Naturally Occurring Anhydro-colour Bases

by
T.R. SESHADRI

Recent Work on Some Natural Phenolic Pigments

by
K. VENKATARAMAN

Structure and Chemical Topography of the Active Site of Aspartate Aminotransferase

by
E.S. SEVERIN
and
N.N. GULYAEV

Asymmetric Syntheses of α-Amino Acids

by
K. WEINGES
and
B. STEMMLE

Synthesis and Biological Activity of Adrenocorticotropic and Melanotropic Hormones

by
K. MEDZIHRADSZKY

AKADÉMIAI KIADÓ, BUDAPEST

RECENT DEVELOPMENTS
IN THE CHEMISTRY
OF NATURAL CARBON
COMPOUNDS

Volume 7

This seventh volume of the series is concerned with various groups of natural compounds, inviting the interest of chemists working in widely different fields of organic chemistry.

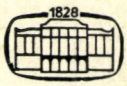
Naturally occurring anhydro colour bases, from their history up to modern structure determinations, are described by T. R. Seshadri (Delhi). Up-to-date methods of structure elucidation of flavonoid and anthraquinoid compounds are also presented in the paper of K. Venkataraman (Poona, India). Actual problems of enzyme chemistry are illustrated by E. S. Severin and N. N. Gulyaev (Moscow) in their comprehensive review on the chemical topography of the active site of aminotransferase. K. Weinges and B. Stemmle (Heidelberg) give a survey of most recent approaches to the asymmetric syntheses of α-amino acids. Adrenocorticotropic and melanotropic hormones are the subject of the part written by K. Medzihradszky (Budapest) reviewing in great detail the structure, chemistry, synthesis and biological effects of these hormones.

AKADÉMIAI KIADÓ
Publishing House of the Hungarian Academy of Sciences
Budapest

RECENT DEVELOPMENTS IN THE CHEMISTRY OF NATURAL CARBON COMPOUNDS

RECENT DEVELOPMENTS IN THE CHEMISTRY OF NATURAL CARBON COMPOUNDS

VOLUME VII



AKADÉMIAI KIADÓ

PUBLISHING HOUSE OF THE HUNGARIAN ACADEMY OF SCIENCES

BUDAPEST 1976

NATURALLY OCCURRING ANHYDRO-COLOUR BASES

by

T. R. SESHADRI

Department of Chemistry, The University of Delhi
Delhi, India

RECENT WORK ON SOME NATURAL PHENOLIC PIGMENTS

by

K. VENKATARAMAN

National Chemical Laboratory
Poona, India

STRUCTURE AND CHEMICAL TOPOGRAPHY OF THE ACTIVE SITE OF ASPARTATE AMINOTRANSFERASE

by

E. S. SEVERIN

and

N. N. GULYAEV

Institute of Molecular Biology, Academy of Sciences
Moscow, USSR

ASYMMETRIC SYNTHESES OF α-AMINO ACIDS

by

K. WEINGES

and

B. STEMMLE

Organic Chemical Institute of the University
Heidelberg, FRG

SYNTHESIS AND BIOLOGICAL ACTIVITY OF ADRENOCORTICOTROPIC AND MELANOTROPIC HORMONES

by

K. MEDZIHRADSZKY

Institute of Organic Chemistry, L. Eötvös University
Budapest, Hungary

AKADÉMIAI KIADÓ

PUBLISHING HOUSE OF THE HUNGARIAN ACADEMY OF SCIENCES

BUDAPEST 1976

Series ISBN 963 05 0702 1
Vol. 7 ISBN 963 05 0784 6

PRINTED IN HUNGARY

CONTENTS

T. R. SESHADRI

NATURALLY OCCURRING ANHYDRO-COLOUR BASES

I. INSOLUBLE REDWOODS

In past centuries a number of redwoods were used as dyestuffs. The more important of them were red sandal, narrawood, barwood and camwood. Since their colouring matters were insoluble in water, they were called "insoluble red" woods as distinguished from those that give soluble extracts like brazil-wood, sappan-wood and logwood. The former provide colouring matters that are substantive to wool and after dyeing cannot be removed from the fibre with alcohol. However, the colours given by these dye woods are somewhat fugitive to light and have therefore been completely superseded by synthetic dyes.

