved in a solvent (e.g. toluene) containing a fluorescent chemical (2,5-diphenyl-oxazole) that emits a flash of light (scintillation) when struck by an ionizing particle (electron). The flash is captured by a photomultiplier tube, transformed into an electric pulse, amplified, routed through a scaler and counted. The counter is usually connected to a computer and the data are processed in a desired manner (e.g. RIA calculation).
- Logit-log calculation:** the most familiar mathematical method in radioimmunoanalysis to linearize the sigmoidal-shaped dose-response curve. The logit [$\text{logit}(Y) = \log_e((Y)/(1-Y))$, where $Y = (B - \text{NSB})/(B_0 - \text{NSB})$] is plotted against log dose, yields a straight line for most assays. This makes the estimation of unknown sample concentrations much easier and more reliable.
- Magnetic particles:** are applied in a solid-phase separation technique in which the antibody is covalently coupled to a magnetizable particle (Fe_3O_4) contained in a matrix (cellulose or plastics). Separation of the bound and free fractions can be easily achieved by the application of a magnetic field.

Mixed anhydride coupling: a chemical method for binding of a hapten to an immunogenic carrier protein. The carboxyl group of the hapten is converted with isobutyl chloroformate to a mixed anhydride, which can then react with the primary amine group of the protein molecule.

Monoclonal antibodies: immunoglobulins derived from a single clone of plasma cells. Since all immunoglobulins produced by a given plasma cell are chemically and structurally identical, these antibodies constitute a pure population with highly specific antigen-binding properties. They can be produced by hybrid cells-formed by fusion of an antibody-producing cell from an immunized animal (rat, mouse) with a myeloma cell-in a cell culture or in vivo (see hybridoma).

Monoiodotyrosine: peptides and proteins containing tyrosine can be labelled with radioactive iodide to give tracers. The iodination should be conducted so as to obtain mainly the monoiodinated product, because this is more stable than the disubstituted analogue.

Non-equilibrium assay: refers to a method in which the reaction is stopped before equilibrium has been attained between the labelled and non-labelled antigen, in order to eliminate or diminish the simultaneous competition between the two types of antigen (see sequential analysis).

Non-specific binding: (NSB) the term denotes the occurrence of non-specific adsorption of labelled free antigen on a protein precipitate or on the tube walls, or of antigen-antibody complex adsorption on charcoal, for example, which leads to misclassification of the evaluation of the binding reaction. With radioactive antigen of appropriate quality, the non-specific binding accounts for less than 5% of the total radioactivity (see blank value).

Precipitation: a general term to describe the process of making the antigen-antibody complex insoluble. Immunoglobulins can be precipitated with inorganic salts and organic solvents by dehydration or immunologically with a second antibody.

Protein A: a major cell-wall component of *Staphylococcus aureus*. It binds rapidly and with high affinity to immunoglobulin G (antibodies) from rabbit, guinea pig and pig. Protein A is utilized to precipitate the antigen-antibody complex in immunoassays.

Quality control: in general terms, the analytical and other steps which must be taken to ensure that the results of assays are reliable and representative of the true concentration of the material to be de-

terminated. This expression is also commonly used to refer to test samples (stored in many aliquots, each in stable form), one aliquot being measured in each assay to assess inter-assay precision.

Quenching: the efficiency of the liquid scintillation counting is reduced by the adsorption of light quanta (caused by beta radiation) in the liquid-scintillation media (chemical, colour and optical quenching). It can be eliminated by quench correction.

Radioactive decay: the disintegration of the nucleus of an unstable nuclide, accompanied by the spontaneous emission of charged particles and/or photons.

Radioactive isotope: an isotope with an unstable nucleus that becomes stable by emitting ionizing radiation.

Radioactivity: the spontaneous disintegration of certain nuclides, accompanied by the emission of one or more types of radiation, such as alpha-particles, beta-particles, and gamma-photons. The unit of radioactivity is Becquerel or Curie (see there).

Radioimmunoassay: (RIA) a competitive protein binding method for the quantitative determination of antigenically active substances that are present in very small amounts, such as hormones. The concentration of an unknown, non-labelled antigen is determined by comparing its inhibitory effect on the binding of radioactively labelled antigen to a specific antibody with the inhibitory effects of known standards.

Radioiodination: preparation of substances containing radioactive iodine (^{125}I , ^{131}I) used as tracers in radioimmunological methods (see iodination).

Radiolabelling: preparation of a tracer compound containing a radioactive isotope (^3H , ^{125}I or ^{131}I) for radioimmunological methods.

Radioligand assay: a general term for an in vitro radioassay based on a radioactive ligand-binding agent reaction.

Radioreceptor assay: a protein-binding method using a cell-associated binding substance (membrane or intracellular receptors) and a radioactive labelled ligand for the determination of biological substances.

Reference preparation: a standard substance which is the basis for comparison in all those assays in which the analyte is not available in pure, synthetic, physicochemically characterized form. Such materials are usually of high molecular weight (isolated from a biological system) with known biological potency. International organizations (e.g. WHO) coordinate the preparation and distribution of this type of standards.

Reproducibility: (precision) a measure of the variation observed between repeated determinations of the same sample. It can be characterized by the mean ($\bar{x}$), standard deviation (S.D.) and coefficient of variation (C.V.).

Reversed-phase chromatography: a technique for the separation of compounds on a supporting phase (silica gel) that has been made hydrophobic by treatment with non-polar reagents (octyl, octadecyl group, etc.). In this type of chromatography (mainly used as HPLC), the elution sequence of the sample compounds is usually the reverse of that in adsorption chromatography (i.e. the most polar component elutes first).

Sandwich immunoradiometric analysis: a radioimmunological test system which uses two antibodies against two different antigenic determinants of the analyte. The antigen is extracted with solid-phase antibodies (immunosorbents) and is then quantified with another, ^{125}I -labelled antibody.

Saturation analysis: a term generally used to imply a competitive type radioassay in which the concentration of the antibody is limited, and the binding sites are saturated at all antigen concentrations ($[\text{Ag}^*] > [\text{Ab}]$).

Scatchard plot: a presentation method for evaluation of the affinity constant of an antigen-antibody reaction based on the Law of Mass Action. In an ideal case, when the quotient bound/free fraction of an antigen is plotted as a function of the concentration of the bound antigen, a straight line results. The slope of this line gives the affinity constant (K_a), and the intercept on the x-axis represents the total concentration of binding sites available. However, antisera are usually polyclonal (antibodies with different affinities and specificities) and therefore a non-linear curve results.

Second antibody: an immunoglobulin directed against the species of the first antibody to yield an insoluble immune complex (see double antibody separation technique). The first and the second antibodies must be produced in different animal species.

Sensitivity: the smallest amount of antigen which can be differentiated from zero.

Separation methods: for determination of the distribution of radioactivity between the bound and free ligand fractions, they have to be separated. The separation methods are based on differences in the physico-chemical and/or immunological properties of the free and bound ligands (see

adsorption method, charcoal, precipitation, solid-phase system, double antibody method).

SHBG: (sex hormone binding globulin) a natural, circulating binding protein for oestrogens and androgens (high concentration in pregnant women). It has been used in the measurement of testosterone or dihydrotestosterone in the competitive protein binding assay.

Solid-phase separation method: the antibodies are coupled (adsorptively or covalently) to insoluble structures (cellulose, glass, polystyrol, polypropylene, etc.), such as powder, discs, tubes, beads or Sepharose. When the immune reaction has taken place (incubation), the separation of bound and free antigen can be achieved by centrifugation or simply decantation.

Specific activity: the radioactivity referred to unit amount of substance (Bq/mol, Ci/mol, Ci/g).

Specificity: the degree of interference encountered from substances other than the one being measured (the selective binding between the antibody and the ligand). This property of an antibody determines whether it can be used for radioimmunological estimation of the antigen or not, because numerous other substances influence the antigen-antibody reaction.

Standard curve: see dose-response curve.

Standards: reference substances used for calibration in a quantitative comparative assay; they should be chemically identical to the substance to be assayed. They are synthetic, well-characterized compounds of small molecular weight or isolated substances of biological origin (usually of high molecular weight).

Titre: (or antibody concentration) that dilution of the antibody which binds fixed quantity of the labelled antigen (usually 50%). It can be defined by the titration curve established when labelled antigen is incubated with serial dilutions of antiserum.

Total radioactivity: the amount of labelled substance (ligand or binding agent) that is added to an assay tube. It will distribute between the bound and free fractions.

Tracer: a labelled antigen used to quantify the distribution of antigen between the free and bound fractions.

Tritium: (^3H) the radioactive isotope of hydrogen, with a half-life of 12.46 years. When it undergoes radioactive decay, it emits weak beta-

particles (18.6 keV) which can be measured by liquid-scintillation counting.

Two-site radiometric assay: an antigen is reacted with a solid-phase antibody, followed by reaction with another, ^{125}I -labelled antibody, which binds another antigenic determinant of the antigen (see "sandwich" IRMA).

Chapter 2

PITUITARY-ADRENAL CORTEX SYSTEM

In the following we are dealing with the pituitary-adrenal cortex system, i.e. with adrenocorticotrophic hormone (ACTH) and hydrocortisone (cortisol).

ADRENOCORTICOTROPIC HORMONE (ACTH, CORTICOTROPIN)

Chemical structure. ACTH consists of 39 amino acids; it is a single-chain polypeptide with a molecular weight of 4,541 dalton. The N-terminal 24 amino acids are responsible for the biological activity. Shorter and longer peptides related to ACTH are to be found in the organism (e.g. α -MSH, β -MSH, lipotropin). Their prohormone is pro-opiomelanocortin (POMC) with a molecular weight of about 31,000 dalton.

Physiological conditions. ACTH exerts its main effect on the function of the adrenal cortex: it stimulates the early phase of steroid synthesis, enhances the blood flow in the adrenals, and increases the weight of the adrenals. Extra-adrenal effects of ACTH have also been reported, primarily on the basis of animal experiments. The question remains open as to whether these are of significance in humans. The enhancement of the pigmentation of the skin has been attributed by a number of authors to ACTH. It is presumably not the ACTH molecule itself, but the related β -MSH that plays a role in this. ACTH secretion displays a marked diurnal rhythm: the highest level is found between 7 and 9 a.m., and the lowest level between 8 and 12 p.m. Besides the diurnal rhythm, ACTH secretion under normal conditions is influenced by the feed-back effects of the corticosteroids and various stress states (insulin, pyrogen, pain, nervousness). All these effects are manifested with the participation of the hypothalamus, with the mediation of corticotropin releasing hormone (CRH, corticoliberin). ACTH circulates in the free state in the plasma, without protein binding, and it disappears rapidly from the blood; its biological half-life is 5-10 min.

Method. For purposes of immunization, the 1-24 and 1-32 ACTH fragments are bound to bovine albumin or to thyroglobulin. The antibody is generally produced in rabbit. A cross-reaction is to be expected with the shorter ACTH fragments (MSH, 1-24 and 1-32 fragments). ¹²⁵I-labelled ACTH is used

for the determination; this is purified by reversed-phase silica gel chromatography. More recently, a stable ACTH analogue too has been prepared for ^{125}I labelling. The plasma sample is extracted with glass powder or silicate (Vycor glass, Quso 32), or minicolumn chromatography. Trasylol is used at 4°C to inhibit the decomposing enzymes. The bound and free fractions are separated with dextran-coated charcoal, with polyethylene glycol and with glass powder. The time necessary for the procedure is 3-4 days. The main technical difficulties are caused by the rapid decomposition, the low plasma level, the unsatisfactory stability of ^{125}I -ACTH, and the fact that it adheres to a glass wall, so that plastic tubes are used.

Clinical applications: An enhanced ACTH production is encountered in primary hypoadrenia, some Cushing's disease patients and ectopic ACTH syndrome. A high ACTH level accompanying a low hydrocortisone concentration is indicative of an adrenocortical enzyme defect. Measurement of the ACTH level after bilateral adrenalectomy is of good value for establishing the correct substitution dose of glucocorticoids. A low level is found in cases of pituitary-hypothalamus damage, adrenocortical tumour and prolonged corticosteroid therapy. ACTH determination may be used as the most important diagnostic tool for adrenocortical tumour and ectopic ACTH syndrome, and in the differentiation of primary and secondary hypoadrenia. The normal range is 10-70 pg/ml (5-11 pmol/l).

Sample taking requires great care. ACTH decomposes quickly *in vitro*, too. Accordingly, the sample must be placed in ice-water immediately, and the formal elements are separated in a cooled centrifuge. Since the hormone adheres to a glass wall, plastic tubes must be used throughout the procedure. Care should be taken to obtain samples in an environment as free as possible from stress and in the appropriate period of the day. If Cushing's disease is suspected, the variations in the diurnal rhythm must also be examined (blood sampling at 8 a.m., 2 p.m., 8 p.m. and 2 a.m.).

HYDROCORTISONE (CORTISOL)

Chemical structure. The corticosteroids have the common chemical feature that there is a 20-keto-21-ol side-chain on carbon atom 17 of the sterane skeleton. Cortisol also contains 11-OH, 17-OH and 3-keto groups (Fig. 4) (molecular weight 362 dalton). Cortisol is produced by the adrenal cortex, in large amount (25 mg/day). 90-95% of the cortisol in the blood is

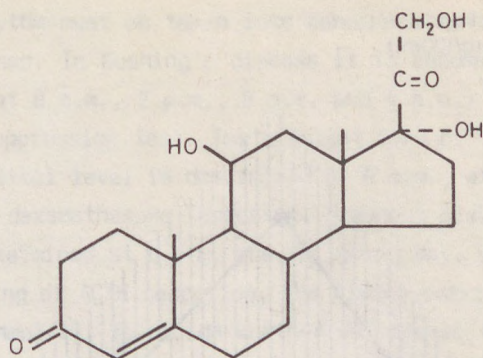


Fig. 4. Chemical structure of cortisol

in the biologically inactive form; it circulates in the state bound to transcortin, belonging to the α -globulin group. Its biological half-life is 1-2 h. Only 1% of the amount of hormone produced is excreted in the urine; the remainder is metabolized in the liver. This has the consequence that the cortisol level may be changed in liver diseases.

Physiological conditions. Cortisol has considerable effects on the metabolic processes in the organism. The name glucocorticoid is an indication that the effect on the carbohydrate metabolism is particularly important: it enhances gluconeogenesis and elevates the blood sugar level. It has a substantial influence on protein synthesis, depressing protein formation through its antianabolic or catabolic effect. Its action on urine excretion, inflammation and the immune system are also well known.

Similarly to ACTH, the level of cortisol exhibits a regular diurnal fluctuation: it is high in the early morning, and low in the evening and at night (Fig. 5). In stress states, the cortisol level is elevated. It blocks ACTH release through its negative feed-back effect. Only free cortisol has a biological effect. However, the measured results may be influenced considerably by a change in the binding capacity: during pregnancy and when taking oral contraceptives, the transcortin level and the plasma total cortisol level increase; in parallel with a low serum protein level, a decrease is observed in the cortisol concentration.

Method. Initially fluorimetry, and later a procedure based on transcortin binding was used. More recently, RIA has become widespread. Cortisol-21- or 11-hemisuccinate or cortisol-6-carboxymethyl oxime is used to

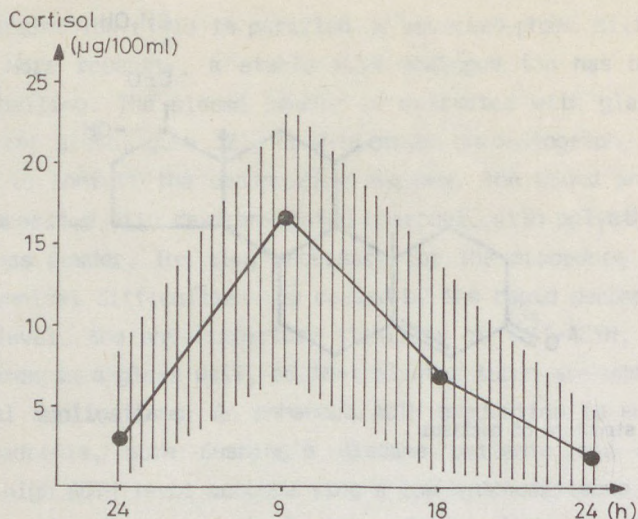


Fig. 5. Diurnal fluctuation in cortisol level

prepare the antigen. Immunization is most often performed in rabbits. Cross-reactions are to be expected with 11-deoxycortisol, cortisone and prednisolone. Because of the high plasma cortisol level, however, the disturbing effect of the cross-reactions is not appreciable. Tritiated cortisol or ^{125}I -labelled cortisol is applied as labelled hormone.

The serum samples need not be extracted. Reaction between the antigen and the antibody is fast; the incubation period is a few hours. In our laboratory, incubation is carried out at 45 min at 37°C . The bound and free fractions may be separated with dextran-coated charcoal, with the use of a second antibody, or with the solid-phase antibody method.

Clinical applications. In Cushing's disease, the plasma cortisol level is high and the diurnal rhythm disappears. The hyperplastic and tumorous origins may be distinguished through the use of suppression tests. Similarly to the tumorous case, the high cortisol level in the ectopic ACTH syndrome cannot be suppressed. A low cortisol level is found in Addison's disease, in hypopituitarism, after chronic corticoid therapy and in congenital enzyme defect (a high ACTH level!). The normal levels: 80-150 ng/ml (224-420 nmol/l) at 8 a.m., and 30-120 ng/ml (84-336 nmol/l) at 8 p.m.

The diurnal rhythm must be taken into consideration when the time of sampling is determined. In Cushing's disease it is recommended to examine the entire rhythm (at 8 a.m., 2 p.m., 8 p.m. and 4 a.m.) and to carry out the dexamethasone suppression test. The simplest form of this is the overnight test: the cortisol level is determined at 8 a.m., at 11 p.m. the patient receives 1 mg dexamethasone (Oradexon, Organon) orally, and the cortisol level is redetermined at 8 a.m. the following day. Under normal conditions, with blocking of ACTH secretion, the plasma cortisol level is less than 60 ng/ml (168 nmol/l). To decide whether an adrenal tumour or ectopic ACTH syndrome is involved, 8 mg dexamethasone is administered daily to the patient for 6 days, and the corticosteroid secretion is examined in the second half of the treatment. A substantial decrease (50%) does not result with these two syndromes. If hypoadrenia is suspected, an ACTH stimulation test is performed: synthetic 1-24 ACTH is injected i.v. in a quantity of 0.2 IU/kg body weight. In a normal case, the cortisol level is doubled within 30-60 min; in Addison's disease, this change does not occur.

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Chapter 3

PITUITARY-THYROID SYSTEM

The components of this system that will be considered are thyrotropic hormone (TSH), thyroxine (T₄), triiodothyronine (T₃) and the T₃-binding capacity.

In thyroid diagnostics, there has recently been an advance of **in vitro** tests, at the expense of the **in vivo** methods. A five-level regulatory mechanism serves to maintain the normal thyroid function: 1. hypothalamus (TRH), 2. pituitary (TSH), 3. thyroid (T₄, T₃), 4. thyroid-binding globulin (TBG), and 5. target organ (specific cell receptors).

THYROTROPIC HORMONE (TSH, THYROTROPIN)

Chemical structure. This hormone belongs to the group of glycoproteins. Its molecular weight is about 28,000 dalton. It is comprised of α - and β -subunits. The α -chain is not specific: it may also be found in other hormones (FSH, LH, hCG). The β -subunit is characteristic of this compound.

Physiological conditions. TSH stimulates the functioning of the thyroid; in its absence, the thyroid atrophies. It influences hormone formation at several levels: it enhances the transport of iodine towards the thyroid, it promotes the organic binding of iodine and the storage of hormone by the thyroid, and **via** the proteolysis of thyroglobulin it promotes T₄ and T₃ secretion. Through a negative feed-back regulatory mechanism, the thyroid hormone level has a significant influence on TSH secretion: an elevated T₄ level induces a decrease in TSH secretion, while a decreased T₄ concentration induces an enhancement of TSH secretion. The thyrotropin releasing hormone (TRH, thyroliberin), besides TSH, stimulates prolactin secretion. TRH is a simple tripeptide (pyroglutamyl-histidyl-prolinamide) widely employed in thyroid diagnostics. The biological half-life of TSH in the plasma is about 1 h; the corresponding time is shorter in hyperthyroidism, and longer in hypothyroidism.

Method. Immunization is carried out in rabbit, through repeated administration of the β -chain of TSH. Cross-reactions are to be expected

with LH, FSH and hCG. Monoclonal antibodies produced in mouse have also been used recently. ^{125}I is employed for labelling.

Extraction is not applied before the determination. Only 24 h is required for the incubation. The standards of the calibration curve are dissolved in TSH-free serum. The bound and free fractions are separated by means of a second antibody, with a solid-phase antibody technique, or with polyethylene glycol. Problems may be caused by the contamination of the reference TSH and the disturbing effects of cross-reactions. The sensitivity of the method is $0.1\text{--}1.0\ \mu\text{U/ml}$ (lower than the normal value). Using the ultrasensitive method its sensitivity is $0.05\ \mu\text{U/ml}$. Recent procedures include the use of monoclonal antibodies, immunoradiometric assay (IRMA), and magnetic separation (MAIA).

Clinical applications. An elevated TSH level is observed in primary hypothyroidism and in certain malignant diseases (hydatidiform mole, choriocarcinoma). A pituitary adenoma producing TSH is very rare. Depressed levels are found in hypopituitarism (together with decreases in the levels of other pituitary hormones) and in active hyperthyroidism. In the latter cases, it is recommended to carry out the TRH test: 30 min after the i.v. administration of $200\ \mu\text{g}$ synthetic TRH, the basal TSH level is increased several-fold (Fig. 6). The TSH concentration does not rise in hyperthyroidism and hypopituitarism. TSH does not display a diurnal rhythm. Stress and pregnancy do not cause changes. The normal value is $1\text{--}4\ \mu\text{U/ml}$ ($1\text{--}4\ \text{mU/l}$). It does not decompose in the serum even at room temperature, and if quick-frozen it can be stored for years.

THYROXINE (T₄)

Chemical structure. The formula of T₄ is presented in Fig. 7. The molecular weight is 777 dalton. It is not species-specific, and has the same composition in mammals. It is stored in the form of thyroglobulin in the follicles, from where it is released through active hormone proteolysis.