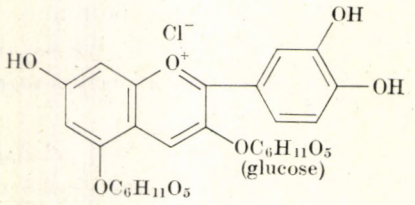
Among the above mentioned woods the most important is red sandalwood obtained from *Pterocarpus santalinus*, a papilionaceus tree grown in tropical Asia. The heartwood is of a dark red colour, and has a faint aromatic odour like that of orris root and hence it got the common name. The red colouring matter is markedly astringent and therefore the wood powder finds use for the healing of wounds and as an astringent. It forms a lead salt and can thus be precipitated.

Narrawood is also obtained from *Pterocarpus* species; it is well known in the Philippines and has constituents very similar to those of red sandal. Barwood is derived from a large, fine tree, *Baphia nitida*, common on the west coast of Africa; it is somewhat similar in colour and has no odour. Another variety of *Baphia nitida* yields camwood which produces deeper shades on dying.

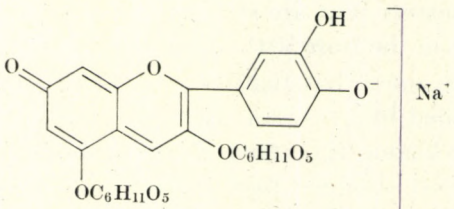
Though the earliest investigation of red sandalwood was made as early as 1832 and the others somewhat later, a clear idea of the chemistry of their components has been obtained only recently. This was due to the complexity and novelty of structure of their molecules. Some simpler compounds of this group of colouring matters that were successfully investigated in the earlier part of this century cleared the way for the study of the more complex colouring matters of the woods.

II. ANHYDRO-COLOUR BASES

In their classical study of naturally occurring anthocyanins, Willstätter and his co-workers (1913–1916) obtained these compounds as deep red flavylium salts (chlorides). They also noticed that under mild basic conditions, these salts underwent change into colour bases (anhydro-bases) which were violet and gave blue solutions with aqueous caustic alkali. This observation was used to explain the blue colour of the cornflower and the red colour of the rose, both of which contain the same pigment, cyanin. The red colour was attributed to the flavylium salt **I** (acid salt of the colour base), and the blue to an alkali salt (**II**) of the colour base. Since the sap of the blue cornflower is actually more acidic than that of the red rose, Robinson later suggested that the blue colour was due to a complex formed by the association of the pigment with a colloid. This has been supported by more recent work.