Physiological conditions. Its most important biological effect is to enhance the metabolism. It increases the frequency of the heart, the cardiac output, and hence the blood pressure. It elevates the blood glucose level. T₄ is produced in the epithelial cells of the acinus. In the circulation, it occurs predominantly (99.97%) bound to plasma proteins, mainly

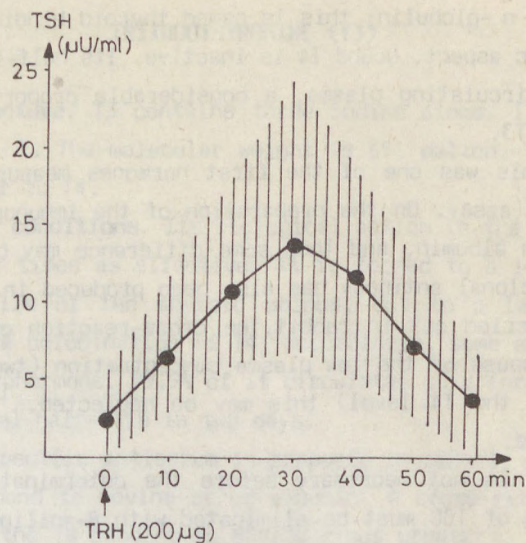


Fig. 6. Variation in plasma TSH level following i.v. administration of TRH

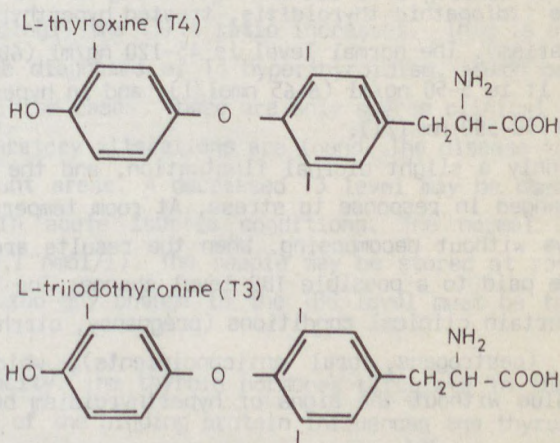


Fig. 7. Chemical structures of L-thyroxine (T₄) and L-triiodothyronine (T₃)

bound to inter- α -globulin; this is named thyroid binding globulin (TBG). From a metabolic aspect, bound T4 is inactive. Its half-life is about eight days. In the circulating plasma, a considerable proportion of the T4 is transformed to T3.

Method. This was one of the first hormones measured by competitive protein-binding assay. On the preparation of the immunogen, it is coupled to bovine serum albumin, and thus some difference may be made between T4 and T3. A monoclonal antibody has also been produced in mouse. The immunization is carried out in rabbit. The cross-reaction of T3 is to be expected, but because of the low plasma concentration (two orders of magnitude less than the T4 level) this may be neglected. ^{125}I -T4 is used as labelled hormone.

Extraction is not necessary before the determination, but the disturbing binding of TBG must be eliminated with 8-anilinonaphtholsulphonic acid (ANS). Incubation lasts a few hours. T4-free serum is used for the calibration curve. The bound and free fractions are separated with a second antibody, with the solid-phase antibody technique, or with polyethylene glycol. The sensitivity of the method is 10-15 ng/ml.

Clinical applications. An elevated T4 level may be encountered in hyperthyroidism, possibly in association with diffuse goitre, multinodular struma and toxic adenoma. A low T4 level is found in hypothyroidism of various origins (idiopathic thyroiditis, treated hyperthyroidism, congenital hypopituitarism). The normal level is 45-120 ng/ml (60-150 nmol/l); in hypothyroidism it is 5-50 ng/ml (6-65 nmol/l); and in hyperthyroidism it is 120-460 ng/ml (150-580 nmol/l).

There is only a slight diurnal fluctuation, and the T4 level is essentially unchanged in response to stress. At room temperature, it can be stored for days without decomposing. When the results are evaluated, attention must be paid to a possible TBG level change. The TBG level may be increased by certain clinical conditions (pregnancy, cirrhosis, hepatitis) or pharmacons (oestrogens, oral anticoncipients), which leads to an elevated T4 value without the signs of hyperthyroidism being observed in the patient.

TRIIODOTHYRONINE (T3)

Chemical structure. T3 contains three iodine atoms. Its structure is to be seen in Fig. 7. The molecular weight is 651 dalton. It binds to TBG in a similar manner to T4.

Physiological conditions. Its biological action is the same as that of T4, but it is four times as effective. It is formed to a lesser extent in the epithelial cells of the thyroid acinus, but to a larger extent it originates from the deiodination of T4; accordingly, some authors consider T4 to be only a prohormone. 99.9% of it circulates in a form bound to protein. Its biological half-life is two days.

Method. The specific antiserum is prepared in rabbit, by means of the conjugate of T3 bound to bovine serum albumin. A cross-reaction is to be expected with T4 (the T4 binding is 80-100 times greater). ^{125}I -T3 is used as labelled hormone. Without extraction, the serum is incubated for 1-3 h. The bound and free fractions are separated with dextran-coated charcoal, solid-phase antibody, a second antibody or polyethylene glycol. TBG interference is eliminated with ANS.

Clinical applications. A high T3 level may be measured even in the early phases of hyperthyroidism. In the event of a thyroid hyperfunction, the T3 concentration is generally enhanced to a greater extent than that of T4, and accordingly the T3/T4 ratio increases. This is of considerable importance in the diagnosis of T3 hyperthyroidism, which occurs in about 5% of hyperthyroidism cases. There are only sparse clinical symptoms here, and no other laboratory alterations are found. The disease is more frequent in iodine-deficient areas. A decreased T3 level may be observed in hypothyroidism and in acute febrile conditions. The normal level is 0.8-2.0 ng/ml (1.2-3.1 nmol/l). The sample may be stored at room temperature for 24 h. Here, too any change in the TBG level must be taken into consideration.

Binding capacity. The thyroid hormones circulate in the bound state. The concentration of the binding protein influences the thyroid hormon level. The T3-binding capacity is determined indirectly. ^{125}I -labelled T is used for the procedure (T3 has a lower affinity for the binding protein). The labelled T3 is taken in a medium containing resin + the blood plasma to be examined. If much T3 binds to the protein, little remains for the resin, and *vice versa*. The ratio of the two may be utilized to calculate the T3-binding capacity, which is normally 35-45%. Care must be taken to ensure the appropriate

temperature and incubation time. From the total T4 level and the T3-binding capacity, the free T4 index may be calculated (this is a number characterizing the free T level). However, recent data suggest that the T4 index is not a reliable parameter.

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Chapter 4

GROWTH HORMONE (SOMATOTROPIN); HUMAN GROWTH HORMONE (hGH)

Chemical structure. This is a species-specific hormone containing a single chain of 191 amino acids. As concerns its structure, it is somewhat similar to prolactin. Its molecular weight is 21,500 dalton. The essentially larger "big" hGH, with a molecular weight of 40,000-45,000 dalton, can be regarded as a prohormone from which hGH is formed.

Physiological conditions. It enhances growth, lipolysis, and glycogen storage in the liver. In the skeletal muscles, it exerts an insulin antagonist effect. It stimulates protein synthesis in the liver and the kidney, and it stimulates the growth of the epiphyseal disks **via** somatomedin. It is produced in special cells of the adenohypophysis (in the older nomenclature: acidophilic cells). In the serum, its basal level is extremely low. Stimulation tests are used to measure the hGH reserve. hGH secretion is enhanced by fasting, physical activity, amino acid infusion, hypoglycaemia, stress and sleep. The hGH level is depressed by hyperglycaemia (glucose loading) and certain tranquillants (barbiturate, chlorpromazine). From a regulatory aspect, the hypothalamic control is decisive. Growth hormone releasing hormone (GH-RH, somatoliberin), containing 44 amino acids, was recently isolated. This stimulates growth hormone secretion. Somatostatin, containing 14 amino acids, has been known for a longer time. It inhibits hGH secretion. hGH circulates free in the plasma. Its half-life is 30 min. Its decomposition takes place in the liver, and it is not excreted in the urine.

Method. It is itself a good immunogen. High-purity hGH is administered to guinea-pig, goat or rabbit. It usually gives a cross-reaction with hPL, but this is of significance only in pregnancy. Monoclonal antibodies have also been prepared. ¹²⁵I-labelling is employed. High-purity hGH from the WHO or the National Pituitary Agency is used as standard. hGH-free plasma is required for preparation of the calibration curve. The incubation takes only 3-24 h. The bound and free fractions are separated with a second antibody technique or by means of polyethylene glycol. The sensitivity is 0.1-1.0 ng/ml.

Clinical applications. A decreased hGH secretion is found in isolated growth hormone insufficiency, and in hypopituitarism extending to several

hormones. The early diagnosis of pituitary dwarfism is of great importance. If treatment with hGH is begun at the age of 3-4 years, normal height may be attained. Hypopituitarism may be induced by a tumour (craniopharyngeoma, chromophobic adenoma), histiocytosis, Sheehan's syndrome and surgical hypophysectomy. We speak of secondary hypopituitarism in the event of a severe mental crisis or in states of depression. Psychosocial low growth is recognized; a certain hGH deficiency can be demonstrated in this state. Decreased hGH secretion has also been observed in other endocrine diseases, such as hyper- and hypothyroidism. Acidophilic adenoma of the pituitary gives rise to acromegaly in adults, and to gigantism before pubescence. In both conditions the basal hGH level is elevated; the tumour generally displays automatism. A high hGH level has been described in diabetic ketosis, in absorption disturbances, in protein deficiency, in African pygmies (peripheral resistance), in some cases of anorexia nervosa, in Laron dwarfs (disturbance of somatomedin formation), and in hypothalamic tumours. Ectopic hGH production has been reported in a pulmonary tumour.

If a deficiency of the hormone is suspected, the basal hGH level is not informative. Stimulation is performed: the hGH level is elevated by insulin, arginine, L-dopa, apomorphine, clonidine, physical activity, and sleep for one hour.

PROVOCATIVE TESTS

Insulin hypoglycaemia. We consider this to be the most reliable (80-90%) procedure. After the i.v. injection of 0.1 IU insulin/kg body weight, the glucose level is recorded (a decrease of at least 50% must be attained) and blood samples are taken for hGH determination. Thirty min after the administration of insulin, the serum hGH level is significantly elevated; if there is no hGH reserve, this elevation does not take place. In order to avoid hypoglycaemic coma, it is advisable to maintain an infusion for 90 min and, if necessary, to administer glucose **via** the infusion.

Arginine loading. Within 30 min following the i.v. infusion of 0.5 g/kg arginine-HCl, the serum hGH level is elevated. The patients tolerate arginine better than insulin hypoglycaemia.

L-Dopa test. 0.5 g/kg is given orally. The growth hormone level in the

serum 45-90 min after L-dopa administration is increased to 2-3 times the initial level. No such increase occurs in cases of pituitary dwarfism.

Combined tests are also carried out, with insulin + argine, or with insulin + L-dopa. The view is generally accepted that an hGH deficiency can be diagnosed only on the basis of agreeing results to several stimulation tests.

An inhibitory test may also be performed in acromegaly. After the oral administration of 50 g glucose, the plasma hGH level normally decreases, whereas it frequently increases in acromegaly. The normal range is 1-10 ng/ml (45-454 pmol/l). There is not a marked diurnal rhythm, but hGH secretion is enhanced during sleep. Samples can be kept for several hours at room temperature, and for a long period at -20°C .

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Chapter 5

PITUITARY-GONAD SYSTEM

Here we must consider the gonadotropic hormones (follicle stimulating hormone (FSH), luteinizing hormone (LH)), sex steroids (oestradiol, progesterone, testosterone) and prolactin (PRL).

The regulation proceeds on three levels: the hypothalamus (LH-RH), the pituitary (FSH, LH) and the peripheral gland. The hCG produced by the placenta is a hormone similar to LH. Luteinizing hormone releasing hormone (LH-RH, gonadoliberin) is a peptide consisting of ten amino acids, which passes from the hypothalamus **via** the portal vessels into the pituitary, where it enhances the secretion of LH and FSH. By acting on the ovary and the testis, these latter hormones increase the production of sex steroids and gametogenesis. A complex functional interrelationship can be demonstrated between certain members of the gonad system. In women, their most important activity is the control of the cyclic changes (follicle maturation, ovulation, corpus luteum formation). A part is played in the regulation of these processes by the oestradiol feed-back effect, which is always negative (high oestradiol level and decreased FSH secretion) as concerns FSH. As far as the secretion of LH is concerned, the regulation is not so clear-cut: both a high and a low oestradiol level may be accompanied by a decreased LH secretion, and **vice versa**. For example, the oestradiol and LH levels are both high in the middle of the cycle, and it is then that ovulation occurs. As a consequence of its negative feed-back effect, the high oestradiol concentration later decreases the LH secretion. Progesterone does not have a direct influence on the secretion of FSH and LH, but it enhances the effect of oestradiol. During pregnancy, choriogonadotropic hormone, too (hCG) plays a role in the regulation: it decreases FSH and LH secretion, which is attributed to the pituitary inhibitory effect of hCG. LH-RH does not enhance LH and FSH secretion during pregnancy.

GONADOTROPIC HORMONES

Follicle-stimulating hormone (FSH, follitropin), luteinizing hormone (LH, lutropin)).

Chemical structure. Both hormones are two-chain glycoproteins. The biologically inactive α -chain is identical with the corresponding part of TSH and hCG. The β -subunit, which exhibits weak biological activity, is hormone-specific. The full biological activity is obtained when the two chains (α and β) are connected together. The molecular weight is 26,000-30,000 dalton. The most important carbohydrate residue is the sialic acid. The biological effect depends on the carbohydrate content. The hormones have a considerable species-specificity, for which the α -chain is responsible. In order to obtain specific antiserum, immunization is performed with the β -subunit. If immunization is carried out with the α -chain or with the entire hormone, a cross-reaction is to be expected with FSH, LH, TSH and hCG.

Physiological conditions. In women, FSH induces follicle maturation. FSH takes part together with LH in the induction of ovulation and in the development of the corpus luteum. LH ensures the maintenance of the corpus luteum. During pregnancy, the role of LH is taken over by hCG. In men, FSH promotes spermiogenesis. By acting on the Leydig cells, LH enhances testosterone secretion.

FSH and LH are produced in the anterior lobe of the pituitary. This function was earlier attributed to the basophilic cells. More recently, as a consequence of the application of the electron microscope and immune staining, cells producing gonadotropic hormones and cells producing TSH are differentiated.

The basal levels of FSH and LH depend on the age and on the menstrual cycle. In childhood, the FSH-LH level is low (Figs 8 and 9), in pubescence it increases to the adult level in women, and in the menopause the concentrations of both hormones are substantially increased (FSH more markedly). Such a sharp correlation cannot be demonstrated in men. As concerns the menstrual cycle (Figs 10 and 11), moderately elevated FSH and LH levels can be detected in the follicular phase; in the middle of the cycle (at the time corresponding to ovulation), in one day the LH is increased 5-20-fold, and the FSH 2-3-fold. In men, there is no cyclic variation.

The gonadotropic hormones circulate without protein binding in the plasma. Their biological half-life is 0.5-4 h. They are excreted in the

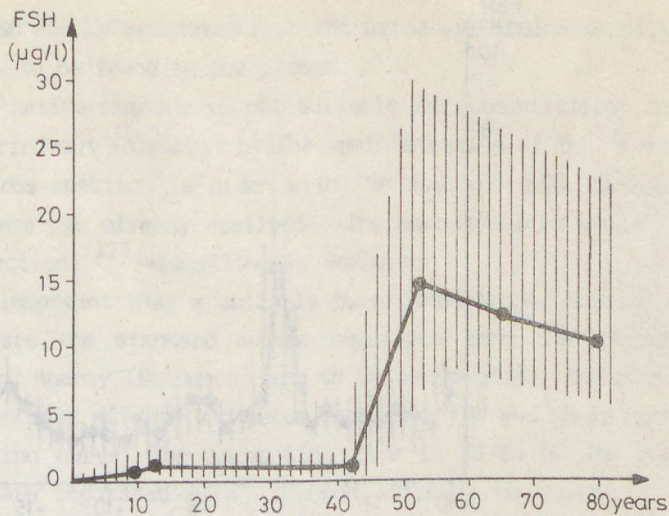


Fig. 8. Change in plasma FSH level in healthy women as a function of age

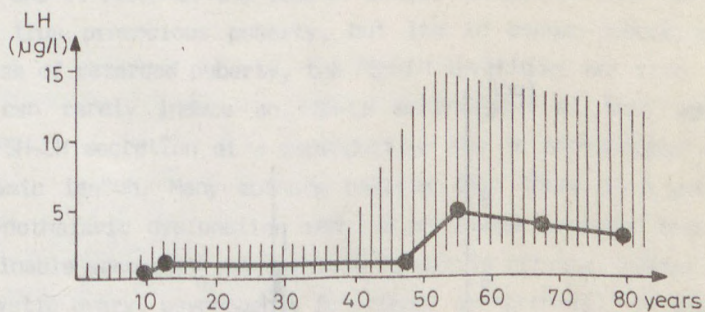


Fig. 9. Change in plasma LH level in healthy women as a function of age

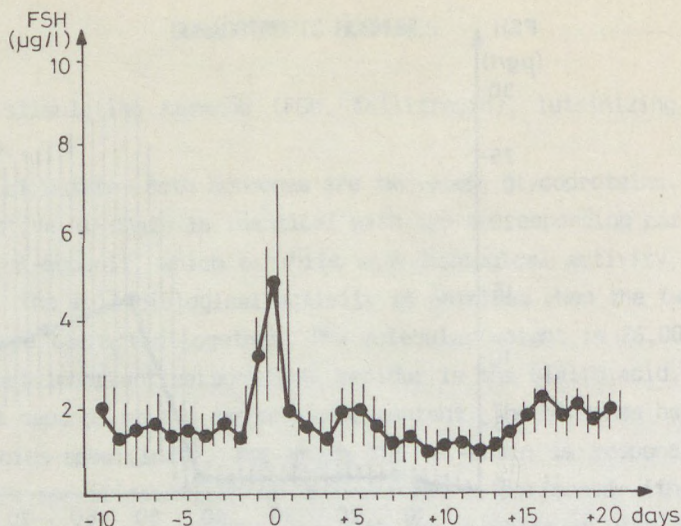


Fig. 10. Change in plasma FSH level during the menstrual cycle. Days counted from ovulation.
(Data from the Department of Gynaecology, Szent-Györgyi Albert University Medical School, Szeged)

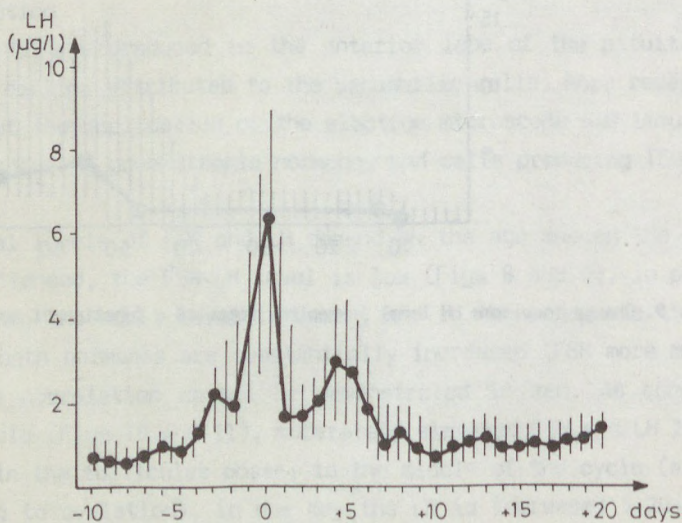


Fig. 11. Change in plasma LH level during the menstrual cycle. Days counted from ovulation.
(Data from the Department of Gynaecology, Szent-Györgyi Albert University Medical School, Szeged)

urine, but the FSH and LH separated from the urine are immunologically different from those to be found in the plasma.

Method. The native hormone is not suitable for immunization. Antibody formation is carried out in rabbit by the administration of the β -subunit. In general, a cross-reaction is given with TSH and with hCG, but specific FSH and LH antisera are already available. The monoclonal LH antiserum has a lower cross-reaction. ^{125}I -labelling is employed.

It is very important that a suitably pure gonadotropic hormone should be used to prepare the standard curve; substance from the WHO and the National Pituitary Agency (Bethesda) are to be recommended. The plasma has only a low non-specific effect, and serum free from FSH and LH is not needed for the calibration curve. The incubation time is 16-24 h. The free and bound fractions are separated with a second antibody technique, a solid-phase antibody technique, or by means of polyethylene glycol. Difficulties may arise because of the preparation of the standard curve, the cross-reactions, and the decomposition of the iodinated hormone.

Clinical applications. From the aspect of the clinical evaluation, primarily the age must be taken into consideration. The FSH-LH level is low before pubescence, and it rises at the time of sexual maturation. The FSH-LH level is high in true precocious puberty, but low in pseudo-precocious puberty. In the case of retarded puberty, the FSH-LH level does not rise. A malignant tumour can rarely induce an FSH-LH enhancement at this age.

A decreased FSH-LH secretion at a reproductive age is encountered in cases of hypothalamic lesion. Many authors believe that there is a connection between hypothalamic dysfunction and the syndromes included among the not easily definable secondary amenorrhoeas (anorexia nervosa, postpill amenorrhoea, polycystic ovary, psychogenic functional amenorrhoea). In these syndromes, the FSH-LH level may be low, normal or even elevated. However, there is one common feature: the customary LH peak in the middle of the cycle is not observed. A decreased FSH-LH level is found in cases of pituitary damage (surgical hypophysectomy, X-ray irradiation, craniopharyngeoma, chromophobic adenoma, metastasis, histiocytosis, Sheehan's syndrome, empty-sella syndrome) and in some extrahypothalamic, extrapituitary diseases (congenital adrenal hyperplasia, infection, non-endocrine tumour, hypo- and hyperthyroidism).

In syndromes in which the FSH-LH level is expected to be low, the gonadotropic reserve capacity of the pituitary must be determined. An i.v. injection of $25\text{ }\mu\text{g}$ synthetic LH-RH (Relisorm, Serono) is administered to our

patients, and the FSH-LH levels are examined after 0, 30 and 60 min. We consider the response to be positive if the hormone level rises by at least 50%. FSH secretion is known to be less easily stimulated by LH-RH than LH secretion.

Enhanced gonadotropic secretion can be observed in normal menopause and in diminished peripheral gland function (Turner's syndrome, Klinefelter's syndrome).

Blood samples may be kept for a short period at room temperature, but they must be stored in a refrigerator for periods in excess of 24 h.

Normal values	FSH	LH
Men:	1-3 ng/ml	1-3 ng/ml
Women: prepuberty	1-3 "	1-3 "
fertility	1-3 "	1-3 "
mid-cycle	2-9 "	5-60 "
menopause	5-29 "	2-8 "

The FSH and LH levels generally vary in parallel, but in certain cases there may be a difference between the concentrations of the two hormones: (i) after castration, the levels of both FSH and LH increase, but the change in the FSH concentration is much more pronounced (5-12-fold); (ii) at the middle of the cycle, the LH peak is the larger; (iii) an LH level increase can be demonstrated in pregnancy; (iv) the secretion of FSH increases in early puberty; (v) in the Stein-Leventhal syndrome, an elevated LH level is often observed while the FSH level remains unchanged or decreases.