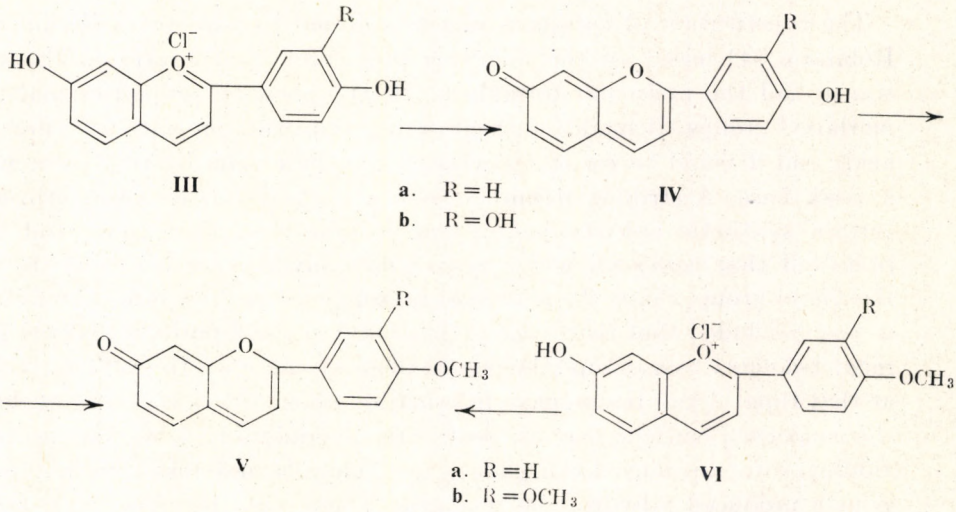
I



II

The colour bases can be prepared by the treatment of flavylium salts with cold aqueous sodium acetate or pyridine, or even with boiling water. Their colours may vary considerably and depend on the structure of the compounds. The large majority of natural anthocyanins, which are flavonol analogues, give violet-coloured compounds, but the more stable colour bases occurring in nature are related to flavones and are deep red. The ready formation of the colour base has been conveniently used by Pratt and Robinson [1] for the conversion of hydroxyflavylium iodides, obtained in the course of synthesis, into the chlorides. The iodides are converted by treatment with sodium acetate or pyridine into the colour bases and these, when dissolved in hydrochloric acid, yield the chlorides. For the formation of a colour base it is necessary to have a free hydroxyl in the 5-, 7- or 4′-position of the parent flavylium salt. Among the possible isomeric forms of the resulting quinonoid structure the 7-keto is the most favoured and most stable. This point has been examined in some detail by Arora *et al.* [2] with

particular reference to the more important 7- and 4′-positions. The main considerations were: (*i*) the known naturally occurring colour bases have the keto group in the 7-position and (*ii*) the synthetic 7-keto compounds have greater stability and give deep red colour like the natural products. This proposal was confirmed by a study of two examples: the colour bases obtained from 7,4′-dihydroxy- and 7,3′,4′-trihydroxyflavylium chlorides. When these compounds were methylated they were found to yield the 4′-methyl and 3′,4′-dimethyl ethers, respectively, whose constitutions were established by alkali fission and also by comparison with the synthetic ethers (*cf.* **III**—**VI**).



III. THE COMPONENTS OF CARAJURA

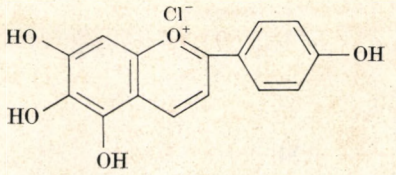
Almost at the beginning of the anthocyanin studies of Willstätter, the first quinonoid anhydro-base, carajurin was isolated by A. G. Perkin in 1914. However, it took more than a decade to establish fully its chemical constitution. The source was carajura, a rare cosmetic pigment prepared by the original inhabitants of Rio Meta and Orinoco in South America from the leaves of *Bignonia chica*. The yield was low and the preparation tedious, hence the pigment appears to have been very costly. It caused a deep red stain on the skin and was, therefore, used as a skin paint. Perkin [3] obtained a sample of carajura from a firm of curio-dealers in London and found it to contain a small quantity of the calcium lake of two colouring matters which had been precipitated on, or intermingled with, a substance

having the nature of ground bark or peat. After treatment with hot dilute hydrochloric acid, alcohol removed the colouring matters in the form of a resin, and from this carajurin was extracted by means of boiling benzene. The compound separated in ruby red crystals, m.p. 204–206°C; it was soluble in boiling dilute alkali with a red colour, and rapidly formed oxonium salts with mineral acid. The minor component insoluble in benzene yielded carajurone.

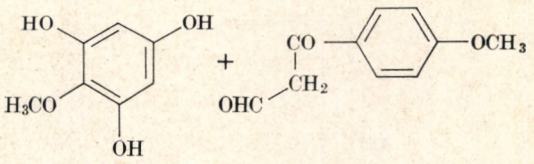
1. CARAJURIN

(*i*) *Constitution*

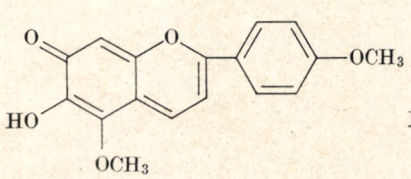
The constitution of carajurin was established by Chapman, Perkin and Robinson [4] based on the following important considerations. The substance had the molecular formula $C_{17}H_{14}O_5$, phenolic properties and two methoxyl groups. It readily formed orange-red oxonium salts with mineral acids and it could be easily recovered from these salts by treatment with a weak base. A striking resemblance was noticed between carajurin and certain synthetic anhydro-benzopyranols known at that time, and this indicated that carajurin was a quinonoid anhydro-base belonging to the flavylium group. Since the salts had no tendency to pass into pseudobase, it was concluded that there was no hydroxyl in the 3-position and the pigment belonged to a group (flavone analogues) unknown to occur in nature at that time. Alkali fission gave information about the side phenyl nucleus; the condensed benzene part was lost in the degradation, being too unstable. Boiling with aqueous alkali gave *p*-acetylanisole and fusion with caustic potash produced *p*-hydroxybenzoic acid. There was, therefore, a methoxyl in the 4′-position. Demethylation with hydriodic acid yielded carajuretin hydriodide ($C_{15}H_{11}O_5I$) which gave the colour base, carajuretin ($C_{15}H_{10}O_5$) when acted upon by cold pyridine. Carajuretin hydrochloride was found to be identical with scutellareinidin chloride (**VII**). The location of the second methoxyl was settled in the following manner: 5,7-dihydroxy-6,4′-dimethoxyflavylium chloride (**VIII**), synthesized from iretol (**X**) and anisoylacetaldehyde, was found to be different from carajurin hydrochloride. The latter gave characteristic ferric reaction, whereas the former did not. Hence the 6-position was ruled out. The choice between the 5- and 7-positions could not be made so definitely. However, earlier work with simple benzopyrylium salts indicated that the colour base of a 5-hydroxy compound could be expected to be unstable and blue or violet, whereas that derived from a 7-hydroxy compound could have a red colour and be stable. The red colour of carajurin and its remarkable stability lent support to the structure **IX**.