SEX STEROIDS

OESTRADIOL

Chemical structure. This is the most important oestrogen. It contains 18 carbon atoms (Fig. 12) and has a molecular weight of 272 dalton. Besides the ovary, the adrenal cortex and the testis possess enzyme systems necessary for oestrogen biosynthesis. In the blood, oestradiol binds to sex

hormone binding globulin (SHBG), and only 30% of it circulates free in biologically active form. It is inactivated in the liver, and is excreted bound as ester (sulphate, glucuronide) in the urine.

Physiological conditions. It is mainly produced by the theca interna granulosa cells in the ovum. It acts primarily on the endometrium, the vagina and the mammary gland. The oestradiol level depends on the stage of the menstrual cycle (Fig. 13): it progressively increases in the phase of follicle maturation, the peak is reached in the days prior to ovulation, it suddenly decreases after ovulation, and in the phase of development of the corpus luteum it again rises (second peak). The oestradiol level is low in childhood and in the menopause.

Method. The antigen necessary for immunization is prepared with albumin coupled to carbon atoms 3, 6, 11, 15 or 17. Specific antiserum cannot be obtained with oestradiol-antigen conjugated on the 3-OH or 17-OH group, but high specificity is ensured by conjugation on carbon atoms 6, 11 and 15. Cross-reactions are given by oestrone and oestriol. Immunization is generally carried out in rabbit. Oestradiol labelled either with ^3H or with ^{125}I is used as labelled hormone. Iodination may be effected after coupling of the side-chain of histamine, tyrosine or tyrosine methyl ester to the sterane

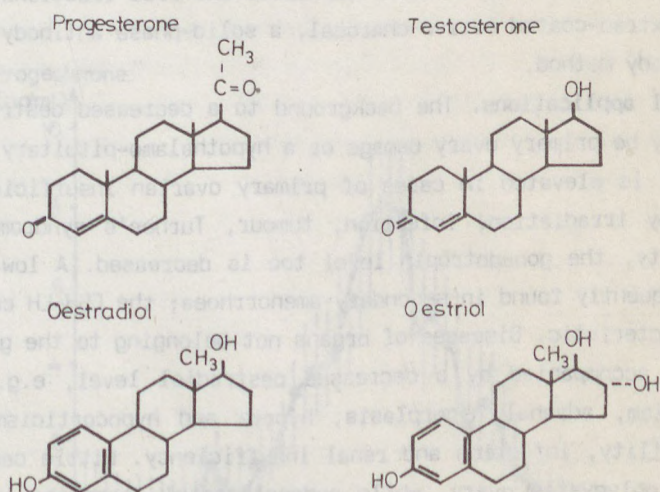


Fig. 12. Chemical structures of progesterone, testosterone, oestradiol and oestriol

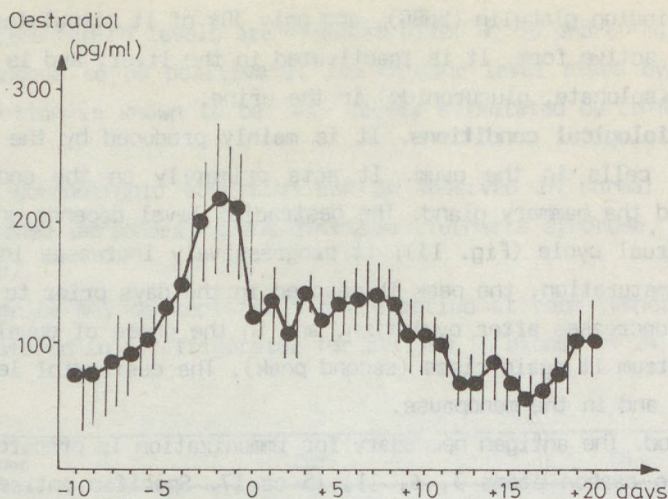


Fig. 13. Change in plasma oestradiol level during the menstrual cycle. Days counted from ovulation. (Data from the Department of Gynaecology, Szent-Györgyi Albert University Medical School, Szeged)

skeleton. Determination is performed after extraction with freshly distilled diethyl ether or chromatography (Celite, Sephadex LH 20, Sep-Pak). The time required for incubation is 1-24 h. The bound and free fractions are separated with dextran-coated active charcoal, a solid-phase antibody method or a second antibody method.

Clinical applications. The background to a decreased oestradiol level in adults may be primary ovary damage or a hypothalamo-pituitary lesion. The FSH-LH level is elevated in cases of primary ovarian insufficiency (castration, X-ray irradiation, infection, tumour, Turner's syndrome). In retarded puberty, the gonadotropin level too is decreased. A low oestradiol level is frequently found in secondary amenorrhoea; the FSH-LH concentration is not characteristic. Diseases of organs not belonging to the gonad system may also be accompanied by a decreased oestradiol level, e.g. hypo- and hyperthyroidism, adrenal hyperplasia, hyper- and hypocorticism, anorexia nervosa, debility, infection and renal insufficiency. Little oestradiol is produced in polycystic ovary, while androstenediol secretion is enhanced.

A pathologically elevated oestradiol secretion can primarily be demonstrated in certain ovarian tumours: granulosa theca cell tumour, arrhenoblastoma, lipid cell tumour (enhanced androgen secretion).

Normal values	pg/ml	pmol/l
Children:	2- 20	7.34- 73.4
Adult men:	10- 60	36.7 - 220
Adult women:		
follicular phase	20-100	73.4 - 367
preovulation	200-600	734 - 2202
luteal phase	100-300	367 - 1101
menopause	5- 50	18.35- 183.5

PROGESTERONE

Chemical structure. This plays an important role in the early stage of steroid synthesis. The formula is given in Fig. 12. The molecular weight is 314 dalton. It is produced mainly in the corpus luteum, but it is also formed in the adrenal cortex and in the testis.

Physiological conditions. Progesterone is bound to transport protein in the blood. Its main biological effects are to develop the endometrium of the uterus, and to promote sticking of the ovum (if there is no fertil-

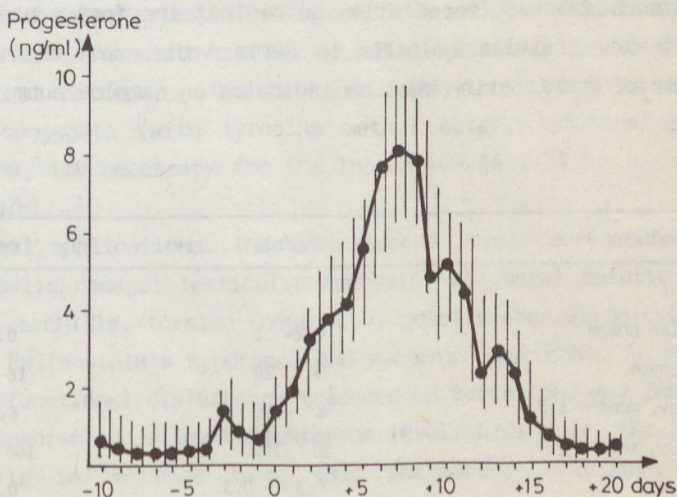


Fig. 14. Change in plasma progesterone level during the menstrual cycle. Days counted from ovulation. (Data from the Department of Gynaecology, Szent-Györgyi Albert University Medical School, Szeged)

ization, the corpus luteum atrophies). The progesterone level is low throughout the follicular phase, following ovulation it rises sharply, and it peaks on the 20th-25th day (Fig. 14). If pregnancy ensues, the progesterone level rises progressively until the end of pregnancy (a level 20 times as high as the postovulation peak may be attained).

Method. Measurements were earlier made after petroleum ether or chromatographic extraction; nowadays, a direct determination is performed. The antigen necessary for immunization is provided by albumin bound to carbon atom 3, 6, 11, 20 or 21. Rabbit, sheep or goat may be used for antibody production. Cross-reactions are given by 17 α -hydroxyprogesterone, pregnenolone and deoxycorticosterone. Monoclonal antibody produced in mouse has also been described. Progesterone labelled with either ^3H or ^{125}I is applied as labelled hormone. The bound and free fractions are separated with dextran-coated active charcoal, a double antibody or a solid-phase antibody. The incubation time is 1-24 h. The sensitivity is 5-20 pg/ml.

Clinical applications. An elevated progesterone level is found in folliculoma, lipoid cell tumour and congenital adrenal hyperplasia. The progesterone concentration is low in all cases involving an impaired corpus luteum function. If ovulation does not occur, the premenstrual progesterone elevation cannot be demonstrated either. The success of ovulation induction may be controlled by measurement of the progesterone level. If the progesterone level falls rapidly during pregnancy, it may be concluded that the placenta has suffered a lesion or that the foetus has died. The progesterone level similarly falls in certain diseases of the adrenal.

The time of menstruation must be indicated on samples submitted to the laboratory.

Normal values	ng/ml	nmol/l
Fertile women:		
follicular phase	0.2- 1	0.6- 3.2
luteal phase	5 - 20	16 - 64
pregnancy, months 1-3	2 - 50	6.4-160
pregnancy, months 3-9	50 -150	160 -480
Men:	0.1- 0.3	0.3- 1.0

TESTOSTERONE

Chemical structure. This is the main androgen. It is similar in structure to progesterone (see Fig. 12). The molecular weight is 288 dalton. Its most important precursor is androstenedione. In the target cells it is transformed to dihydrotestosterone, which is the most effective androgenic compound.

Physiological conditions. Testosterone is responsible for the development of a male appearance. It has a considerable anabolic effect: it enhances nitrogen retention, promotes cell growth, and accelerates ossification. The main site of its production is in the Leydig cells of the testis, but it is also formed in the adrenal cortex and in the ovary. The vast majority of it circulates bound to protein (SHBG). Of the gonadotropic hormones of the pituitary, LH exhibits a stimulatory action on testosterone secretion; in men, this may be referred to as interstitial cell stimulating hormone (ICSH). A certain diurnal rhythm may be observed in testosterone secretion: the testosterone level is higher in the early morning than at other times of the day.

Method. Binding to SHBG was earlier used for the determination (CPBA). More recently, it is bound to bovine serum albumin for purposes of immunization. The binding site is generally carbon atom 3, 7 or 15. Immunization is most effective in rabbit or goat. Cross-reactions are to be expected with dihydrotestosterone, androstenedione and other androgens.

Extraction with diethyl ether or petroleum ether is normally applied, but a direct procedure, too is known. Its tritiated form or its ^{125}I labelled conjugate (with tyrosine methyl ester, tyramine) is used as tracer. The time necessary for the incubation is 1-24 h. The sensitivity is 1-30 pg/ml.

Clinical applications. The testosterone level decreases in cases of primary testis damage: testicular agenesis, bilateral ablation, X-ray irradiation, orchitis, torsion (necrosis), genetic aberration (Klinefelter's syndrome, Reifenstein's syndrome) and myotonic dystrophy. A hypothalamic-pituitary functional disturbance (reduced LH secretion) may induce secondary hypogonadism and a low testosterone level. Similarly, the testosterone level falls in response to certain pharmacons (androgen, oestrogen, progesterone, chlorpromazine, Metopiron). The testosterone concentration is not characteristic in impotence: it depends on what causes the potency disturbance. A high testosterone level is frequently observed in psychic impotence.

An elevated testosterone level is rarely found in men (gonadotropic hormone producing tumour, adrenocortical tumour, idiopathic precocious puberty). An enhanced testosterone formation in women leads to androgenization, the dominant sign of which is hirsutism. It occurs in Cushing's disease, adrenocortical tumour, congenital adrenocortical enzyme defect, testicular feminization, ovarian tumours (arrhenoblastoma, lipoid cell tumour) and polycystic ovary syndrome. In idiopathic hirsutism the plasma testosterone level is normal, but the androgen-forming enzyme system of the skin is hyperactive.

Normal values	ng/ml	nmol/l
Men:	2.5-8.5	8.75-29.8
Before puberty:	< 0.5	< 1.75
Women:	0.2-0.8	0.69- 2.8

PROLACTIN (PRL)

Chemical structure. The exact amino acid composition of human PRL is not known. It contains about 190 amino acids and has a molecular weight of about 22,000 dalton. Its structure is similar to those of hGH and hPL.

Physiological conditions. Its main biological effect is the stimulation and maintenance of lactation. It has only a limited direct influence on the mammary gland. The gland is stimulated to lactate by oestrogen and progesterone; PRL plays a role in the initiation of lactation. The significance of PRL in men is unknown. It is produced and stored by the adenohypophysis. The hypothalamus is the most important regulator of PRL; hormone secretion is decreased by PRL inhibitor factor (PIF). It is assumed that PIF is identical with dopamine. PRL secretion is enhanced during breast-feeding and as a consequence of the mechanical stimulation of the breasts. PRL displays a diurnal rhythm: the peak level (twice the basal level) is observed 2-3 h before waking, and the PRL concentration is lowest at midnight. In early pregnancy the diurnal rhythm is retained, but later the PRL level progressively increases; at the end of pregnancy, it may be ten times the initial level. Following delivery, there is at first no difference between the PRL levels of lactating and non-lactating women, but later PRL level remains high only in breast-feeding mothers.

PRL secretion is stimulated by various pharmacons: oestrogens (oral contraceptives), insulin, amino acids, reserpine, α -methyldopa, phenothiazine, amphetamine, heroin and antidepressants. The PRL reserve capacity is measured by means of TRH; the procedure is the same as in the examination of TSH secretion (see p. 48). The peak in the PRL level increase is to be observed 20-30 min following administration. The PRL concentration may decrease 30-90 min after treatment with L-dopa (0.5 g) or apomorphine. Bromocriptine (2 α -bromoergocriptine, Parlodel, Sandoz) not only inhibits PRL secretion as an acute effect, but is also suitable for long-term therapy.

Method. PRL itself is a good immunogen; the problem is its purification. The antibody is produced in rabbit, but a monoclonal antibody too has been produced in mouse. An antiserum specific to hPRL has recently been produced against the 1-13 C-terminal fragment; the fragment is preliminarily bound to bovine serum albumin. ¹²⁵I-PRL is used as labelled hormone. It can be kept only with difficulty, and needs purifying from time to time. The greatest difficulty is caused by isolation of the PRL necessary for the standard (National Health Institute, Bethesda). The time required for incubation is 5-24 h. The bound and free fractions are separated with a second antibody or with a solid-phase antibody technique.

Clinical applications. An enhanced secretion of PRL occurs in non-endocrine tumours (lung, kidney), hypothalamus disturbances and pituitary tumours (30% of pituitary tumours are prolactinomas). The PRL level is high in 20-40% of acromegalic patients. The pharmacons mentioned above must be taken into consideration in the course of differential diagnoses. Hyperprolactinaemia frequently accompanies fertility disturbances and menstrual abnormalities; there is an elevated PRL level in about 15% of secondary amenorrhoeas. Chronic hyperprolactinaemia leads to galactorrhoea-amenorrhoea syndrome and the development of prolactinoma. The patients can be cured with bromocriptine, or possibly surgically. Hypoprolactinaemia is rarely observed: such a phenomenon has been described in total pituitary destruction and in empty sella syndrome. The basal PRL concentration in women is 3.5-20 ng/ml (158-900 pmol/l) (between 10 a.m. and midnight), and in men is 2.0-10 ng/ml (90-450 pmol/l). Stimulation is performed with TRH.

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Chapter 6

PLACENTA AND FOETUS

This section is devoted to human chorionic gonadotropin (hCG), human placental lactogen (hPL) and oestriol.

A good reflection of the state of the foetus and of the placenta is given by the levels of the hormones in the blood; accordingly, study of these hormones permits conclusions as to the condition of the foetus. The concentrations of the placental hormones depend on the following factors: (i) the functional state of the hormone-producing organs, (ii) the size of the hormone-producing organs, and (iii) the possibility that the substance may cross the placental barrier.

HUMAN CHORIONIC GONADOTROPIN (hCG)

Chemical structure. This is a two-chain glycoprotein. Structurally, it resembles FSH, LH and TSH. The α -chain component is the same in these four hormones. The β -chain in hCG is larger than in the other hormones; it contains about 145 amino acids. Its molecular weight is 37,000-59,000 dalton. Presence of the carbohydrate group is indispensable for the biological effect. The carbohydrate content is about 30%. The structure of hCG is closest to that of LH, with which there is therefore a cross-reaction.

Physiological conditions. hCG maintains the corpus luteum function in early pregnancy; additionally, a trophic effect has been observed as concerns the ovary and the adrenal. It is produced by the trophoblasts of the placenta. Its concentration is 400-800 times higher in the maternal serum than in the foetus. It has a long biological half-life: 5 h in the fast phase, and 24 h in the slow phase. The hCG level increases exponentially from the 9th-10th day of fertilization (Fig. 15). It decreases slightly from the 10th week, but a high level can be measured in the maternal plasma up to the end of pregnancy, and even for a further month after the delivery.

Method. Immunization is performed in rabbit, through administration of the β -subunit. A cross-reaction is to be expected with FSH, LH and

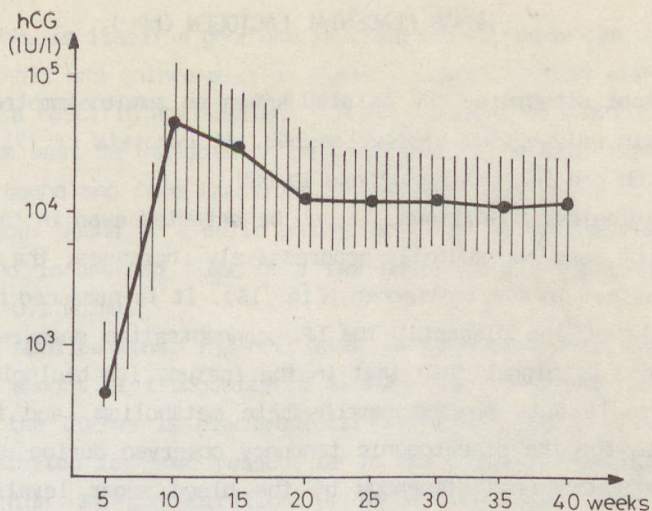


Fig. 15. Variation of plasma hCG level during pregnancy

TSH, but a specific antiserum, too is available. ^{125}I -hCG is used as labelled hormone. The calibration curve must be prepared with highly-purified hCG (WHO standard). Incubation procedures lasting from 30 min to 12 h are known. The bound and free fractions may be separated by application of a second antibody or a solid-phase antibody. The sensitivity is 0.5-5 mU/ml. Measurements may be made from the 9th day of pregnancy.

Clinical applications. hCG can hardly be detected in non-pregnant women (< 5 IU/l). Its level is high in early pregnancy (from the 9th day), in hCG-producing tumours (teratoma, dysgerminoma, chorioepithelioma), and in hydatidiform mole (25-30 IU/l). A high hCG level persisting beyond one month after the delivery raises the suspicion of mole. Choriocarcinoma occurs 1000 times more frequently in hydratidiform mole. A low hCG concentration during pregnancy is indicative of ectopic pregnancy, or it may be a delayed sign of the interruption of pregnancy. It displays a fairly high stability in the serum, and can also be demonstrated in the urine.

In clinical practice, it is considered advantageous to determine hCG in the urine; the hormone is excreted in the urine, from which it can readily be extracted, and thus hydatidiform mole or choriocarcinoma can be diagnosed much sooner.

HUMAN PLACENTAL LACTOGEN (hPL)

Chemical structure. hPL is also known as somatomammotropin. It is a single-chain polypeptide similar to hGH. It consists of 191 amino acids, 161 of which are identical to those in hGH.

Physiological conditions. It can be detected even in the early phase of pregnancy, and subsequently progressively increases; the hPL level attains a maximum in the last month (Fig. 16). It is produced by the trophoblast cells of the placenta. The hPL concentration measured in the maternal blood is higher than that in the foetus. Its biological half-life is 20 min. It acts on the carbohydrate metabolism, and is considered responsible for the diabetogenic tendency observed during pregnancy. The hPL concentration is influenced by the blood sugar level: in hyperglycaemia the hPL level decreases, while in hypoglycaemia it rises. Similarly to hGH, hPL acts on the protein metabolism: it enhances the storage of nitrogen, and increases the amino acid transport across the placenta.

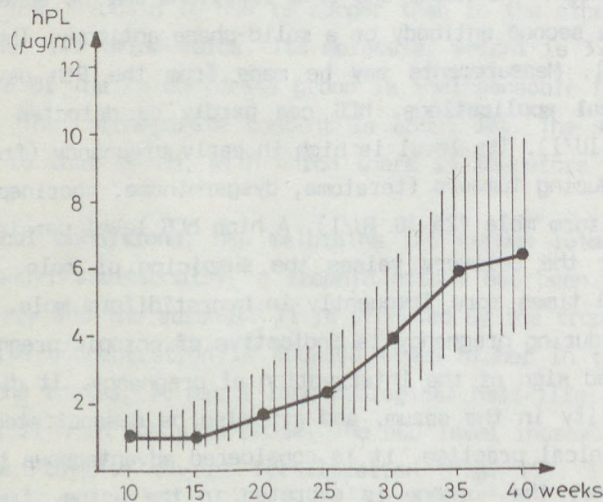


Fig. 16. Variation of plasma hPL level during pregnancy

Method. hPL is itself a good antigen. An immune serum can be produced with it in rabbit and guinea-pig; it gives a cross-reaction with hGH, but there is also a specific anti-serum. ¹²⁵I-hPL is used as labelled hormone. hPL-free serum must be utilized to prepare the calibration curve. Separation of the bound and free fractions is possible with a second antibody, with an antibody bound to a solid phase, or with the polyethylene glycol technique. The incubation time is a few hours. The sensitivity of the method is 0.1-0.2 ng/ml.

Clinical applications. The hPL level is connected with the function of the total amount of trophoblastic tissue. The hPL level falls during pregnancy if the uterus is evacuated, if the intrauterine growth of the foetus is inhibited for some reason, or in the event of placental insufficiency. A high hPL concentration is found in multiple pregnancy, in diabetic pregnant women, in cases of Rh incompatibility, and in trophoblastic tumours (high level). Under normal conditions, it can be detected only in traces in non-pregnant women. In the final week of pregnancy, the hPL concentration is above 4 μ g/ml; a lower level raises the suspicion of placental insufficiency.

OESTRIOL

Chemical structure. Oestriol differs from oestradiol in that there is an OH group at position 16, in addition to those on carbon atoms 3 and 17 (see Fig. 12). Its molecular weight is 288 dalton. Oestriol is produced by the placenta; in non-pregnant women, it occurs only as a metabolite of oestradiol.

Physiological conditions. During pregnancy it enhances protein synthesis and the growth of the uterus and placenta, and it exerts an effect on the connective tissue, thereby facilitating the delivery. The oestriol level progressively increases from the 7th-8th week of pregnancy (Fig. 17). Oestriol is to be found in both conjugated and non-conjugated forms in the blood, whereas it can be detected only in conjugated form (sulphate, glucuronide) in the urine. The diurnal fluctuation is slight; the oestriol level is low in the afternoon.

Method. The oestriol must be liberated from the sulphate and glucuronide conjugates by means of hydrolysis. Bovine serum albumin coupled to carbon atom 3 or 6 is used for immunization, generally in rabbit.

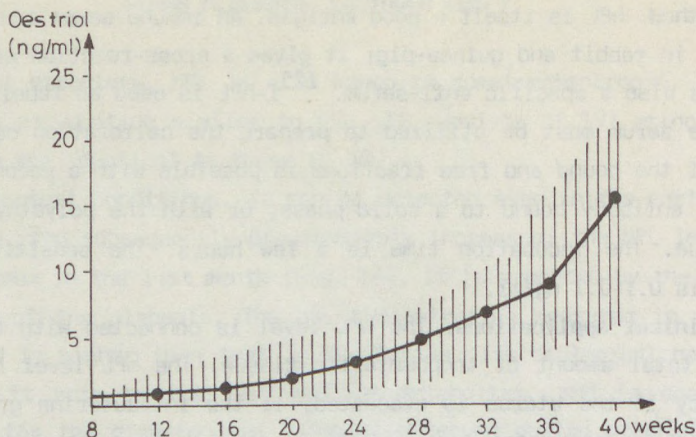


Fig. 17. Variation of plasma oestriol level during pregnancy

Oestradiol and oestrone may give cross-reactions. Tritiated or ^{125}I labelled group containing oestriol is applicable as labelled hormone. A short incubation of 1-3 h is needed. The bound and free fractions may be separated with dextran-coated charcoal, antibody bound to a solid phase, or polyethylene glycol. The sensitivity is $5 \mu\text{mol/ml}$.

Clinical applications. The variations in the oestriol level permit conclusions as to the function of the foetus and the placenta. The range of normal values during pregnancy is 7-25 ng/ml (24.5-87.5 nmol/l). A higher level than this is measured in the case of a large foetus. A lower concentration is to be found in pre-eclampsia and in retardation of the foetus, Rh incompatibility, placental insufficiency, maternal diabetes, hydatidiform mole and during loss of the foetus.

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Chapter 7

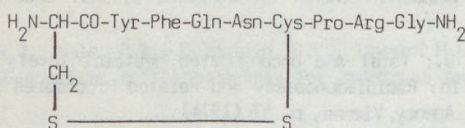
POSTERIOR PITUITARY HORMONES

The hormones to be considered here are vasopressin, oxytocin and neurophysin.

VASOPRESSIN

Chemical composition. This peptide consists of 9 amino acids (Fig. 18). In the majority of mammals (man, rat, horse, sheep, dog and monkey), arginine is to be found at position 8; this gives rise to the name arginine-vasopressin. The molecular weight is 1,084 dalton.

Vasopressin



Oxytocin

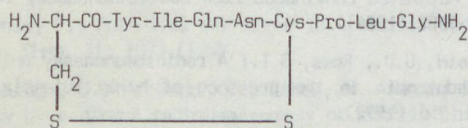


Fig. 18. Chemical structures of arginine-vasopressin and oxytocin

Physiological conditions. Its main effect is enhancement of the reabsorption of water in the epithelial cells of the distal tubule in the kidney; it is therefore also known as antidiuretic hormone. It increases the blood pressure, enhances the smooth muscle contraction, promotes sodium excretion, stimulates the secretion of ACTH, and also influences the memory function of the cerebral cortex. The plasma vasopressin level varies, depending on the water intake; in the event of normal hydration it is

1-3 pg/ml. It is produced in the magnocellular portion of the hypothalamus, and is stored in the posterior lobe of the pituitary. It is bound to a specific blood protein (neurophysin II), but can also be detected in free state in the plasma. It is rapidly decomposed by the aminopeptidase to be found in the blood. Its biological half-life in man is 15-20 min. Tissue inactivation is performed by the kidney and the liver. The most important factor as concerns regulation of its secretion is the osmotic condition in the plasma: hyperosmosis (hypertonic NaCl solution, thirsting) gives rise to a vasopressin outflow through osmoreceptors in the anterior hypothalamus, while hyposmosis (water intake) suppresses vasopressin secretion. An exception is a decrease in volume of the plasma, which (in spite of the hyposmosis) is a significant stimulus for vasopressin secretion. Nervous effects (pain, emotion) and certain chemical substances (histamine, nicotine, acetylcholine, morphine, barbiturate, ferritin, bradykinin, β -endorphin) also enhance the concentration of vasopressin in the blood.

Method. The antigen necessary for immunization is produced by coupling arginine-vasopressin to thyroglobulin. Immunization is usually carried out in rabbit or goat. Cross-reactions are to be expected with lysine-vasopressin, vasotocin and oxytocin. Vasopressin is labelled with ^{125}I ; the labelled hormone is purified by chromatography on a Sephadex G-25 column or by HPLC. Before RIA, the vasopressin is extracted from the plasma samples with thermally activated glass powder, from which it may be eluted with 60% aqueous acetone. Recently the extraction is performed by mini-column chromatography. The extraction and the RIA are performed at $+4^{\circ}\text{C}$. The incubation lasts 24-48 h. The bound and free hormone fractions are separated with dextran-coated charcoal or with polyethylene glycol.

Clinical applications. A low vasopressin level is measured in central diabetes insipidus (organic damage to the hypothalamus or the pituitary stalk) and in psychological polydipsia (hyperhydration arises as a consequence of the enhanced water intake following the central stimulus of thirsting). Plasma vasopressin levels of 0.5-2.6 pg/ml have been reported for healthy subjects (0.54-2.81 fmol/ml). More information is provided by the concentration change observed in the course of loading tests than by the basal vasopressin level. The vasopressin reserve may be measured with the following tests:

1. **Thirsting test.** The vasopressin level is determined after thirsting which has lasted 6-8 h. The vasopressin level rises above 10 pg/ml if there is a normal reserve; it is unchanged in cases of diabetes insipidus.

2. **Hypertonic (2.5%) NaCl infusion test.** After appropriate prehydration (20 ml water/kg body weight, orally), a hypertonic 2.5% NaCl intravenous infusion is administered (0.10 ml/kg body weight/min for 120 min), and measurements are made of the quantity and specific gravity of the urine at 15 min intervals, and of the plasma vasopressin level at 30 min intervals. A normal vasopressin reserve is indicated by a decrease in the urine fractions, and by increases in the specific gravity and the plasma vasopressin level.

3. **Histamine loading.** The vasopressin level is increased 2-5-fold 3, 10 and 20 min after the subcutaneous administration of 0.5 mg histamine (Peremin, Chinoin). The absence of such a response is indicative of an osmoreceptor defect.

An elevated vasopressin concentration is found in hyperaduretinism (Schwartz-Bartter syndrome), which develops in paraneoplastic endocrine syndrome (ectopic vasopressin formation in tumours of the lung, duodenum, pancreas, thyroid or thymus), in the case of pathological or physiological circulating volume stimulation (pneumonia or a postcommisurotomy state), in cerebral disturbances (meningitis, encephalitis, skull trauma, haemorrhage, abscess or brain tumour), and in the action of pharmacons (clofibrate, carbamazepine or chlorpropamide). Diagnosis of the syndrome, which involves hyponatraemia, hyponatriuria and cerebral oedema, is extremely important. It happens infrequently that the vasopressin level is not elevated in the Schwartz-Bartter syndrome. In this case, water loading is carried out; in response to this, the plasma vasopressin level does not fall if the patient has the Schwartz-Bartter syndrome.

OXYTOCIN

Chemical structure. This was the first polypeptide for which the structure was elucidated. Its chemical composition resembles that of vasopressin (see Fig. 18); there are differences in only two amino acids: isoleucine is present instead of phenylalanine at position 3, and leucine instead of arginine at position 8. Its molecular weight is 1,008 dalton.

Physiological conditions. Oxytocin is produced in the magnocellular portion of the hypothalamus (nucleus paraventricularis). It stimulates contraction of the uterine musculature, and therefore plays an important role in the mechanism of delivery and in the contraction of the uterus

following delivery. It enhances the contraction of the smooth muscle cells of the mammary gland, and thereby promotes the outflow of milk. In the circulation, it is to be found in part in a form bound to a specific binding protein (neurophysin I), and in part in a monomeric form not bound to protein, which can be dialysed. Its half-life is very short (less than 5 min). Its decomposition begins in the plasma: a proteolytic enzyme, cystine aminopeptidase, cleaves off the amide group and opens the ring, the hormone thereby losing its biological activity. The kidney and the liver may also play a role in the break-down.

Method. The antigen necessary for the immunization is prepared by coupling oxytocin to thyroglobulin or to bovine serum albumin, similarly as in the case of arginine-vasopressin. The animal most widely used for immunization is the New Zealand white rabbit. The radioisotope used to label oxytocin is ^{125}I . The labelled hormone is purified by high-pressure liquid chromatography (HPLC) or on a Sephadex G-25 or DEAE-Sephadex A-25 column. Before RIA, the oxytocin is extracted from the plasma samples by means of minicolumn chromatography or thermally activated glass powder, fuller's earth or acetone. Certain authors apply direct methods, mainly to samples taken in the course of pregnancy or during delivery. The bound and free hormone fractions are separated with dextran-coated charcoal, precipitation with a saturated solution of ammonium sulphate, or a second antibody. The course of the assay is the following: After preincubation with cold hormone, the labelled hormone is added to the system, a further incubation follows, and the bound and free fractions are then separated. The fasting plasma level is $1.5 \mu\text{U/ml}$ (2.86 fmol/ml) in healthy men and $1.4 \mu\text{U/ml}$ (2.67 fmol/ml) in healthy women. Literary data agree as to the basal values, but those reported in pregnancy and during delivery differ.

Clinical applications. The oxytocin level increases in response to breast-feeding. Oxytocin may also be produced by non-endocrine tumours. After destruction of the posterior lobe of the pituitary (diabetes insipidus), the patient does not possess an oxytocin reserve, but even in this case the delivery generally proceeds spontaneously. In obstetrics oxytocin is used only for enhancing contraction of the uterine musculature in weak labour.

NEUROPHYSIN

Chemical structure. The term neurophysin refers to a group of proteins displaying structural similarities. These proteins have been isolated from the neurohypophysis of codfish and various mammals, e.g. sheep, cow, pig, rat, guinea-pig and man. In all species examined so far, including man, at least two forms of neurophysins have been identified: neurophysin I (oxytocin-neurophysin or oestrogen-stimulated neurophysin), and neurophysin II (vasopressin-neurophysin or nicotine-stimulated neurophysin). These differ from each other in physico-chemical properties, in amino acid compositions and in antigenicities. The molecular weight of the neurophysin monomer is about 10,000 dalton. To date, the amino acid sequences of neurophysins from 9 different mammals have been totally or partly established. Bovine neurophysin consists of 96 amino acids. It is rich in cysteine and in disulphide bonds. The amino acid composition of the peptide part between the amino acids in positions 10 and 70 is fairly constant in the different species. In contrast, the N-terminal 1-9 peptide part and the C-terminal 75-96 peptide part exhibit very appreciable structural differences from species to species. As concerns the molecular part between amino acids 38 and 57, it is assumed that this contains ionic bonding for the posterior pituitary hormones.

Physiological conditions. The primary biological function of the neurophysins is the transport of oxytocin and vasopressin in the hypothalamo-posterior pituitary system. Neurophysins are synthesized together with vasopressin and oxytocin in the perikarya of the magnocellular neurons. Brownstein and co-workers named the common precursor propressophysin or prooxyphysin. The neurophysin bound to the corresponding nonapeptide is enclosed in the vesicles and migrates to the axon terminal. Neurophysins leave the neuron together with nonapeptides but they circulate isolated from them in the plasma. The various neurophysins are secreted into the plasma independently of each other, and the factors regulating their secretion also differ. The plasma level of vasopressin-neurophysin (neurophysin II) is increased considerably by administering nicotine, as is the secretion of oxytocin-neurophysin (neurophysin I) by administering oestrogen.

Method. With RIA, neurophysins can be measured in the plasma. A number of RIA methods have been developed for their measurement but, because of the close structural similarity between the various neurophysins, it is

difficult to prepare an antibody which is specific for only one neurophysin. The antisera produced in the various laboratories differ from one another in specificities. Robinson and colleagues succeeded in producing specific antisera against human neurophysin I and II, which makes possible to measure the two neurophysins separately.

The immune serum against neurophysin is produced in rabbit.

The neurophysin is labelled with radioactive iodine by the chloramine T method. The iodinated product is purified on Sephadex G-25 and G-75 columns. If stored at 4°C, the diluted tracer is stable for three months.

The RIA of the neurophysins is performed on a 50-100 μ l plasma sample without preliminary extraction of the hormone. A non-equilibrium system is applied in the course of the procedure. The plasma samples are incubated for two days at room temperature with diluted antiserum in assay buffer (0.5% BSA in 0.1 M barbiturate buffer, pH 8.6), then tracer is added (about 2,000 cpm/assay tube), and incubation is continued for a further five days. The bound and free hormone fractions are separated by means of zirconyl phosphate. With a view to control of the aspecificity, neurophysin-free plasma (treated with charcoal) with the same volume as that of the unknown plasma samples is added at every point of the calibration curve. The sensitivity of the neurophysin RIA is 0.5 ng/ml.

Clinical applications. The plasma concentration of neurophysin I is 1.1 ± 0.7 ng/ml ($n = 320$) in healthy women, and 1.0 ± 0.7 ng/ml ($n = 107$) in healthy men. The administration of oestrogen elevates the plasma neurophysin I level in women to 7.0 ± 5.2 ng/ml ($n = 136$). At the end of the first trimester of pregnancy it rises to 7.2 ng/ml, and remains at this level throughout. At delivery, its concentration has been reported to be 9.1 ng/ml. The plasma neurophysin II level is 0.9 ± 0.3 ng/ml in healthy women and 0.6 ± 0.3 ng/ml in healthy men. Oestrogen does not influence the plasma concentration of neurophysin II, but nicotine may rise it more than 6 times.

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Chapter 8

HYPOTHALAMIC HORMONES

The substances that will be dealt with here are thyrotropin releasing hormone (TRH), luteinizing hormone releasing hormone (LH-RH), corticotropin releasing factor (CRF), growth hormone releasing factor (GRF) and somatostatin (SRIF).

THYROTROPIN RELEASING HORMONE (TRH, THYROLIBERIN)

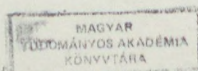
Chemical structure. TRH was isolated and characterized by two independent research teams in 1966, from porcine and bovine hypothalamus. This was the first releasing hormone for which the chemical structure was elucidated. TRH consists of three amino acids: pyroGlu-His-Pro-NH₂. Its molecular weight is 363 dalton. TRH received its name from its effect, causing the release of TSH; it was later demonstrated that it is similarly active in inducing prolactin secretion.

Physiological conditions. TRH is to be found predominantly in the central nervous system, but its extraneural occurrence is also known (pancreas, gastrointestinal tract, gonads). TRH immunoreactivity has been demonstrated in urine, too. A proportion of the immunoreactivity that can be detected by means of RIA in the extraneural tissues is not due to the natural hormone. 30% of the TRH activity in the central nervous system is to be found in the hypothalamus: primarily in the median eminence, the pituitary stalk, the nucleus posterior and the anterior hypothalamus. 70% of the neural TRH activity is to be found outside the hypothalamus: in the cortex, the motor nuclei, the medulla oblongata, the spinal cord, and even an appreciable amount in the retina. The bulk of the hypothalamic TRH is synthesized in the hypothalamus itself, while a small proportion is of peripheral origin: the TRH content of the hypothalamus is decreased by deafferentation.

In the organism, TRH probably has endocrine (thyrotropic), neurocrine (neurotransmitter) and paracrine effects. Its stimulation of TSH secretion has been demonstrated to be of physiological significance, but

it is highly likely that it also plays a role in the enhancement of PRL secretion during breast-feeding. TRH reaches the thyrotropic cells of the pituitary **via** the portal circulation, but certain studies have indicated that some of the adenohypophyseal TRH are synthesized in the anterior lobe itself. TRH is bound to the cell membrane receptors of the thyrotropic cells, and its effect is exerted **via** the activity of adenylcyclase. Physiologically, its action on the thyrotropic cells is blocked by dopamine and somatostatin. A short-loop feed-back probably exists as concerns TSH-TRH; the thyroid hormones primarily act at the pituitary level, and their effect on TRH has not been demonstrated. In the TSH-TRH short-loop feed-back mechanism, modulatory roles may be played by growth hormone, cortisol and the sex steroids. TRH-positive nerve fibres terminate in the posterior lobe of the pituitary too, and it is not excluded that TRH plays a part physiologically in the secretion of vasopressin. The regulation of extrahypothalamic neural TRH occurs outside the hormonal feed-back mechanism; its level is influenced by noradrenaline, dopamine, histamine and serotonin. The cerebral TRH has neurotransmitter and neuromodulator functions, depending on the regions of the brain. It is possible that the extraneural TRH (pancreas, gastrointestinal tract) plays a part in the gastrointestinal regulation under physiological conditions. The function of the TRH in the gonads is at present unknown.

Method. The development of the RIA methods was facilitated by the preparation of synthetic TRH, which is coupled to bovine serum albumin in one step with bis-diazotized-benzidine. Rabbits are immunized with the resulting complex and complete Freund's adjuvant. The specificity of the antibody obtained is generally not too high, because of the nature of the relatively short, apolar peptide antigen; accordingly, in measurements on the various biological samples it is usually necessary to confirm the authenticity (excess antibody, parallel work with dilution series, chromatography, etc.). Increase of the specificity of the antibody may be attempted through coupling of the peptide to dinitrophenylene. Either tritiated or iodinated tracer may be used for the RIA; the sensitivity of the method may be improved considerably by the application of mono-¹²⁵I-His₂-TRH (separated by absorption or HPLC). Extraction is definitely necessary for the determination of TRH in tissue samples and peripheral blood (the active charcoal-acetone-methanol combination is the most frequently used method, the recovery usually being above 60%). In this way, it emerged that only 0.5% of the immunoreactivity in the peripheral blood originates from the natural peptide.



Clinical applications. A number of difficulties impede the widespread use of the RIA of TRH in clinical practice. The acquisition of information on the hypothalamic function would necessitate the determination of TRH in the portal blood, this method being applicable to patients at best when pituitary surgery is carried out. Its determination in the peripheral blood is hampered by the relatively low level of TRH, for the peptide is rapidly inactivated by serum enzymes, and its renal extraction is too fast. The application of benzamidine may be of value to inhibit the loss of the immunoreactivity; this substance can probably block deamination of the peptide. The plasma level of TRH immunoreactivity in healthy subjects varies from team to team (depending on the method used), in the range 19.8 ± 3.1 to 137 ± 53 pg/ml (54.5 ± 8.5 to 377 ± 146 fmol/ml). Guignier et al. (1981) found the serum TRH immunoreactivity level to be lower than normal in hyperthyroidism, and higher than normal in hypothyroidism and cold nodular goitre, and hence they presumed that the method might well be of potential value in the diagnosis of thyroid diseases.

LUTEINIZING HORMONE RELEASING HORMONE (LH-RH, GONADOLIBERIN)

Chemical structure. LH-RH activity was demonstrated in the hypothalamus of experimental animals at the beginning of the 1960s; FSH-RH activity, too was found later. LH-RH-like activity was first detected in man by Schally (in hypothalamus extracts). It was initially thought that two different substances are responsible for the release of FSH and LH, but this could not be confirmed. The exact chemical structure of LH-RH was established through the processing of hundreds of thousands of hypothalami. It contains ten amino acids: pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂. Its molecular weight is 1,183 dalton.

With combinations of substitutions in positions 6 and 10, analogues with 30-60 times the effectiveness of LH-RH can be obtained, these being long-acting compounds, too. The stronger effect can probably be explained by the better binding to the LH-RH receptors of the pituitary, and the long-acting effect by the resistance to enzymes. LH-RH analogues of inhibitory type have also been prepared; these display a high affinity for the receptor, but are biologically inactive. Their application allows blockade

of the FSH-LH surge and ovulation, and hence they are potential contraceptives.

Physiological conditions. LH-RH causes the release of FSH and LH in rat and other experimental animals. **In vitro** investigations have indicated that, besides the release, LH-RH is also able to enhance the synthesis of FSH and LH. Both the natural and the synthetic decapeptide induce FSH and LH secretion in humans, and it is improbable that FSH-RH is a separate substance. Rat experiments have shown, however, that the i.v. injection of a synthetic LH-RH bolus leads to an LH increase, while an appreciable FSH increase, too can be detected following the chronic infusion of LH-RH. LH-RH is in all probability not species-specific; it gives rise to an FSH-LH increase and ovulation in numerous species.

In humans, puberty is introduced by nocturnal pulsatile gonadotropin secretion, which in fact is induced by LH-RH secretion. Accordingly, a result may be attained in the treatment of retarded puberty by the pulsatile application of low doses of LH-RH. High doses of long-acting analogues deplete the gonadotropin reserves, and they may therefore cause reversible castration in the treatment of precocious puberty and prostate cancer. The sex steroids fundamentally exert a negative feedback effect on the secretion of LH-RH, but a positive feedback too operates towards the hypothalamus. The exact mechanism of action of LH-RH is not yet fully known; this peptide, too probably enhances the activity of intracellular adenylcyclase. However, Ca^{2+} is also necessary for development of the effect. The advances in immunohistology permitted the detection of LH-RH immunoreactivity in extrahypothalamic regions of the central nervous system: the mesencephalon, cortex and pineal gland. LH-RH probably plays an important role in sexual behaviour.

LH-RH is degraded rapidly: enzymes in the plasma cleave off the N-terminal dipeptide pyro-Glu-His, which is excreted in the urine. The enzyme set necessary for degradation is possessed primarily by the cells of the liver, the anterior lobe of the pituitary and the kidney.

Method. A number of RIA methods are known for the detection of LH-RH. The various teams use different antigen preparations, e.g. LH-RH adsorbed by polyvinylpyrrolidone or Al_2O_3 , but coupling to bovine serum albumin is also possible. ^{125}I -LH-RH is commonly suitable as labelled antigen. The bound and free fractions are separated with dextran-coated charcoal or with second antibody technique. The level of LH-RH in the peripheral blood is very low, as a consequence of the relatively fast plasma degradation and renal excretion.

The measurement usually demands extraction. Characterization by means of chromatography and electrophoresis suggests that the peptide itself is responsible for most of the immunoreactivity to be found in the plasma and the cerebrospinal fluid. Recently the plasma sample is extracted by mini-column chromatography.

Clinical applications. A clear-cut assessment of the clinical applications of LH-RH RIA cannot be given at present. Certain authors state that the immunoreactivity detectable in the peripheral blood, the cerebrospinal fluid or the urine does not reflect the hormone level in the pituitary portal blood, and thus its measurement is not suitable for an evaluation of the hypothalamic function. Others consider that such a connection does exist, and that the RIA of LH-RH has a potential role in hypothalamic diagnostics.