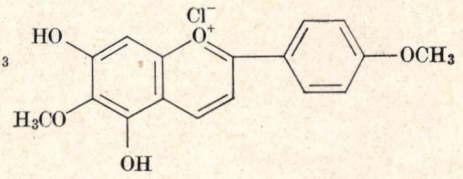
VII



X



IX

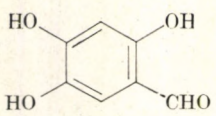


VIII

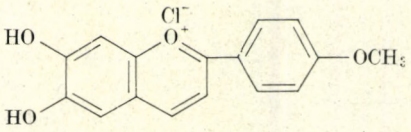
The most important piece of evidence among those mentioned above, was derived from the action of hydroiodic acid which yielded scutellareinidin iodide. However, it remains valid only if the possibility of isomeric change during this demethylation can be excluded. In the related group of flavones the change from 5,7,8-hydroxy compounds into the corresponding 5,6,7-hydroxy isomers invariably takes place by this treatment. Hence this possibility had to be investigated.

One method of approach was to study closely related simpler models. For this purpose 6,7-dihydroxy-4′-methoxyflavylium chloride [5] (**XI**) was prepared from 2,4,5-trihydroxybenzaldehyde (hydroxyquinolaldehyde) (**XII**) and *p*-methoxyacetophenone and, similarly, 7,8-dihydroxy-4′-methoxyflavylium chloride [6] (**XIII**) was synthesized using pyrogallolaldehyde. The latter (**XIII**) gave a prominent emerald green colour with ferric chloride and a violet colour base, and stained the skin black. On the other hand, the former (**XI**) gave a violet colour with ferric chloride and a red colour base capable of staining the skin red just like carajurin. Using 2,4,5-trihydroxy-6-methylbenzaldehyde (5-hydroxyorcylaldehyde) and *p*-methoxyacetophenone, 6,7-dihydroxy-4′-methoxy-5-methylflavylium chloride (**XIV**) was also prepared [7]. It resembled carajurin very closely in colour reactions and properties; the presence of a methoxyl or a methyl group in the 5-position did not seem to make any marked difference.

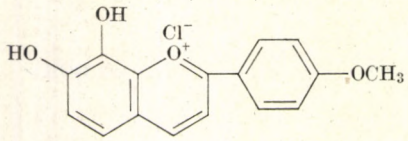
As more important, the possibility of isomeric change during demethylation was examined by Ponniah and Seshadri [8]. They prepared 5,8,4′-trimethoxy-7-hydroxyflavylium chloride (**XV**) from 2,4-dihydroxy-3,6-dimethoxybenzaldehyde (**XVI**) and *p*-methoxyacetophenone, and carried out its