Miyake et al. (1980) measured the peripheral plasma LH-RH immunoreactivity in women with a normal cycle (4.5 ± 0.45 pg/ml; 3.8 ± 0.38 fmol/ml), together with simultaneous determination of FSH, LH, oestradiol and progesterone, and stated that an LH-RH peak (42 ± 11.4 pg/ml; 35.5 ± 9.6 fmol/ml) can be detected at the middle of the cycle in most women. The LH-RH peak emerged following the serum oestradiol increase, and did not coincide exactly with the LH peak.

Immunoreactive LH-RH can be detected in the urine too, its level rising in direct proportion to the age. Both before puberty and in the period of pubescence, a positive correlation has been observed between the amount of immunoreactive LH-RH in the urine and the levels of FSH and LH in the plasma. In the course of chemical characterization of the measurable LH-RH immunoreactivity, it was found that the urine contains mainly the 2-10 LH-RH nonapeptide fragment.

CORTICOTROPIN RELEASING FACTOR (CRF, CORTICOLIBERIN)

Chemical structure. It was first reported by Slusher and Roberts that hypothalamus extracts contain activity inducing the release of ACTH. Schally and Guillemin later isolated and partially characterized substances with CRF activity from the posterior lobe of the pituitary: α -CRF exhibited chemical similarities to α -MSH, and β -CRF to vasopressin. The characterization of these peptides was subsequently not continued. The iso-

lation of CRF and its structural determination were hampered and delayed mainly by the extremely low concentration of the peptide in the hypothalamus. The break-through came in 1981, when Vale et al. isolated a 41 amino acid polypeptide from sheep hypothalamus; even in very low concentration, this was capable of selectively inducing the release of ACTH. Its chemical structure: H-Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Thr-Lys-Ala-Asp-Gln-Leu-Ala-Glu-Glu-Ala-His-Ser-Asn-Arg-Lys-Leu-Leu-Asp-Ile-Ala-NH₂. Its molecular weight is 4,673 dalton.

The synthetic CRF is about ten times more effective than the native peptide, which is probably a consequence of the methionine sulfoxide content of the purified molecule. An intact C-terminal part is indispensable for the biological effect to be displayed. The N-terminal region probably varies in structure from species to species. CRF exhibits structural similarities to various known peptides: angiotensinogen, sauvagine and urotensin I.

Physiological conditions. CRF activity is to be found in greatest concentration in the median eminence of the hypothalamus, but appreciable activity can also be detected in the nucleus arcuatus, the nucleus paraventricularis, the nucleus dorsomedialis and the nucleus ventromedialis. Hypothalamic CRF is able to stimulate both the synthesis and the secretion of ACTH and peptides related to ACTH. However, CRF (or a substance chemically very similar to it) can also be detected in the adrenal medulla and the lung. Although the action of CRF is very specific *in vitro* and *in vivo*, it is currently considered that it does not influence alone the secretion of ACTH under physiological conditions.

It is highly probable that a part is played in regulation of the secretion of ACTH (and related peptides) not only by CRF, but also by another substance to be found in the posterior lobe of the pituitary and by vasopressin; this substance can be well differentiated from vasopressin, but has not yet been characterized exactly. CRF secretion is stimulated by serotonin and acetylcholine; the action of the former can be regarded as a specific effect. The secretion of CRF is inhibited by hexamethonium, noradrenaline and glucocorticoids. The peptide disappears from the circulation relatively slowly, for it has a fairly high affinity for the plasma proteins (as concerns the hypothalamic peptides, the plasma has the longest half-life, and the slowest metabolic clearing rate). The physiological significance of the CRF occurring extraneurally (adrenal, lung) is not well known.

Method. The antiserum is generally prepared by the immunization of rabbit. The conjugate of synthetic oCRF with bovine serum albumin or thyroglobulin is used as antigen. A monoclonal antibody suitable for RIA has also been prepared: synthetic oCRF is coupled to rabbit myosin by means of N-succinimidyl 3-(2-pyridyldithio)-propionate, mice are immunized with the complex, and mouse splenocytes are fused with plasmacytoma cells. The end-dilutions of the currently used antisera differ greatly (ranging from 1/10,000 to 1/180,000), as do the sensitivities of the methods. ^{125}I -CRF or ^{125}I -Tyr-CRF may be applied as labelled antigen; the iodination is generally performed with the Hunter-Greenwood technique. The labelled antigen is usually purified by gel chromatography or HPLC. The method is specific; a cross-reaction is normally observed only with sauvagine.

Clinical applications. The RIA of CRF has comparatively small clinical importance. The concentration of CRF in the peripheral blood in normal subjects and in pathological cases, too (Nelson's syndrome, Cushing's disease) is below the sensitivity threshold even of the relatively sensitive methods. A fairly high plasma concentration can be detected in the third trimester of pregnancy (67.3 ± 22.8 pg/ml; 14.4 ± 4.9 fmol/ml); this immunoreactivity is given by a substance very similar to CRF. The physiological significance of this fact is still unknown. A high plasma immunoreactive CRF level can sometimes be observed in cases similar to ectopic ACTH syndrome (ectopic CRF formation). The immunoreactive CRF concentration is particularly elevated in the cerebrospinal fluid (16.3 – 53.2 pg/ml; 3.5 – 11.4 fmol/ml); this is regulated by the peripheral glucocorticoid level.

GROWTH HORMONE RELEASING FACTOR (GRF, SOMATOCRININ)

Chemical structure. Human pancreas growth hormone releasing factor (hpGRF) was isolated and characterized by two research team at virtually the same time from two pancreatic tumours giving rise to acromegaly. The peptide described by Guillemin et al. (1982) contains 44 amino acids: Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asu-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Glu-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Glu-Asp-Ile-Met-Ser-Arg-Glu-Glu-Gly-Glu-Ser-Asu-Glu-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu-NH₂ (hpGRF₄₄). Its molecular weight is 5,041 dalton. The molecule isolated by Vale and coworkers is identical with the first 40 amino acids of the above sequence (hpGRF₄₀). By means of HPLC,

bioassay (pituitary cell culture) and RIA, it was later demonstrated that the GRF produced by pancreatic tumour is extremely similar (or identical) to the peptide to be found in the human hypothalamus. hpGRF_{40} and hpGRF_{37} , which could likewise be isolated from pancreatic tumour, are probably enzyme-degradation products of hpGRF_{44} . After elucidation of the amino acid sequence, the name somatocrinin was proposed instead of GRF. Somatocrinin displays a structural homology with the PHI (peptide- NH_2 -His-C-Ile- NH_2), VIP, secretin and glucagon family. Porcine and bovine GRF similarly consist of 44 amino acids, and exhibit a considerable structural homology with human somatocrinin. Following synthesis of the peptide, structure-activity studies demonstrated that the most biologically active form is $\text{hpGRF}_{44}\text{-NH}_2$. Amidation of the C-terminal generally increases the biological activity. The biologically active moiety is probably the 2-24 amino acid segment (N-terminal). The currently accepted view is that the N-terminal part in all probability plays a role in the binding to the receptor, while the C-terminal regulates the biological activity of the peptide.

Physiological conditions. Somatocrinin occurs in highest amount in the median eminence. In experimental animals and in humans, the peptide selectively induces the release of GH, without influencing the secretion of the other anterior lobe hormones. By this means, human GH deficits of pituitary and hypothalamic origin can be clearly distinguished from each other. Non-specific GH stimulants (e.g., a noradrenergic stimulant) have been found to exert their effects **via** this peptide. Somatocrinin is secreted into the portal blood together with somatostatin; the diurnal rhythm of GH is maintained by their dynamic equilibrium. The diurnal rhythm of GH can be suspended with antiserum produced against somatocrinin. To date, other physiological effects of somatocrinin are not known. **In vitro** experiments indicate that its effect is mediated by Ca^{2+} , adenylycyclase and, in part, PGE_2 .

Method. Antiserum against somatocrinin has been produced by a number of teams. Coupling to either bovine serum albumin or haemocyanin is suitable for enhancement of the antigenicity. The antisera produced in this way may be used with an end-dilution ranging from 1/120,000 to 1/200,000. Not even the peptides of the chemically related glucagon family give essential cross-reactions with these antisera ($< 0.2\%$). Antisera produced against hpGRF_{44} and hpGRF_{40} give appreciable cross-reactions with the two peptides. ^{125}I -GRF is used as labelled antigen; it is prepared by the Hunter-Greenwood technique (chloramine T). The determination of immuno-

reactive somatocrinin in the peripheral plasma generally necessitates extraction because of the nature of the plasma components causing the interference.

Clinical applications. The spreading of the RIA of somatocrinin in clinical practice is primarily impeded by the fact that the normal plasma level of the immunoreactive peptide is less than the sensitivities of the currently used assays. Accordingly, at best it may be utilized for diagnosis of a (hypothalamic or ectopic) GH excess (acromegaly) brought about by the not too frequent hyperproduction of somatocrinin. An elevated immunoreactive GRF level can be measured in carcinoid tumours, pancreatic tumours and small-cell pulmonary cancers. It is very difficult to distinguish between ectopic GRF production and that of hypothalamic origin. In these cases, the GH responses to various exogenous stimuli (TRH or L-dopa) do not usually differ. If a somatocrinin deficit is presumed (dwarfism), pharmacological tests with synthetic somatocrinin may be of help.

SOMATOSTATIN (SOMATOTROPIN RELEASE INHIBITING FACTOR, SRIF)

Chemical structure. This hypothalamic factor inhibiting the secretion of growth hormone was discovered as a side-product in the isolation of luteotropin releasing factor. The final characterization of somatostatin was achieved by Burgus and Ling, who applied a miniaturized stepwise Edman degradation technique and mass-spectrography. Somatostatin contains 14 amino acids: Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys. As a consequence of the presence of the two Cys, the structure includes a disulphide bridge. The molecular weight is 1,638 dalton. The biological activity of the synthesized somatostatin is the same as that of the native peptide. Numerous analogues too have been prepared. Besides growth hormone, somatostatin influences the secretion of glucagon and insulin. Accordingly, on the basis of both theoretical and practical considerations, attempts have been made to prepare analogues which exhibit a certain selectivity in their effects. Des-Asn⁵-somatostatin effectively inhibits the secretion of insulin, but has little effect on the secretion of glucagon. D-Cys¹⁴-somatostatin strongly inhibits glucagon secretion, but exerts relatively little action on the secretion of insulin. Synthetic analogues have also been prepared which are more potent and longer-acting

than the native hormone with respect to the suppression of growth hormone secretion: D-Trp⁸-somatostatin and D-Ala⁸-somatostatin are 8 and 20 times more effective, respectively, than the native peptide. The incorporation of fluorine into the molecule is another valuable means of enhancing the bioactivity. When Bauer et al. prepared certain analogues in 1982, they set out from an extremely simplified starting compound which itself has only a very slight biological activity; this compound contains the active centre of somatostatin: amino acids 7-10. Two Cys molecules were coupled to this tetrapeptide, these Cys units forming a disulphide bridge with one another. The D stereoisomer of Trp was incorporated; this resulted in enzyme-resistant derivatives with long biological activity.

Physiological conditions. Somatostatin effectively inhibits growth hormone secretion. Somatostatin has no effect on basal FSH and LH secretion, but it can block TRH-induced release of TSH without influencing the PRL secretion enhancement. In acute experiments, it decreases a pathologically high ACTH level; from this aspect, its point of attack is probably suprahypophyseal. As concerns the effects of the peptide on the pituitary, the GH and TSH effects are probably of physiological importance. It has a number of extrahypophyseal effects, too; it can block the secretion of insulin, glucagon, gastrin, secretin, VIP, renin, motilin and cholecystokinin. It induces an antisecretory effect by blocking the parietal and peptic cells of the stomach. It may be regarded as a local inhibitor as concerns the pituitary, pancreas, duodenum, stomach, kidney and salivary gland. Its experimental and clinical application is of promise from this respect. Not all of these latter effects have proved to be of physiological importance. Somatostatin is also to be found extrahypothalamically in the central nervous system. Somatostatin activity occurs in high concentration in the preoptic area, the amygdala, the brainstem and the spinal cord. It is highly likely that the peptide fulfils neurotransmitter and neuromodulator functions in these parts of the central nervous system.

Method. Use of the synthetically prepared somatostatin permitted the production of antiserum and the development of RIA. The first specific and sensitive RIA for somatostatin was reported by Arimura et al. The antigenicity was enhanced by coupling to human α - and β -globulin (glutaraldehyde method). Other authors successfully utilized conjugation to thyroglobulin, haemocyanin or bovine serum albumin. The majority of the currently used antisera exhibit affinity for the C-terminal part of the

peptide. Since somatostatin contains neither Tyr nor His, its labelling with iodine is not possible with the conventional techniques. Tyr¹-somatostatin, Tyr¹¹-somatostatin or N-Tyr-somatostatin may be employed for iodination. These labelled antigens have similar affinities for the antiserum, but ¹²⁵I-N-Tyr-somatostatin is more stable than the other two compounds. The RIA of somatostatin in not particularly complicated tissue extracts, cell culture media or organ perfusates is well reproducible. Its determination in plasma is not easy, for numerous plasma components display a markedly good affinity for most antisera. The measurement of somatostatin in blood plasma therefore generally demands a preliminary extraction. Chromatographic studies have confirmed that the bulk of the somatostatin immunoreactivity obtained from peripheral blood by extraction originates from the native peptide (or from a substance chemically very similar to this). Another method suitable for measurement of the plasma somatostatin level is affinity chromatography. The RIA may also be made suitable for measurements without extraction if two types of antiserum are combined with two labelled antigens.

Clinical applications. Since somatostatin may be produced by numerous tissues, similarly to other hypothalamic peptides its determination in the peripheral blood provides little information on the state of the hypothalamus. No really essential difference has been demonstrated between the plasma somatostatin levels of healthy subjects and patients with cerebral obesity, diabetes or acromegaly (40-110 pg/ml; 24.4-67.2 fmol/ml). Determination of the plasma somatostatin may be clinically useful for diagnosis of the not too frequent somatostatinoma, or for assessing (or following) the therapy. An elevated serum somatostatin immunoreactivity may also be detected in some patients with medullary thyroid carcinoma or small-cell lung cancer. As a complementary method for other procedures, the examination may likewise be of help as a tumour marker.

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Chapter 9

NEUROPEPTIDES

β-ENDORPHIN, ENKEPHALIN

Chemical structure. The stereospecific cerebral opiate receptors were demonstrated in 1973. Intensive research subsequently began to identify the endogenous opiates binding to them. As a result of this work, the opioid peptides are at present classified into three groups, each group originating from one common polypeptide precursor. The common polypeptide molecule is synthesized in the cell body; then, on the action of post-translational enzyme processes, the corresponding end-products are to be found in the nerve endings. The three groups are as follows:

1. The first group contains the endorphins originating from pro-opiomelanocortin (POMC) (Fig. 19). Cleavage of this precursor leads to the formation of ACTH and β-lipotropin (β-LPH). The former is transformed to α-MSH in the melanotropic cells, while the latter is converted to β-endorphin at various rates in the corticotropic and melanotropic cells. β-Endorphin is identical with the C-terminal amino acid sequence 61-91 in β-LPH. Its molecular weight is 3,465 dalton. Besides the endocrine cells of the anterior and central lobes of the pituitary, POMC can be detected in the mediobasal hypothalamus (nucleus arcuatus), in the brainstem (nucleus tractus solitarius), and in a number of peripheral organs (placenta, pancreas, lung, gonads and leukocytes). The main source of the β-endorphin to be found in human plasma is the adenohypophysis.

Pro-opiomelanocortin (POMC)

N-terminal	ACTH	β-lipotropin(β-LPH)		
γ-MSH	α-MSH	CLIP	γ-LPH	β-endorphin (β-LPH ₆₁₋₉₁)

Fig. 19. Chemical structure of pro-opiomelanocortin (POMC)

	5.
(Met)-enkephalin	Tyr-Gly-Gly-Phe-Met
	5.
(Leu)-enkephalin	Tyr-Gly-Gly-Phe-Leu

Fig. 20. Chemical structures of (Met)-enkephalin and (Leu)-enkephalin

2. The precursor of the next opioid family is proenkephalin A, cleavage of which leads to the formation of the enkephalins (Fig. 20). This group includes two pentapeptides, (Leu)-enkephalin and (Met)-enkephalin, and variants of the latter lengthened in the C-terminal part, e.g. adrenorphin₁₋₈. The sequence of (Met)-enkephalin corresponds to the β -endorphin₁₋₅ sequence; its molecular weight is 574 dalton. In (Leu)-enkephalin, there is a leucine in place of the methionine at position 5; its molecular weight is 556 dalton. The enkephalin family is to be found very extensively in the various regions of the brain (striatum, hypothalamus) and in the spinal cord, at both sites mainly in the interneurons. They also occur in large amount in the chromaffinic cells of the adrenal medulla (stored together with catecholamines) and in the mesenteric plexus of the gastrointestinal tract. The (Met)-enkephalin in the plasma probably originates from the adrenal medulla.

3. The dynorphin family originating from the third precursor, proenkephalin B, is the most populous (Fig. 21). Six peptides may be listed here: α -neoendorphin₁₋₁₀, β -neoendorphin₁₋₉ (differing only in the C-terminal tenth amino acid), dynorphin₁₋₈, dynorphin₁₋₁₇ (previously known as dynorphin A), rimorphin (previously dynorphin₁₋₁₃ or dynorphin B), and

α - Neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys
β - Neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro
Dynorphin ₁₋₈	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile
Dynorphin ₁₋₁₇	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu- -Lys-Trp-Asp-Asn-Gln
Rimorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
Leumorphin	Rimorphine-Arg-Ser-Gln-Glu-Asp-Pro-Asn-Ala-Tyr-Tyr- -Gln-Glu-Leu-Phe-Asp-Val

Fig. 21. Chemical structure of dynorphin derivatives

leumorphin (the N-terminal of which contains the dynorphin sequence). One common property linking these peptides is the composition of the first five amino acids: each of them begins with the (Leu)-enkephalin structure. The dynorphin derivatives are to be found in the various regions of the brain. Their concentrations are highest in the neurohypophysis and the magnocellular nuclei (together with vasopressin), and in the submucosal plexus of the gastrointestinal tract.

Physiological conditions. In spite of a considerable amount of information on the biosynthesis and localization of the opioid peptides, little is known as to their physiological roles. They exert their effects mainly as neurotransmitters or neuromodulators; in certain peripheral organs they have paracrine effects. Besides the classical analgetic effect (role in pain perception), they take part in the regulation of pituitary hormone secretion, and in the modulation of autonomous nervous system processes (food intake, water metabolism, heat regulation, cardiovascular effects, glucose homeostasis, sympathetic activity, reproductive processes, etc.). Their effects on learning and memory mechanisms and on behavioural reactions indicate that they may be of pathogenetic importance in psychiatric disorders (schizophrenia, endogenous depression, alcoholism).

Method. The quantitative determination of the various opioid peptides in the homogenates of the different brain regions and organs, in the cerebrospinal fluid and in the plasma is carried out by RIA. Of the opioid determinations on plasma, the best-developed procedure is β -endorphin RIA. Fewer publications are available on the measurement of (Met)-enkephalin, and there are only isolated reports on dynorphin RIA.

Although the methods applied in the measurement of the opioid peptides do not differ essentially from the general principles of RIA techniques, a number of difficulties must be overcome. Because of the short half-lives of the peptides, and their rapid decompositions, blood samples must be collected in EDTA-containing tubes in an ice-bath; centrifugation and acidification of the plasma must follow immediately. Immunization is generally performed in rabbit, with a fairly large amount (several mg) of synthetic opioid peptide coupled to thyroglobulin or to bovine serum albumin.

Extraction of the opiates from the samples is necessary in order to eliminate the disturbing effect of aspecific protein binding and to inhibit proteolytic processes, and also because of the low concentration of the peptides in the plasma. Of the various procedures used (talc, glass powder, acetic acid, acetone, etc.), the best appears to be extraction

(based on the principle of reversed-phase liquid chromatography) on a Sep-Pak C₁₈ column packed with octadodecasilyl silica gel (ODS); this is fast, convenient, and ensures the necessary recovery, with minimal aspecific contamination.

Because of the similarities between the structures of the opioid peptides, the antisera give significant cross-reactions with the individual peptides. For example, the cross-reaction between β -LPH and β -endorphin is almost quantitative; the antiserum produced against (Met)-enkephalin gives marked cross-reactions with (Leu)-enkephalin and the shorter endorphin fragments. Before RIA, therefore, the various peptides must be separated from the extracts. This may be achieved by means of Sephadex G-50 or G-75 column chromatography, affinity chromatography or HPLC. Following this, the RIA is carried out on the eluate fractions running in the same way as standards. In the case of β -endorphin (β -LPH), after the separation the measurement may be made with the antiserum produced against the C-terminal, or in parallel with antisera recognizing the C- or the N-terminal of β -LPH. The latter does not give a cross-reaction with β -endorphin; only the N-terminal part of β -LPH, γ -LPH, is measured.

Without extraction and chromatography, the considerable cross-reactions mean that the values found for the plasma concentrations of the individual opioid peptides will be much higher than the actual values; accordingly, the value of the results obtained is questionable. ¹²⁵I is generally used to label the standards for the opioid peptides; ¹³¹I may also be applied. The labelled substance must be purified on Sep-Pak or by HPLC. The RIA is carried out in a non-equilibrium incubation system. A second antibody method is usually employed to separate the bound and free fractions.

Clinical applications. The normal level of β -endorphin in the plasma is 14-35 pg/ml (4-10 fmol/ml), while that in the cerebrospinal fluid is 173-350 pg/ml (50-100 fmol/ml). The corresponding levels of (Met)-enkephalin are 12-34 pg/ml (20-60 fmol/ml) and 6-12 pg/ml (10-20 fmol/ml), respectively. The immunoreactivity of dynorphin in the plasma is 10-32 pg/ml (5-15 fmol/ml). The plasma level of β -endorphin is elevated in pregnancy and in Nelson's syndrome, and a pathological dexamethasone suppression test has been observed in endogenous depression (similarly to cortisol). The level of β -endorphin in the cerebrospinal fluid is depressed in migraine syndrome and in chronic alcoholics. Elevated plasma (Met)-enkephalin levels have been measured in various forms of shock.

B-Endorphin kits are commercially available from New England Nuclear and Immuno Nuclear Co. With the latter, chromatographic extraction of the samples is necessary.

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RENIN ANGIOTENSIN AND ALDOSTERONE

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graph TD
    A[Plasma renin substrate  
(α-globulin)] -- "Renin  
(kidney)" --> B[Asp-Arg-Val-Tyr-Ileu-His-Pro-Phe-His-Leu-COOH]
    B -- "Converting enzyme  
(lung)" --> C[Asp-Arg-Val-Tyr-Ileu-His-Pro-Phe-COOH]
    C --> D[Angiotensin II]
    
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Fig. 22. Pathway of formation of angiotensin II

nature, α -globulin (plasma renin substrate) is cleaved proteolytically to angiotensin I, which is decomposed further to angiotensin II by the converting enzyme. Angiotensin II has a direct effect on the blood pressure and enhances aldosterone secretion. Angiotensin II and aldosterone enhance sodium retention and elevate the blood pressure.

RENIN ANGIOTENSIN

Chemical structure. Renin is a proteolytic enzyme with a molecular weight of 57,000 dalton. It cleaves α -globulin into angiotensin I, which contains ten amino acids. The large quantity of converting enzyme to be found in the lung effects further cleavage, splitting off the two amino

acids (His-Leu) before the terminal carboxy group of angiotensin I; the resulting octapeptide is named angiotensin II. The presence of the COOH group is indispensable as concerns the biological activity. Angiotensin I and II differ in the various animal species; this is due to variations in the first five amino acids.

Physiological conditions. Renin is produced by the juxtaglomerular apparatus of the kidney. The main stimulus of its secretion is a decrease in the renal perfusion, which is induced by a fall in blood pressure, or a reduction in the plasma volume (dehydration, sodium loss, plasma protein decrease, constriction of a renal artery). Renin secretion is also enhanced after the administration of catecholamines. There is a decrease in renin secretion in response to angiotensin II (direct effect) or adrenergic blockers. The regulatory centre of renin secretion is to be found in the sodium flux in the vicinity of the dense spot and in the baroreceptors (juxtaglomerular apparatus). The half-life of renin is 10 min.

Angiotensin II is the most effective pressor agent. Its effects in this direction are exerted both directly and **via** enhancement of aldosterone secretion. It is decomposed extremely quickly by angiotensinase. Its biological half-life is 1 min.

Method. The antigen necessary for immunization is prepared by coupling angiotensin I to bovine serum albumin. The antibodies are raised in rabbit. ^{125}I -angiotensin I is used as labelled hormone. The results differ, depending on the individual laboratory procedures. It is important that the incubation be carried out at appropriate pH (7.4) and temperature (37°C). The enzyme reaction may be changed by the dilution. An enzyme inhibitor must be added to the samples, in order to prevent the decomposition of angiotensin I; the most frequently used compound is ethylenediamine-tetraacetate (EDTA). The incubation time is 2-3 h. The angiotensin I varies linearly with the length of the incubation period. The bound and free fractions may be separated with dextran-coated charcoal.

Clinical applications. Renin secretion is enhanced in all those diseases in which the renal perfusion is reduced: congestive heart disease, cirrhosis, nephrosis, primary nephropathy, malignant hypertension and renovascular hypertension. In the latter case, arteriography and isolated sampling from the renal vein are recommended. An elevated renin level is similarly to be expected in certain cases of essential hypertension (PRA level increased), and also during pregnancy and after the taking of oral contraceptives.

A low renin level is found in primary aldosteronism (tumour or hyperplasia), in about 30% of cases of essential hypertension, after the taking of exogenous mineralocorticoids, and in response to β -adrenergic blockers.

In connection with the sampling, it must be borne in mind that the renin level varies diurnally, its secretion being influenced by the diet (sodium content), fasting and standing-up. Accordingly, a single sampling cannot be regarded as reliable. It is necessary to perform provocation tests. If the renin level is low, a sodium restriction is imposed for 5 days (10 mEq/day), or furosemide is given in an oral dose of 120 mg, and blood samples are taken 3-4 h later.

Normal levels:

PRA:	2.86 ng/ml/h	(2.34 nmol/ml/h)
PRC:	44.00 μ U/ml	(44.0 mU/l)
PRS:	1.56 ng/ml	(1.28 nmol/ml)

The main indications for determination of the renin level are as follows:

1. Differentiation of hyperaldosteronism of adrenocortical origin (renin level decrease) and of non-adrenocortical origin (renin level increase).

2. Establishment of a diagnosis of renal arterial stenosis.

3. Differentiation of essential hypertension associated with an enhanced or with a decreased renin level, from the aspect of the selection of the correct therapy. In hypertension with an elevated renin level, a favourable result is to be expected from the administration of β -adrenergic blockers; in cases with a decreased renin level, the administration of diuretics is to be considered.

ALDOSTERONE

Chemical structure. The structure is well known: there are keto groups on C-3 and C-20 of the sterane skeleton, with an aldehyde group on C-18 (Fig. 23). The molecular weight is 360 dalton.

Physiological conditions. Aldosterone is produced in the zona glomerulosa of the adrenal. It exerts a direct effect on the distal tubules of the kidney, enhancing sodium reabsorption and potassium excretion.

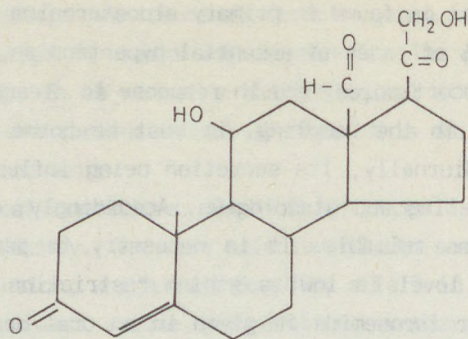


Fig. 23. Chemical structure of aldosterone

Various factors play roles in aldosterone regulation: increases in the angiotensin II and the plasma potassium levels, and decreases in the ACTH and the plasma sodium concentrations, lead to an enhanced secretion of aldosterone.

Its diurnal rhythm is similar to that of renin: the aldosterone level is higher in the morning than in the afternoon. It is hardly bound to transcortin; it mainly circulates free in the blood, from which it disappears quickly. Its biological half-life is 20-30 min. It is metabolized in the liver and the kidney, and is excreted in the urine, predominantly in the form of the tetrahydro and 18-glucuronide derivatives.

Method. Immunization is performed in rabbit or sheep, with the conjugate of aldosterone-18,21-diacetate-3-carboxymethyl oxime bound to bovine serum albumin. Experience is also very good with the antigen to aldosterone-21-hemisuccinate-bovine serum albumin.³H or ¹²⁵I labelled side-chain containing aldosterone is used as labelled hormone.

The determination was earlier carried out after extraction with dichloromethane; more recently, a direct method is also available. The incubation time is 24 h. The sensitivity is 10 pg/ml. The bound and free fractions are generally separated with dextran-coated charcoal.

Clinical applications. The diseases associated with enhanced aldosterone secretion are classified into the groups of primary and secondary aldosteronism. Primary aldosteronism may develop as a consequence of adrenocortical adenoma or bilateral hyperplasia. A third subgroup, too is

distinguished: primary aldosteronism that can be suppressed with exogenous mineralocorticoid or with glucocorticoid. Besides the elevated aldosterone level, primary aldosteronism is characterized by a low renin level, an increased sodium concentration and hypopotassaemia.

Secondary hyperaldosteronism occurs in oedematous conditions: cirrhosis, nephrosis and cardiac insufficiency. This group also contains Bartter's syndrome (with hyperplasia of the juxtaglomerular apparatus in the background) and malignant hypertension. In secondary aldosteronism, both the renin and the angiotensin II levels are elevated.

A reduced secretion of aldosterone is observed in Addison's disease; if this disease is untreated, vomiting, diarrhoea, sweating, sodium loss and a blood pressure fall may occur, and without appropriate and rapid intervention the outcome is fatal. In the Addisonian crisis, the serum sodium level is depressed and the potassium level and renin concentration are increased. A low aldosterone level may also be measured in congenital adrenal hyperplasia, in certain adrenal tumours and in certain types of essential hypertension.

When samples are taken, attention must be paid to the diurnal rhythm of aldosterone secretion. In the early morning (before the subject gets out of bed) the aldosterone level is low; after 2-3 h in the upright position, the level is elevated considerably. In hyperaldosteronism and in hypoaldosteronism, aldosterone samples taken in the recumbent and in the upright positions, respectively, yield the more information. In the stimulation test, sodium withdrawal (10 mEq/day Na^+ , 80 mEq/day K^+) is imposed for five days, while in the course of suppression sodium loading (150-200 mEq/day Na^+ , 80 mEq/day K^+) is carried out, similarly for five days, and the serum aldosterone and renin levels are measured. Mineralocorticoid suppression is performed by the administration of 200 μg fludrocortisone acetate (three days) or 2 x 10 mg i.m. DOCA (three days).

Normal aldosterone level:

Recumbent:	40-102 pg/ml	(112-285 pmol/l)
After walking for 2 h	100-305 pg/ml	(280-854 pmol/l).

If the aldosterone level is elevated, it must be decided whether primary or secondary aldosteronism is involved. If the case is one of primary aldosteronism, it must be established whether the disease is caused

by an adrenocortical tumour or by hyperplasia. The renin level is the deciding factor in the differentiation of primary and secondary aldosteronism. If suppression is possible with mineralocorticoid or glucocorticoid, this is evidence against a tumour. If the aldosterone level is increased 2-4-fold after walking, hyperplasia is probable; a tumour is suggested if the aldosterone concentration is unchanged (presumably as a consequence of automatism).

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Chapter 11

PARATHYROID HORMONE AND CALCITONIN

The Ca^{2+} homeostasis is of significance as concerns the bone structure, but it also plays an important role in connection with muscle contraction, discharge of the various secretions, the energy balance and neurotransmission. The following three hormones participate in its regulation:

1. Parathyroid hormone (PTH) elevates the Ca^{2+} level. Its secretion (via a negative feed-back mechanism) is stimulated by a low free Ca^{2+} level. It exerts its action on the small intestine, the kidney and the bone system.
2. Calcitonin reduced the Ca^{2+} level. Its secretion is promoted by a high serum Ca^{2+} level.
3. Vitamin D enhances the absorption of Ca^{2+} and the mobilization of Ca^{2+} from the bones.

PARATHYROID HORMONE (PTH, PARATHYRIN)

Chemical structure. It consists of 84 amino acids. The molecular weight is 9,500 dalton. Four different PTH molecules have been described: (i) molecular weight 11,500 dalton: presumably a prehormone; (ii) molecular weight 9,500 dalton: the active and stored form; (iii) molecular weight 7,000 dalton: this contains a COOH end-group, and has no biological activity; (iv) molecular weight 4,500 dalton: this has a terminal amino group and is biologically active.

Physiological conditions. It decreases the resorption of phosphate by the renal tubules, and enhances osteolysis, the absorption of Ca^{2+} in the intestines, and the excretion of phosphate and Ca^{2+} . The biological activity is provided by the fragment consisting of the N-terminal 33-34 amino acids. It is produced by the dark principal cells of the parathyroid gland. The serum Ca^{2+} level plays an important part in its regulation: PTH secretion is enhanced (within 10 min) as a consequence of a serum Ca^{2+} decrease. A close (inverse) correlation can be demonstrated with the phosphate level. The half-life of the PTH with a molecular weight of 7,000 is 2-4 h, while that of the PTH with a molecular weight of 9,500 is 30 min.

Method. PTH originating from other species may be used for immunization; thus, guinea-pig is treated with bovine PTH 1-84, but the C-terminal fragment of human PTH may also be applied as antigen. Cross-reactions are to be expected with the PTH fragments. The labelled hormone is prepared by labelling bovine PTH with ^{125}I . Hormone decomposition is prevented with an enzyme inhibitor (Trasylol) and quick-freezing. PTH undergoes appreciable adsorption on a glass surface, and samples are therefore collected in plastic tubes. The incubation time is 2-6 days. The bound and free fractions are separated with dextran-coated charcoal or with polyethylene glycol.

Clinical applications. PTH is of value in the differential diagnosis of hypercalcaemias. The classification of Hamilton may be taken as basis:

A. Neoplastic origin

1. Primary hyperparathyroidism

- a. adenoma
- b. hyperplasia
- c. carcinoma

2. Non-endocrine tumour

- a. bronchial carcinoma
- b. mammary carcinoma
- c. lymphoma
- d. malignant myeloma
- e. leukaemia

3. Bone metastasis, bone tumour

B. Non-tumorous origin

- 1. Sarcoidosis
- 2. Vitamin D intoxication
- 3. Milk-alkali syndrome
- 4. Addison's disease
- 5. Thiazide diuresis

Normocalcaemic hyperparathyroidism occurs, too. PTH cannot be detected in other hypercalcaemias (of non-parathyroid origin). A high PTH level may similarly be observed in chronic renal insufficiencies and in certain malignant diseases. Secondary hyperparathyroidism, with an accompanying Ca^{2+} loss, is likewise associated with an enhanced plasma PTH level. The diurnal rhythm of PTH (lower in the early morning, a peak in the

evening) must be taken into consideration when samples are taken. Conclusions can be drawn as to the location of an adenoma if samples are taken from the thyroid veins with a catheter. The normal PTH level is 1.1-2.5 ng/ml (120-260 pmol/l).

CALCITONIN

Chemical structure. This polypeptide contains 32 amino acids. Its molecular weight is 3,400 dalton. The entire molecule is necessary for the biological activity. A definite species-specificity can be demonstrated.

Physiological conditions. Its main biological effects involve decreases of the plasma Ca^{2+} level, the phosphate level and the bone resorption. It is produced by the parafollicular cells of the thyroid in mammals, and by the ultimobranchial organ in fish. It is to be found in human urine. The calcitonin level is elevated by an enhanced serum Ca^{2+} level (above 9 mg/100 ml). As a consequence of its inhibitory action on bone construction, it may be applied effectively in the treatment of Paget's disease.

Method. Immunization is carried out with human calcitonin in guinea-pig or rabbit. Calcitonin is usually labelled with ^{125}I . The time required for incubation is 1-5 days. The bound and free fractions are separated with dextran-coated charcoal or with polyethylene glycol or second antibody technique.

Clinical applications. The most important early sign of thyroid medullary carcinoma is a calcitonin level increase, which may attain even 3 $\mu\text{g/ml}$. By this means, the background may be clarified and the effectiveness of treatment can readily be followed. The normal level is 0.02-0.4 ng/ml (58.6-117 pmol/l). A high level may be observed in malignant tumours, due to ectopic calcitonin formation. It can similarly be detected in pseudohypoparathyroidism, chronic hypocalcaemic conditions and carcinoid disease.

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Chapter 12

INSULIN

Chemical structure. This is a two-chain polypeptide: the A chain contains 21, and the B chain 30 amino acids. Two disulphide bridges are to be found in the molecule. The molecular weight is 6,000 dalton. The insulin molecules in the various species are very similar: porcine insulin differs from human insulin in only one amino acid, while bovine insulin differs from human insulin in three amino acids. Insulin originating from a different species may be employed in RIA, but it is preferable to use human insulin for the standard curve. The prohormone of insulin is the C peptide (connecting peptide), the secretion of which is slower.

Physiological conditions. Insulin has a considerable influence on the carbohydrate and lipid metabolisms. Its primary effect is to promote the penetration of glucose into the cells; as secondary effects, it diminishes lipolysis and glyconeogenesis, and enhances lipogenesis and glycogen formation. It is produced by the β -cells of the pancreas, 20% of it in the form of proinsulin. It circulates in free form in the blood. Its biological half-life is only 4 min. About half of it is secreted **via** the portal system of the liver. The half-life of the C peptide is longer. The main regulatory factor is the plasma glucose level. Besides hypoglycaemia, insulin secretion is enhanced by glucagon, secretin, gastric inhibitor peptide, vagus stimulation and the administration of amino acids. The insulin level decreases in response to fasting or the administration of catecholamine or somatostatin. The response to stimulation occurs in two phases: (i) an early phase (4-8 min), and (ii) a late phase (protracted). The basal level depends on the nourishment (carbohydrate uptake).

Method. The procedure is well developed and has been applied for a long time. High-purity insulin originating from other species may also be used for immunization. The antigen is generally administered to rabbit or guinea-pig. A cross-reaction is given with proinsulin (C peptide). However, since the blood level of proinsulin is only about 20% of that of insulin, and since its binding to the antibody is also lower, the disturbing effect of proinsulin may be neglected. ^{125}I -insulin is used as labelled hormone. The calibration curve is prepared with the application of human insulin and insulin-free plasma. The incubation time is 8-16 h. The sensitivity is

1-2 $\mu\text{U/ml}$. The bound and free fractions are separated by means of a second antibody, a solid-phase antibody or dextran-coated charcoal.

Clinical applications. An enhanced insulin secretion is most often caused by an adenoma originating from the β -cells of the pancreas. However, it also arises in other cases, e.g. non-pancreatic tumours in neonates and elderly subjects (retroperitoneal sarcoma, lung carcinoma). The basal insulin level is moderately elevated in obesity, and a higher insulin response may be observed to stimulation.

A diminished insulin production may be observed in diabetes mellitus of juvenile type; a lower response is then obtained to glucose loading, too. In adult diabetes the diagnostic value of insulin is slight. Its importance has recently increased in prediabetes; the response to glucose loading is lower in these patients and the members of their families. Both basal and stimulated insulin levels are examined.

The following stimulation tests are performed:

1. Oral glucose loading test: after the administration of 100 g (or 1.75 g/kg) glucose, blood is taken at definite times (0, 10, 20, 30, 45, 60, 90, 120 and 180 min) for the determination of the serum glucose and insulin.

2. Intravenous glucose loading: adults receive 25 g (or 0.5 g/kg) glucose, fat patients more than this, and infants less. Blood samples are taken after 0, 4, 6, 10, 20, 30, 45 and 60 min.

3. Intravenous tolbutamide test (1 g or 20 mg/kg), or 1 mg glucagon. Blood samples are taken as in test 2.

Fasting for 24-72 h reduces the insulin level and corresponds to an inhibition test.

The plasma samples must be stored at -20°C . The insulin results may be distorted by heparin. Besides the establishment of a diagnosis of hyperinsulinism, the tests may be used to assess the insulin reserve. If there is no insulin response to oral glucose loading, insulin must be introduced in the treatment of diabetes mellitus. The normal basal insulin level is 5-25 mU/l (36-180 pmol/l); there is a wide variation after stimulation: 50-200 mU/l (358-1434 pmol/l).

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Chapter 13

GASTROINTESTINAL HORMONES

Various gastrointestinal hormones are known: gastrin, cholecystokinin, secretin, vasoactive intestinal peptide (VIP), gastric inhibitor peptide (GIP), motilin and enteroglucagon. In clinical practice, mainly the RIA of gastrin has become widespread; those of CCK, secretin and VIP are applied less widely. The main problems are the isolation of the hormones and appropriate immunization. We shall deal here only with the RIA of gastrin, cholecystokinin and secretin.

GASTRIN

Chemical structure. Several molecules with gastrin effects exist: (i) big-big gastrin, in which the number of amino acids is not known; (ii) big gastrin, containing 34 amino acids (G-34); (iii) little gastrin containing 17 amino acids (G-17); and (iv) mini gastrin, containing 13 amino acids (G-13).

Sulphated (G-II) and non-sulphated (G-I) gastrin-17 are distinguished. The molecular weight of the latter is 2,098 dalton. The C-terminal moiety consisting of four amino acids (Trp-Met-Asp-Phe-NH₂) is biologically active. This observation was utilized as the starting point in the synthesis of the similarly active pentagastrin (β -Ala-Trp-Met-Asp-Phe-NH₂).

Physiological conditions. The most important effect of G-17 is considered to be its enhancement of the secretion of gastric acid. Further, (i) it increases the secretion of pepsin, the intrinsic factor and the pancreatic juice; (ii) it enhances the muscular tonicity of the alimentary canal (lower part of the oesophagus, stomach, small and large intestines and gall-bladder); (iii) it induces relaxation of Oddi's sphincter; and (iv) it stimulates growth of the gastric mucosa and enhances the blood flow of the stomach, pancreas and small intestine.

It is produced mainly in the antrum of the stomach and in the proximal end of the duodenum. The secretion of gastrin is increased by vagus stimulation, insulin (hypoglycaemia), protein intake and Ca-infusion, and decreased by the presence of gastric acid. Gastrin is primarily metabolized

in the kidney. The biological half-life depends on the chain length: for G-17 it is 3 min, for G-34 9 min, and for big-big gastrin 90 min.

Method. The antigen necessary for immunization is prepared by coupling human G-17 to bovine serum albumin. The antigen is administered to rabbit or guinea-pig. Cross-reactions are to be expected from G-34, big-big gastrin and the fragments. ^{125}I -G-17 is used as labelled hormone. The incubation time varies from 3-4 h to several days, depending on the method. The sensitivity is 5-10 pg/ml. The bound and free fractions are separated with charcoal, with anion-exchange resin or by means of a second antibody technique.

Clinical applications. Gastrin RIA is mainly of use in the diagnoses of Zollinger-Ellison syndrome; this is caused by a non- β -cell tumour of the pancreas, and is associated with enhanced gastric acid secretion and severe ulcerative complications. In other diseases (pernicious anaemia, atrophic gastritis, duodenal and ventricular ulcer, renal insufficiency and vagotonia), the gastrin level elevation is lower in extent and is not always characteristic. Various provocation tests may be used for differentiation:

1. **Protein administration** (180 g), followed by blood sampling at 15-min intervals for 2 h. The serum gastrin level normally rises by 50-150%. In the Zollinger-Ellison syndrome there is no change.

2. **Ca^{2+} infusion.** Ca-gluconate is given in an infusion in a quantity of 5 mg/kg/h for 3 h, with blood sampling at 15-min intervals.

3. **Secretin stimulation** in a dose of 1-2 U/kg in a fast infusion. Blood samples are taken at 5-min intervals for 30 min and then at 10-min intervals for a further 30 min. In the Zollinger-Ellison syndrome the gastrin level is elevated by 50%, or by 200 pg/ml.

4. **Glucagon test.** 1 mg is administered intravenously, and blood samples are taken at 5-min intervals for an hour. The serum gastrin level normally falls moderately, but in the Zollinger-Ellison syndrome a considerable increase may be observed, with a peak after 5-15 min.

The normal basal gastrin level is 20-160 pg/ml (9.4-75.2 pmol/l). 180 g protein results in a 50-150% increase in the level. After secretin or glucagon, there is no change or possibly a moderate decrease. A Ca^{2+} infusion leads to an increase of less than 500 pg/ml.

Gastrin determination is important (i) in severe, recurring ulcerative disease involving high acidity, particularly if there is associated diarrhoea, and (ii) in recurrent ulcer after gastrectomy. The suspicion of

the Zollinger-Ellison syndrome arises in both cases. The basal gastrin level in such cases is 300-350 pg/ml; it does not respond to normal stimulation; the administration of secretin or glucagon results in a paradox reaction (a mild increase). In response to Ca^{2+} infusion the level increases by 500 pg/ml.

A decreased gastrin level is found after gastrectomy and after the removal of the adenoma causing the Zollinger-Ellison syndrome.

CHOLECYSTOKININ (CCK)

Chemical composition. CCK is a polypeptide containing 33 amino acids. Its molecular weight is 3,918 dalton. Its C-terminal fragment containing eight amino acids is the octapeptide CCK-8, which carries the biological effect; the molecular weight of CCK-8 is 1,064 dalton. The N-terminal part of CCK differs slightly from species to species, and it has hardly any CCK-like biological effect. The C-terminal 5 amino acids are identical with those in gastrin. The biological effect of CCK is also similar to that of gastrin; the difference is expressed only in the strength of the effect. Gastrin causes only a minimal gall-bladder contraction; CCK slightly enhances acid secretion in the stomach.

Physiological conditions. CCK enhances the enzyme-secreting activity of the pancreas and, via the secretion of secretin, it increases the bicarbonate content of the pancreas. Through contraction of the gall-bladder and relaxation of Oddi's sphincter, it ensures the entry of bile into the intestine. It promotes the secretion and motility of the duodenum. Its action on the cerebrocortical function has been described, too. It is produced by the duodenum mucosa cells. The most important factors from the aspect of the regulation of CCK are the digestion of lipids and protein. If these two types of substance are administered, the secretion of CCK may be enhanced.

Method. A measurement problem is caused by the fact that the last five amino acids of CCK-8 and the C-terminal peptide of gastrin, the biologically active pentagastrin, exhibit the same sequence. Accordingly, two groups of RIA methods have been developed for CCK: (i) N-terminal CCK RIA (a cross-reaction is not given with gastrin, but it is not the biologically active part that is measured); (ii) C-terminal CCK RIA (there is a full cross-reaction with gastrin; if the result of a simultaneously performed

specific gastrin RIA is subtracted from the result of the C-terminal CCK RIA, the value corresponding to the biologically active CCK may be obtained).

N-terminal CCK RIA. The immune serum used is produced in rabbit against CCK₁₋₃₃ bound to bovine serum albumin. CCK₁₋₃₃ serves as standard and hormone to be labelled. Labelling is effected with ¹²⁵I. RIA is performed in an ice-bath. Incubation lasts 72 h. The bound and free fractions are separated with a second antibody during a further 24 h incubation. The basal level of CCK in the plasma with this method is 58 pg/ml (14.8 pmol/l).

C-terminal CCK RIA. Immune serum is produced in rabbit against synthetic CCK-8 bound to bovine serum albumin with glutaraldehyde. For enhancement of the specificity of the RIA, the CCK is extracted from the blood plasma samples with immune serum specific to C-terminal CCK bound on Sepharose activated with CNBr. ¹²⁵I-CCK-8 or ¹²⁵I-G-17 is used as labelled hormone. The bound and free hormone fractions are separated with ion-exchange resin. With this method, the fasting normal plasma concentration is 0.8 pg/ml (0.2 pmol/l), while 45 min after eating in healthy subjects it is 1.1 pmol/l. In the plasma of patients who have undergone Billroth I gastric resection and vagotomy it is 2.9 pmol/l, while in coeliac disease it is not elevated.

Clinical applications. Through the administration of CCK a gall-bladder contraction can be induced. The failure of this to occur is indicative of chronic cholecystitis, cholecystic accretion or cholecystic tumour. The "CCK-deficiency syndrome" was described in 1974. The essence of this is that the well-filling gall-bladder does not contract in response to an appropriate physiological stimulus (e.g. feeding) if there is a lack of endogenous CCK. After CCK administration, however, a normal contraction may be observed. An absolute or relative CCK deficiency is primarily to be assumed in sprue patients. Experimental observations suggest that CCK RIA is of promise in the differentiation of organic and functional stenoses of Oddi's sphincter; by this means, a number of unnecessary papillotomy operations might be avoided. Its therapeutic application may promote the spontaneous loss of small stones remaining after cholecystectomy. As a consequence of its enhancement of the small bowel motility, it may also be considered for the treatment of paralytic ileus.

SECRETIN

Chemical structure. This is a polypeptide containing 27 amino acids. Its molecular weight is 3,056 dalton. Its structure resembles that of glucagon. Fourteen of the amino acids are common in the two hormones. They are also somewhat similar in biological effect.

Physiological conditions. It is produced by the duodenum mucosa cells. The main stimulus of its secretion is an enhancement of gastric acid production. It increases the bicarbonate concentration and volume of the liver and the pancreatic juice. It has a similar effect to that of glucagon on the carbohydrate metabolism. It reduces the acid secretion of the gastric mucosa, and also the motility of the stomach and the duodenum.

Method. Immunization is carried out in rabbit with secretin coupled to bovine serum albumin. ^{125}I -secretin is used as labelled hormone after purification on a Sephadex G-25 or Amberlite CG column. Before RIA, the hormone is extracted from the plasma samples by means of a resin column (XAD-2), from which it is eluted with HCl/methanol. After incubation for 3-4 days, the bound and free fractions are separated with a second antibody against rabbit gamma globulin produced in goat.

Clinical applications. By increasing bicarbonate secretion, an enhanced secretion of secretin results in severe diarrhoea that is difficult to influence. An elevated secretin level can rarely be demonstrated. The normal value is 5.7 pg/ml (1.9 pmol/l). An infusion of 0.1 N HCl into the duodenum for 30 min (at a rate of 0.22 mmol/min) was reported to raise the level to 25.4 pg/ml (8.47 pmol/l). In untreated sprue syndrome, the basal level is 4.1 pg/ml; after HCl infusion here, the secretin level is only 5.8 pg/ml (1.93 pmol/l).

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Chapter 14

HAEMATOPOIETIC SYSTEM

The substances that will be considered here are vitamin B₁₂, folic acid and ferritin.

VITAMIN B₁₂

Chemical structure. This compound is a glycoprotein. Its molecular weight is 1,200 dalton. Important constituents of the molecule from the aspect of the biological activity are cobalt and CN; these explain the nomenclature cyanocobalamin. It is a labile compound: on the action of light, it is oxidized to aquocobalamin.

Physiological conditions. Vitamin B₁₂ is necessary for growth, for haematopoiesis, for the development of the epithelial cells, and for maintenance of the function of the myelin in the central nervous system. Vitamin B₁₂ forms a complex with the intrinsic factor (IF), a glycoprotein produced by the parietal cells of the stomach; the molecular weight of this complex is 55,000 dalton. Two molecules of vitamin B₁₂ and two molecules of IF form a tetramer in the small intestine, from where the vitamin B₁₂ passes into the blood. In the bloodstream it circulates in a form bound to protein. The transport of vitamin B₁₂ is ensured by various plasma proteins: transcobalamin I, an α -globulin, is responsible for maintenance of the vitamin B₁₂ level; transcobalamin II, a β -globulin, promotes the entry of vitamin B₁₂ into cells; and transcobalamin III, an inter- α -globulin, plays a role in vitamin B₁₂ transport.

Vitamin B₁₂ is secreted rapidly **via** the kidney. It is stored for a long time (one year) in the liver. The minimum daily requirement is 0.1 μ g; the recommended daily dose for adults is 2 μ g, for children 0.3 μ g, and for pregnant or breast-feeding women 2.5 μ g.

Method. The intrinsic factor was initially applied as binding protein. More recently, the conjugate of vitamin B₁₂ bound to bovine serum albumin had been used for immunization; it is generally administered to rabbit. Vitamin B₁₂ was previously labelled with ⁵⁷Co or ⁶⁰Co, but labelling with

¹²⁵I is currently the most widespread procedure. For increase of the stability, a cyanide compound or albumin is added to the sample. The incubation time may range from 30 min to several hours. The bound and free fractions may be separated with active charcoal or with an ion-exchange resin. The sensitivity of the method is 10-30 pg/ml.

Clinical applications. A low vitamin B₁₂ level can be measured when there is an absorption disturbance (pernicious anaemia, gastric resection). In such cases, the parietal cells of the stomach are unable to secrete IF, and thus the complex necessary for absorption is not formed. The absorption of vitamin B₁₂ is similarly inhibited in certain diseases: ileal inflammation, surgical removal of the terminal ileum, bacterial overgrowth in the intestines, parasites (*Diphyllobothrium latum*), blind loop formation, following Billroth I gastric resection. A vitamin B₁₂ deficiency may also occur as a consequence of an incorrect diet, e.g. in vegetarians or in infants consuming only mother's milk for too long.

An elevated vitamin B₁₂ level is encountered comparatively rarely. It may be observed in liver diseases (hepatitis) and in myeloproliferative diseases (myeloid leukaemia).

The normal plasma value is 100-900 pg/ml (74-664 pmol/l). A level below 100 pg/ml may be regarded as a considerable vitamin B₁₂ deficiency. Experience indicates that there are fairly large differences between vitamin B₁₂ concentrations obtained by biological determination and those measured by means of RIA.

FOLIC ACID

Chemical structure. Folic acid (pteroyl triglutamic acid) is rapidly reduced *in vivo* to tetrahydrofolic acid, the methylated form of which comprises the main coenzyme, the latter being measured by RIA. The molecular weight is 441 dalton.

Physiological conditions. Folic acid plays an important role in the protein metabolism; it participates in the synthesis of DNA through the formation of purine and pyrimidine nucleotides. Folic acid is not produced in the organism; an external source is necessary. The daily requirement in adults is 50 µg, in children is 100 µg, in pregnant women is 400 µg, and in breast-feeding women is 300 µg. Liver, green vegetables and fruits are rich sources of folic acid, but the folic acid content decreases considerably

during cooking. It is stored in the liver, and metabolized in the intestines. The liver content may be 5-10 mg.

Method. In general, methyltetrahydrofolic acid is measured in the RIA procedure. Immunization is carried out in rabbit with the form of folic acid conjugated to human albumin. As a radioactive derivative, folic acid labelled with ^{125}I is used. The incubation time is several hours. The measurement is more accurate in alkaline medium. The bound and free fractions are separated with a second antibody technique.

Clinical applications. A low folic acid level is found in sprue, in subjects consuming an incorrect diet, in pregnancy, during lactation, in hypothyroidism, in malignant diseases, in haemolytic anaemia, in chronic alcoholics, and after the administration of folic acid antagonists (e.g. methotrexate). The normal range is 5-20 ng/ml (11.3-45.3 nmol/l). It must be borne in mind that the folic acid level is influenced by eating; thus, the blood must be sampled after a period of fasting. Folic acid is sensitive to heat; after separation from the formal elements, therefore, the sample must be cooled quickly.

FERRITIN

Ferritin is an iron-containing protein, and is the most important form in which the organism stores iron.

Chemical structure. Ferritin is a water-soluble compound. At the centre of the ferritin molecule, six iron atoms occupy the vertices of an octahedron. This inorganic nucleus, containing 2,000-4,000 iron atoms and some 74 Å in diameter, is surrounded by a protein sheath, 122 Å in diameter: apoferritin. The molecular weight of apoferritin is about 450,000-480,000 dalton. It consists of 24 subunits (polypeptide chains). The iron-apoferritin complex (ferritin) has immunological and physico-chemical properties that arise mainly from the behaviour of the protein. Ferritin has been isolated from various organs (spleen, bone marrow, liver, testis, kidney, pancreas, ovary, lymph node) of numerous vertebrates (horse, guinea-pig, mouse, rat, rabbit, cat, human), and also from their reticuloendothelial and parenchymal cells, and benign and malignant tissues. A small quantity is also to be found in the blood serum. The amino acid compositions of the ferritins from the various species and their various organs differ slightly from one another; they are known as iso-ferritins. There is not a complete

agreement between the immunological properties of the isoferritins. However, a full immunological cross-reaction is generally obtained between some isoferritin and its apoferritin.

Physiological conditions. About 1,200 mg of iron is stored in adult men, while in women at the reproductive age there is only about 800 mg (due to the higher iron utilization). The iron stored in the tissues is distributed in a ratio of 3:2 in the forms of ferritin and haemosiderin. If the store is increased, both compartments are enhanced, but that of haemosiderin is increased to a greater extent. Haemosiderin is insoluble in water, and it is difficult to mobilize the iron from it. The iron stored in the form of ferritin is more easily accessible: it may be released from it on the action of reductants, xanthine oxidase and chelate formers.

Formation of the iron store protects the organism from being overwhelmed by an excessively large quantity of iron. The synthesis of apoferritin is induced by the iron itself, and the bulk (75%) of the apoferritin is therefore to be found in the form of ferritin. The production of apoferritin may be slowed down with substances inhibiting protein synthesis, but not with substances that block the synthesis of RNA.

The main sites at which iron is stored are the liver, the striated muscles, the spleen and the bone marrow. Although the ferritin content of the blood serum is slight in comparison, the variations in it provide a good reflection of the total ferritin reserves of the organism. Accordingly, the magnitude of the iron stored in the organism may be assessed by determining the serum ferritin level.

Method. Various immunoassay techniques are available for the determination of ferritin: ELISA, IRMA and RIA. All three procedures may be carried out with the use of kits.

Enzyme-labelled immunosorbent assay (ELISA). The principle is as follows. A large amount of antiferritin antibody is bound to the surface of polypropylene tubes. This reacts with the standard ferritin preparation, or with the ferritin content of the serum to be measured. The quantity of ferritin undergoing binding is directly proportional to the ferritin concentration of the serum. Following this, a second antiferritin antibody coupled to alkaline phosphatase is added to the system. The immunologically binding enzyme activity is measured by means of the p-nitrophenol phosphate reaction. The coloured end-product is determined spectrophotometrically.

Immunoradiometric assay (IRMA). This is the most sensitive procedure for the measurement of ferritin. A number of variants are applied. The

principle is as follows. A large amount of antiferritin antibody bound to a solid phase reacts with standard ferritin or with the test serum. The quantity of ferritin binding to the antibody is directly proportional to the ferritin concentration of the serum. The non-bound antigen is removed by washing, and the antigen-antibody complex is then determined on the basis of the immune reaction of a ^{125}I -labelled second antiferritin antibody. The method has the disadvantages that it is time-consuming, consisting of a number of steps, and requires a very large amount of antiferritin antibody.

Radioimmunoassay (RIA) procedures, too have been developed for the measurement of ferritin. Although the sensitivity of these is lower than that of IRMA, they are also suitable for measurements on serum. They are simpler to carry out. The principle of the procedures is as follows. The standard or the serum ferritin competes with the ^{125}I -labelled ferritin for the binding sites of a small amount of antiferritin antibody. The quantity of ^{125}I -ferritin bound is inversely proportional to the ferritin concentration of the serum.

For the procedure to be introduced, above all, ferritin is required. Ferritin may be prepared from various human organs (liver, spleen, heart, etc.). The crude ferritin is precipitated with semisaturated ammonium sulphate from a heated aqueous extract of the homogenized organ. The precipitate is dissolved in physiological NaCl solution, and the NaCl and ammonium sulphate are removed from it by dialysis. Then ferritin is purified by repeated centrifugation (95,000 g) and Sepharose 6-B gel filtration. The preparation obtained this way is used as an immunogen to prepare the antibody, and also as a standard preparation and to produce ^{125}I -ferritin. Efforts have begun to unify the standard preparations used by the different laboratories.

In the immunization, a 1:1 mixture of 50-500 μg ferritin and complete Freund's adjuvant is injected into rabbit, either in a large number of sites in the dorsal skin or i.m. The injections are repeated at three-week intervals. An appropriate titre may be attained within three months. A monoclonal ferritin-antibody has recently been prepared.

The ^{125}I is bound to the ferritin using chloramine T. The reaction is stopped by dilution with sodium metabisulphite and KI. The unbound ^{125}I is removed by chromatography on a Sephadex G-25 or Sepharose 6-B column. Separation with the second antibody may be shortened to 5 min if polyethylene glycol in a final concentration of 20 g/l is added to the RIA mixture at the end of the assay. The sensitivity is 1.5-2.0 μg ferritin/l.

Clinical applications. The serum ferritin concentration is high in neonates (on average $240 \mu\text{g/l}$). It subsequently progressively decreases to about $30 \mu\text{g/l}$ by the age of one year, and remains at this level up to puberty. The serum ferritin concentration in women continues at around $30\text{--}40 \mu\text{g/l}$, whereas that in adult men is $90\text{--}140 \mu\text{g/l}$. The difference between the sexes is smaller in old age.

A decreased ferritin level in the serum raises the suspicion of an iron-deficient condition; a level below $15 \mu\text{g/l}$ is a highly probable indicator of an iron deficiency. In other pathological cases, the ferritin concentration may rise to even $10,000 \mu\text{g/l}$. The cause of this may be the overloading of the organism with iron: following repeated blood transfusions, on the overadministration of iron preparations, or in idiopathic haemochromatosis. However, an elevated ferritin concentration does not necessarily mean an increase in the iron content of the organism. The increase in the ferritin level points to a transfer of the iron reserve of the organism into the iron store. This occurs in anaemias, not based on an iron deficiency, accompanied by an enhanced red blood cell turnover. The phenomenon is also brought about by all those pathological conditions which give rise to an enhancement of the RES function: specific and non-specific inflammations, diseases of the liver and chronic renal insufficiency. In the latter case, a part is further played by the repeated transfusions.

The measurement of ferritin may assist in assessing the malignant haematological diseases and in the follow-up of their therapy. The ferritin level is appreciably elevated on the occurrence of acute leukaemias and their relapses, and at the time of the blastic crisis in chronic myeloid leukaemia. The remission of acute lymphoid and non-lymphoid leukaemias is followed by a decrease in the serum ferritin concentration. The changes in the ferritin level in malignant lymphomas depend on the histological picture of the tumour, too. The highest values are found in Hodgkin's disease and in histiocytic lymphoma; the level is normal in lymphocytic lymphomas, while it is moderately elevated in the mixed histiocytic-lymphocytic lymphomas. These high values normalize during remission of the disease.

Carcinomas of a number of organs are known to be able to produce ferritin in large amounts. Full success has not yet been attained as concerns the detection of isoferritins specific for the organs. It is certain that foetal ferritin and the isoferritins formed by carcinomas are more acidic than the ferritins of the liver and the spleen. Isoferritin produced from the heart has a much closer acidity; hence, it is advisable to make use of

a RIA developed for the isoferritin of the heart if the aim is to follow carcinomas. The determination of ferritin has proved most successful in the case of lung cancer as tumour marker. Increased levels have been found in 64% of lung cancer patients, and in 15% of smokers. In benign lung diseases (tuberculosis, bronchitis, asthma, sarcoidosis, bronchiectasis, pneumonia and benign tumours), the ferritin concentration is usually within the normal range. There is no correlation between the ferritin level and the size of the tumour or its histological picture. The measurement of ferritin may be of use in differential diagnostics and in the monitoring of the effectiveness of treatment, but it is of no help as concerns establishing the stage of the disease or the prognosis. Since the changes in the ferritin and CEA levels do not run in parallel, the certainty of tumour indications can be increased by determining both markers.

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Chapter 15

TUMOUR DERIVATIVES

The substances that will be dealt with here are carcinoembryonic antigen (CEA) and alpha-foetoprotein (AFP).

The detection of specific tumour derivatives is of great importance in the early recognition of neoplasias. Certain tumours are known to produce hormones (Cushing's disease, prolactinoma, paraneoplastic endocrine syndrome), and in non-pregnant women an elevated hCG level is indicative of chorioadenoma or choriocarcinoma. The presence of carcinoembryonic antigen in adults similarly raises the suspicion of malignancy.

The demands made of an ideal tumour marker may be defined as follows: (i) It must be suitable to screen patients at high risk; (ii) It should be specific for the detection of carcinoma; (iii) It should be of assistance in the localization of the tumour; (iv) It should be suitable to follow up tumour therapy.

A tumour marker that is satisfactory from every aspect is not available at present, but some substances may be utilized in the early diagnostics of certain tumours.

CARCINOEMBRYONIC ANTIGEN (CEA)

Chemical structure. CEA is a stable glycoprotein complex. Its molecular weight is 150,000-200,000 dalton. In agar gel electrophoresis, it migrates together with β -globulin. It dissolves well in perchloric acid. In the course of preparation of the antigen, it turned out that there are at least six isomers, the differences mainly being expressed in differences in sialic acid content. It may be extracted from colon carcinoma tissue (20 mg/kg).

Physiological conditions. It is produced in the foetal colon, but is also to be found in the glycocalix of the adult large intestine.

Method. The antigen is obtained from the foetal colon by means of extraction with perchloric acid. It is itself a good immunogen which is administered to rabbit or goat. CEA labelled with ^{125}I is used as radioactive substance. The CEA is extracted from the plasma with perchloric

acid. The extract, antibody and labelled substance are incubated for 1-6 h and the bound and free fractions are then separated with a solid-phase antibody technique.

Clinical applications. CEA is produced to a greater extent by tumour cells. An appreciable increase in the CEA level may be expected during metastasis formation. Tumours of the various organs exhibit a high CEA level; pancreas: 92%, colon: 75%, rectum: 75%, lung: 72%, other gastrointestinal tumours: 67%, and breast cancer: 50%. Elevated CEA levels (to lower extents) are also observed in benign diseases: 50% in pancreatitis, 42% in cirrhosis of the liver, 38% in chronic lung diseases, 21% in inflammations, and 4% in benign mammary tumours. The normal concentration is <2.5 ng/ml after extraction, and <12.5 ng/ml without extraction. A very high level is indicative of malignancy and its dissemination. In such cases, measurement of the CEA level is a suitable means to follow the therapeutic effect: the CEA level falls after surgical removal of the tumour, while it rises again in recurrence.

ALPHA-FOETOPROTEIN (AFP)

Chemical structure. On electrophoresis, AFP runs together with α_1 -globulin. Its molecular weight is 60,000-70,000 dalton. The carbohydrate content is 4%.

Physiological conditions. AFP is to be found in the foetus, where it plays a similar role to that of albumin in adults. Beginning from the 13th week of pregnancy, the function of AFP in the foetus is normally taken over by the serum albumin as the predominant protein. The peak AFP level in the foetus is 3-4 mg/ml, in non-pregnant adults it is 25 ng/ml, and in the 3rd trimester of pregnancy it is 100 ng/ml. A level above 1000 ng/ml in the maternal plasma is indicative of the death of the foetus.

Method. For immunization, human AFP is administered to rabbit. ^{125}I -AFP is used as radioactive substance. The incubation time is 48 h. The calibration curve is prepared with the use of foetal serum or purified AFP. The sensitivity of the method is 1-3 ng/ml. The bound and free fractions are separated with a second antibody.

Clinical applications. AFP can be demonstrated in the plasma even under normal conditions. In liver diseases and in pregnancy, the AFP level is elevated. The normal range is 3-25 ng/ml; in pregnancy it is above

120 ng/ml. An increased AFP level is to be observed in both benign and malignant liver diseases; a level in excess of 1000 ng/ml is indicative of a primary malignant liver disease. Hepatoma, hepatic metastases and teratoma are similarly associated with an enhanced AFP concentration.

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Chapter 16

IMMUNOGLOBULIN E (IgE)

The levels of the majority of the immunoglobulins are sufficiently high to be determined immunochemically. However, the low concentration of IgE in the blood is a serious obstacle to its conventional measurement. A distinction is made between the total IgE concentration and the specific IgE, which increases if a definite allergen is administered. IgE plays a role in allergic states and other immune diseases. Its quantitative determination is of significance in these diseases.

Chemical structure. IgE is a protein containing two heavy and two light chains. Its molecular weight is 200,000 dalton. The chains are linked by disulphide bridges. These are metabolized enzymatically (trypsin, papain).

Physiological conditions. The importance of IgE in non-allergic patients is unknown. As an immune defence system of the organism, the IgE level is elevated in allergic diseases, parasitic infections and other rare diseases associated with an immune deficiency. The IgE fraction (100 $\mu\text{g/l}$) accounts for 1% of the total immunoglobulin concentration (10 g/l).

Method. The procedure depends on whether the total or the specific IgE level is to be determined. The immunosorbent technique is generally used: the antigen (allergen) is coupled to an immunosorbent (e.g. cellulose), and this binds the IgE from the serum of the patient. After the other serum components have been washed out, the bound IgE is combined with ^{125}I -labelled anti-IgE antibody. The unreacted ^{125}I -labelled antibody is washed out of the tube, and the concentration of IgE is assessed from the radioactivity of the adsorbent. The sensitivity of the method is 1-2 ng/ml.

Clinical applications. The total and specific IgE levels are differentiated. The total IgE concentration is elevated in atypical allergy, respiratory diseases (bronchial asthma, obstructive bronchitis, rhinitis), dermatitis (recurrent urticaria) and parasitic infections. An elevated specific IgE level may be observed in various allergic states (allergies relating to grass, trees, pollen, animal dust, foodstuffs, drugs, etc.). In most allergic states, however, *in vivo* provocation tests are required in addition to the *in vitro* tests. The normal IgE level below the age of three

years is 0.01 ng/ml (10 μ g/l), while above the age of ten years it is 0.1-1.43 ng/ml (100-1,430 μ g/l).

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Chapter 17

HEPATITIS B VIRUS

There are two well differentiable clinical forms of hepatitis: (i) infectious hepatitis (hepatitis A), with a high infectivity and a short (30 days) incubation time; (ii) serum hepatitis (hepatitis B), generally with a severe hepatic lesion and a long incubation time (about 100 days). Hepatitis B virus can usually be demonstrated in individuals who have received numerous transfusions; it is also known as Australia antigen, after the site of the discovery. For purposes of RIA, the surface antigen of hepatitis B virus ($\text{HB}_\text{S}\text{AG}$ = hepatitis B surface antigen) is applied.

Chemical structure. Hepatitis B virus is a small, stable infectious particle, which consists of a central nucleic acid core (RNA or DNA) and a protein capsid surrounding this. The diameter of the whole virus (virion) is about 45 nm. The diameter of the $\text{HB}_\text{S}\text{AG}$ used for the RIA test is about 27 nm.

$\text{HB}_\text{S}\text{AG}$ has three differentiable subgroups (a, y and d) and also two further immune subtypes (w and r). The possible variants are ayw, ayr, adr and adw. The most frequent subgroup is the ay.

Hepatitis B is stable against ether; at 60°C it survives for at least 4 h, while there are still survivors after several years at -10 to -20°C . It may be destroyed within 30 min at 100°C , or by ethylene oxide and tricresol.

Physiological conditions. The virus is an intracellular parasite. It exhibits a specific affinity towards certain cell types; hepatitis B causes primarily selective lesion of the liver cells.

Method. A number of serological procedures have been developed for the detection of $\text{HB}_\text{S}\text{AG}$ in human serum. The first RIA was described in 1970: the antiserum is produced in rabbit against purified $\text{HB}_\text{S}\text{AG}$. ^{125}I - $\text{HB}_\text{S}\text{AG}$ is used as labelled substance. The bound and free $\text{HB}_\text{S}\text{AG}$ were initially separated by means of chromatoelectrophoresis, and later by a second antibody technique. The specificity of the antigen is limited because of the difficulties involved in its purification. The determination is generally carried out at 45°C . The serum is first incubated for 2 h with $\text{HB}_\text{S}\text{AG}$ (guinea-pig) bound to a solid phase. The tubes are washed with ion-free water, and ^{125}I -labelled $\text{HB}_\text{S}\text{AG}$ (human) is then added. The sample is in-

cubated for 1 h, and again washed. The radioactivity of each tube is measured and compared with that of a positive control. The HB_sAG level in the positive cases exceeds the serum level in seven definitely negative individuals by a factor of 2.1-fold.

Clinical applications. HB_sAG itself (the protein sheath of the virion) is presumably not infectious, but it is a good indicator of the presence of the virus. Hepatitis B virus may spread as a result of transfusion, with the menstrual blood, **via** the anal or oral route, in the urine, and through sexual contact. After infection, HB_sAG circulates in the blood in the preicteric and icteric phases of the disease. In the 5th-8th weeks after the infection, HB_sAG too is present in the circulation. 10-20% of the adult population, and 35% of children, are chronic carriers. Particularly the non-icteric hepatitis form is of importance since this may lead to chronic hepatitis. Further, it may be demonstrated for a long time in Down's syndrome and in immunosuppressive states. If blood donors are screened by a sensitive RIA, the incidence of all transfusion hepatitis cases can be reduced by 29%, while that of HB_sAG-hepatitis can be decreased by 45%; RIA is therefore an important means of prevention.

The route to be followed in the future will presumably be the development of a more exact procedure for detection of the e antigen and the widespread application of this. This antigen has a molecular weight of about 300,000 dalton; from the aspect of its chemical composition it is a liquid protein. The presence of the e antigen in the blood in conjunction with an active liver disease is characteristic of subjects who earlier contracted hepatitis B virus infection.

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Chapter 18

PHARMACOLOGY

Consideration will be given here to digoxin, barbiturates and morphine.

Ever increasing importance is attached to the determination of various pharmacons with the RIA method. Its use helps the clinician to select and perform the correct therapy, and the possibility also arises for more exact pharmacodynamic examinations. The RIA techniques developed relatively late in this field. This was due to two reasons: (i) the pharmacons and substances consisting of comparatively small molecules, and accordingly the immunization was initially unsuccessful; (ii) the radioactive labelling of the pharmacons also ran into difficulties. At present, however, a very large number of pharmacons can be measured by means of RIA, including new antibiotics, antineoplastic drugs, morphine derivatives, barbiturates and amphetamine derivatives. The method is also of assistance in the diagnostics of pharmacon poisonings. The determination may be carried out on blood or on urine. In clinical practice, predominantly the measurement of digoxin, barbiturates and morphine has become widespread.

DIGOXIN

Chemical structure. The important parts of the digoxin molecule from the aspect of pharmacological activity are a steroid group and a ribosyl residue, which enhances the effect (Fig. 24). The steroid part is digoxigenin while the sugar residue is digitoxose.

Pharmacological effect. Digoxin may be administered orally, intravenously and intramuscularly. Its molecular weight is 781 dalton. The magnitude of the pharmacological effect depends on the mode of administration. It is distributed between the tissues and the serum in a constant ratio of 30:1. It is metabolized in the liver and in the kidney. Normally, its half-life is 33 h, but this period is longer in cases of renal insufficiency. Digoxin acts on the Na,K-activated ATP enzyme of the myocardial plasma membrane and it improves the contractability of the myocardium.

Method. The techniques employed for the detection of digoxin include gas chromatography and procedures based on the ^{86}Rb uptake of the red blood

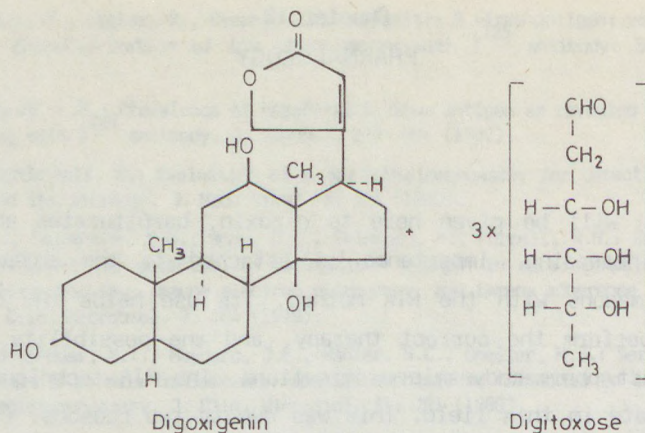


Fig. 24. Chemical structure of digoxin. Digoxigenin forms a glycoside with three molecules of digitoxose, three molecules of H₂O being released

cells and on the Na,K-mediated ATP-ase decrease. However, these methods could not match the precision of RIA. The antigen necessary for the RIA is prepared with a bovine serum albumin conjugate coupled by periodate oxidation to the terminal sugar residue (trisaccharide) of the pharmacon. The antibodies are produced in rabbit or in goat. Digoxin labelled with ³H or ¹²⁵I is used as radioactive substance. The bound and free fractions are separated with a double antibody technique or with dextran-coated charcoal. It is important that the determination be carried out for a definite time at a definite temperature. The digoxin antiserum gives an appreciable cross-reaction with digitoxin, however, the two pharmacons are generally not administered together so that this cross-reaction does not cause disturbance in practice.

Clinical applications. The most important field of application is the detection of digitalis intoxication. The effective and the toxic ranges are well known to lie very close to one another. The signs of poisoning are loss of appetite, nausea, vomiting, diarrhoea, abdominal pain and cardiac arrhythmias (atrioventricular block, ventricular arrhythmia and fibrillation). The sensitivity to digitalis intoxication is enhanced in hypopopassaemia, hypomagnesaemia, hypoxic conditions, alkalosis and hypercalcaemia.

Sampling should be performed 4 h after the administration of digoxin. The compound is fairly stable, but its fast separation from the red blood cells is to be recommended. The normal therapeutic range in the blood serum is 0.8-1.6 ng/ml, while levels above 2 ng/ml lie in the toxic zone. An elevated digoxin level is observed in intoxication and in renal insufficiency (secretion). A low digoxin level is to be found in cases of intestinal hypoxia arising as a consequence of congestion, small bowel disease and weak absorption.

BARBITURATES

Chemical composition and pharmacological effects. A broad scale of barbiturates are used in medical practice. Their neurodepressive effect depends on the chemical composition: tranquillizers and short- and long-acting soporifics are distinguished. The chemical compositions of barbital and phenobarbital are shown in Fig. 25.

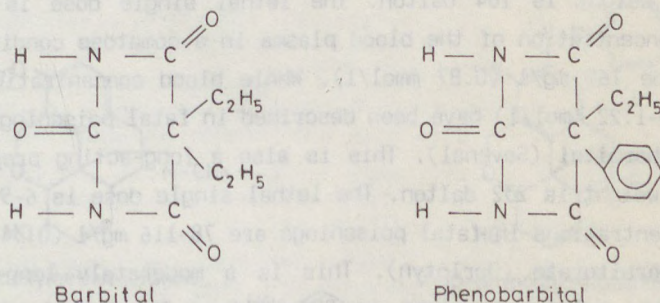


Fig. 25. Chemical structures of barbital and phenobarbital

Method and clinical applications. Fast, sensitive and specific procedures, appropriate for detection of relatively small amounts, are required in practice. Chemical methods were earlier used, but these were difficult to carry out (extraction of the solvent was necessary, frequently with filtration and evaporation) and their sensitivity did not reach the

desired level. The introduction of gas chromatography meant some progress, but even this did not eliminate the need for extraction of the solvent.

5-Allyl-5-(1-carboxy-isopropyl)-barbituric acid is used as barbiturate hapten for the RIA; this is coupled to bovine gamma globulin. Immunization is generally performed in rabbit. High cross-reactions are to be expected between barbital, pentobarbital, phenobarbital and secobarbital. However, the clinician is usually interested in the total barbiturate concentration, and it is therefore not of major importance whether or not the patient takes a number of different barbiturates. ^{14}C -barbital is used as labelled substance. The lower limit of determination is 5 ng/ml barbiturate. The biological half-lives, too have been measured through RIA; these differ considerably in the chemical composition, e.g. 1.7 h for pentobarbital, and 19.3 h for barbital. With regard to the high incidence of barbiturate poisonings worldwide, the exact and rapid determination of the barbiturates is of importance. The sensitivity to barbiturates varies from subject to subject. It depends, among other factors, on the habituation and on the chemical properties of the individual derivatives. For information, we present some data from the toxicological and the forensic medical literature.

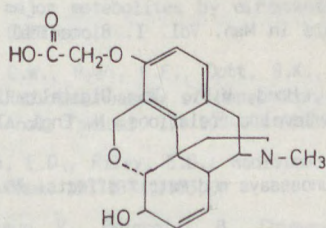
Diethylbarbiturate (Veronal). This is a long-acting preparation. Its molecular weight is 184 dalton. The lethal single dose is 5-20 g. The Veronal concentration of the blood plasma in a comatose condition has been found to be 160 mg/l (0.87 mmol/l). Whole blood concentrations of 90-225 mg/l (0.49-1.22 mmol/l) have been described in fatal poisonings.

Phenobarbital (Sevenal). This is also a long-acting preparation. Its molecular weight is 232 dalton. The lethal single dose is 6-9 g. The whole blood concentrations in fatal poisonings are 78-116 mg/l (0.34-0.50 mmol/l).

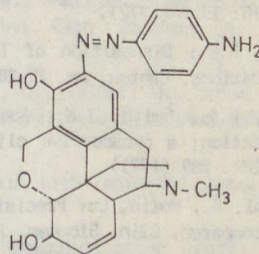
Amylbarbiturate (Dorlotyn). This is a moderately long-acting preparation. Its molecular weight is 226 dalton. The lethal single dose is 2-3 g. The whole blood concentration for soporific action is 8-21 mg/l (0.04-0.09 mmol/l), while that of an overdose is 43-66 mg/l (0.19-0.29 mmol/l). Blood concentrations of 13-96 mg/l (0.06-0.42 mmol/l) have been measured in fatal poisonings.

MORPHINE

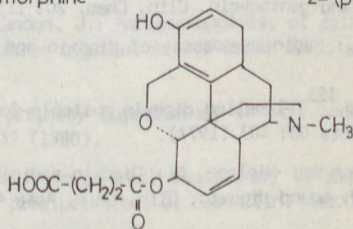
Chemical structure, pharmacological effects and method. Morphine derivatives are widely applied in clinical practice (for the relief of pain and in surgical pretreatment). Unfortunately, its effect in causing euphoria has led to its widespread use as a stimulant for narcotic purposes, too. Its early chemical determination was difficult and gave rise to inaccurate results. The molecular weight of morphine hydrochloride is 376 dalton. Progress was made through the introduction of thin-layer chromatography, but this did not completely eliminate the difficulties either. The development of a RIA technique for morphine necessitated above all the preparation of an appropriate antigen. As a first step, morphine derivatives were prepared (Fig. 26), and a study was conducted, of which compounds can be coupled to protein. It was found that strong binding to bovine serum albumin could be attained through the phenolic OH group in 2-(p-aminophenylazo)-morphine. New Zealand white rabbits were used for immunization. An immune serum against morphine with a suitable titre was produced, but



3-O-carboxymethylmorphine



2-(p-aminophenylazo)-morphine



6-morphenylhemisuccinate

Fig. 26. Chemical structure of morphine derivatives suitable for preparation of antigens required for immunization

this gave cross-reactions with codeine and heroin. As radioactive compound, ^3H -dihydromorphine was used. Even a very low morphine concentration (0.5 ng/ml) can be measured by means of this procedure.

Clinical applications. This technique is used for the demonstration of morphine and its derivatives (codeine and heroin). Its importance lies primarily in the establishment of morphine poisoning. For a person weighing 70 kg, the initial therapeutic dose given i.m. is 10 mg. The oral dose is higher; 30-100 mg. Fatalities have been described after a single dose in excess of 120 mg. The minimum lethal dose is 200 mg, but survival has been reported even after a dose larger than 2,000 mg. In the whole blood, the therapeutic level is around 1 mg/l ($3.5\text{ }\mu\text{mol/l}$), while in fatal cases it is around 40 mg/l ($140.2\text{ }\mu\text{mol/l}$).

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TABLE OF NORMAL VALUES

Adrenocorticotrophic hormone (ACTH)	10-70 pg/ml (5-11 pmol/l)
Aldosterone: supine	40-102 pg/ml (112-285 pmol/l)
after 2 hours' walking	100-305 pg/ml (280-854 pmol/l)
Alpha-fetoprotein (AFP)	3-25 ng/ml
Calcitonin	0.02-0.4 ng/ml (58.6-117 pmol/l)
Carcinoembryonic antigen (CEA), with extraction	< 2.5 ng/ml
without extraction	<12.5 ng/ml
Cholecystokinin (CCK)	58 pg/ml (14.8 pmol/l)
Corticotropin releasing factor (CRF), at the end of pregnancy	traces 67.3±22.8 pg/ml (14.4±4.9 fmol/ml)
in the cerebrospinal fluid	16.3-53.2 pg/ml (3.5-11.4 fmol/ml)
Digoxin (therapeutic zone)	0.8-1.6 ng/ml
Endorphin (β-endorphin)	14-35 pg/ml (4-10 fmol/ml)
Enkephalin ((Met)-enkephalin)	12-34 pg/ml (20-60 fmol/ml)
Ferritin: men	90-140 µg/l
women	30-40 µg/l
neonates	240 µg/l
Folic acid	5-20 ng/ml (11.3-45.3 nmol/l)
Follicle stimulating hormone (FSH): men	1-3 ng/ml (1-3 µg/l)
women, prepuberty	1-3 ng/ml (1-3 µg/l)
fertility	1-3 ng/ml (1-3 µg/l)
mid-cycle	2-9 ng/ml (2-9 µg/l)
menopause	5-29 ng/ml (5-29 µg/l)
Gastrin	20-160 pg/ml (9.4-75.2 pmol/l)
Hydrocortisone (cortisol), 8 a.m.	80-150 ng/ml (224-420 nmol/l)
8 p.m.	30-120 ng/ml (84-336 nmol/l)
Human chorionic gonadotropin (hCG)	< 5 IU/l
Human growth hormone (hGH)	1-10 ng/ml (45-450 pmol/l)
Human placental lactogen (hPL), at the end of pregnancy	traces > 4 µg/ml
Immunoglobulin E (IgE), below 3 years of age	0.01 ng/ml (10 µg/l)
above 10 years of age	0.10-1.43 ng/ml (100-1430 µg/l)
Insulin	5-25 mU/l (36-180 pmol/l)

Table of normal values (cont'd)

Luteinizing hormone (LH): men	1-3 ng/ml (1-3 μ g/l)
women, prepuberty	1-3 ng/ml (1-3 μ g/l)
fertility	1-3 ng/ml (1-3 μ g/l)
mid-cycle	5-60 ng/ml (5-60 μ g/l)
menopause	2-8 ng/ml (2-8 μ g/l)
Luteinizing hormone releasing hormone (LH-RH), beginning of cycle	4.5 $\pm$ 0.45 pg/ml (3.8 $\pm$ 0.38 fmol/ml)
middle of cycle	42.0 $\pm$ 11.4 pg/ml (35.5 $\pm$ 9.6 fmol/ml)
Morphine (therapeutic level)	1 ng/l (3.5 μ mol/l)
Neurophysin I: men	1.0 ng/ml
women	1.1 ng/ml
Neurophysin II: men	0.6 ng/ml
women	0.9 ng/ml
Oestradiol: children	2-20 pg/ml (7.34-73.4 pmol/l)
men	10-60 pg/ml (36.7-220 pmol/l)
women, follicular phase	20-100 pg/ml (73.4-367 pmol/l)
preovulation	200-600 pg/ml (734-2202 pmol/l)
luteal phase	100-300 pg/ml (367-1101 pmol/l)
menopause	5-50 pg/ml (18.3-183 pmol/l)
Oestriol during pregnancy	7-25 ng/ml (24.5-87.5 nmol/l)
Oxytocin: men	1.5 μ U/ml (2.86 fmol/ml)
women	1.4 μ U/ml (2.67 fmol/ml)
Parathyroid hormone (PTH)	1.1-2.5 ng/ml (120-260 pmol/l)
Progesterone: men	0.1-0.3 ng/ml (0.3-1.0 nmol/l)
women, follicular phase	0.2-1.0 ng/ml (0.6-3.2 nmol/l)
luteal phase	5.0-20.0 ng/ml (16.0-64.0 nmol/l)
pregnancy, months 1-3	2.0-50.0 ng/ml (6.4-160 nmol/l)
months 3-9	50-150 ng/ml (160-480 nmol/l)
Prolactin: men	2.0-10 ng/ml (90-450 pmol/l)
women, 10 a.m. to midnight	3.5-20 ng/ml (158-900 pmol/l)
Renin: plasma renin activity (PRA)	2.86 ng/ml/h (2.34 nmol/ml/h)
plasma renin concentration (PRC)	44.0 μ U/ml (44 U/l)
plasma renin substrate (PRS)	1.56 ng/ml (1.28 nmol/ml)
Secretin	5.7 pg/ml (1.9 pmol/l)
Somatostatin (SRIF)	40-110 pg/ml (24.4-67.2 fmol/ml)
Testosterone: men	2.5-8.5 ng/ml (8.75-29.8 nmol/l)
prepuberty	< 0.5 ng/ml (< 1.75 nmol/l)
women	0.2-0.8 ng/ml (0.69-2.8 nmol/l)
Thyrotropic hormone (TSH)	1-4 μ U/ml (1-4 mU/l)
Thyrotropin releasing hormone (TRH), with another method	19.8 $\pm$ 3.1 pg/ml (54.5 $\pm$ 8.5 fmol/ml) 137 $\pm$ 53 pg/ml (377 $\pm$ 146 fmol/ml)
Thyroxine (T4)	45-120 ng/ml (60-150 nmol/l)
Triiodothyronine (T3)	0.8-2.0 ng/ml (1.2-3.1 nmol/l)
Vasopressin	0.5-2.6 pg/ml (0.54-2.81 fmol/l)
Vitamin B ₁₂	100-900 pg/ml (74-664 pmol/l)

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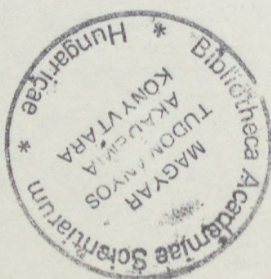
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The introduction of radioimmunoassay (RIA) has brought about fundamental changes in haematological, endocrinological, gastroenterological and cancer research. RIA has an advantage over the previous methods that substances to be measured can be determined with high accuracy and the measurement is reproducible.

In the first chapter the authors summarize the principles of RIA. At the end of the chapter a glossary with the relevant terminology is to be found. The subsequent chapters describe the most important clinical applications of the method. The book is mainly devoted to hormone examinations. Finally the haematopoietic system, tumour derivatives, immunoglobulin E, hepatitis B virus and pharmacology are dealt with.

The individual chapters are lucidly arranged investigating the chemical structure, physiological conditions, methods and clinical application of the material to be discussed. The book is easy to read with numerous figures and is well documented. It may be useful in the work of clinicians, researchers and laboratory assistants.



Ferenc Á. László and Tamás Jankó • GLM-GALAPAGOS ATION OF RAY-DICTIONARY SCAS