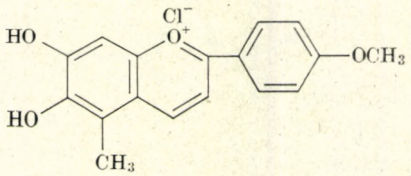
XII



XI

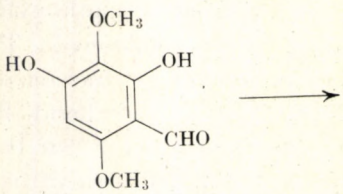


XIII

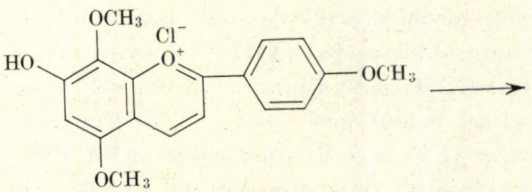


XIV

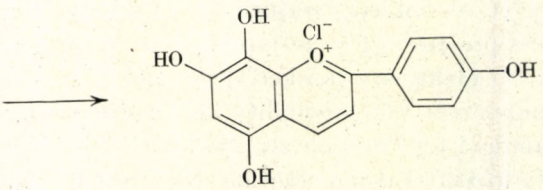
demethylation with hydriodic acid. The resulting flavylium iodide was converted into the colour base which was found to be very different from carajuretin. Its solutions were deep purple in colour; it gave a prominent emerald green ferric reaction and stained the skin black. The hydrochloride should therefore have the structure of 5,7,8,4′-tetrahydroxyflavylium chloride (**XVII**) and hence has been named isocarajuretin hydrochloride. These observations showed that there was no isomeric change during the demethylation of carajurin with hydriodic acid and conclusively supported the constitution as given by Chapman, Perkin and Robinson [4].



XVI



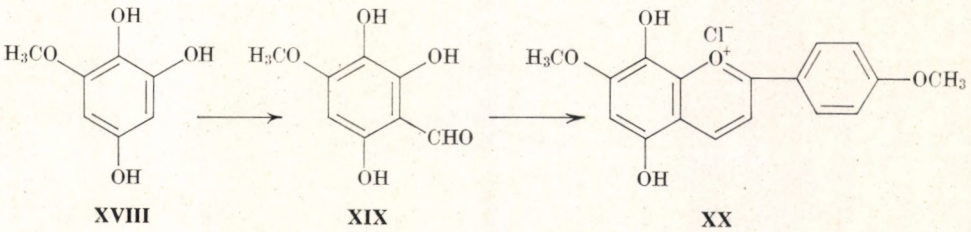
XV

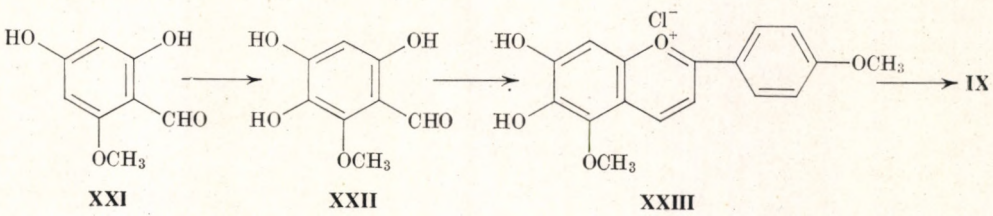


XVII

(*ii*) *Synthesis*

Although a large number of flavylium salts of different types had been prepared earlier, the synthesis of carajurin itself offered difficulties chiefly owing to the presence of the methoxyl group in the 5-position. The synthesis was first attempted by Robinson and Vasey [6]. Starting from 2,6-dimethoxybenzoquinone they made 1,2,4-trihydroxy-6-methoxybenzene (**XVIII**) and subjected it to the Gatterman aldehyde synthesis. Unexpectedly the product was 2,3,6-trihydroxy-4-methoxybenzaldehyde (**XIX**) and on condensation with *p*-methoxyacetophenone it yielded 7,4′-dimethoxy-5,8-dihydroxyflavylium chloride (**XX**); hence this method was unsuccessful. For an unambiguous synthesis of the required 2,4,5-trihydroxy-6-methoxybenzaldehyde (**XXII**) a satisfactory route was provided by the alkaline persulfate oxidation of 6-0-methylphloroglucinaldehyde [9] (**XXI**). Further condensation with *p*-methoxyacetophenone took place satisfactorily, to yield carajurin hydrochloride (**XXIII**).





2. CARAJURONE

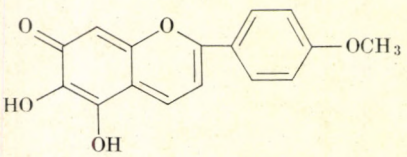
(*i*) *Constitution and Synthesis*

As already mentioned, the benzene-insoluble crystalline component of carajura was named carajurone. Its molecular formula was $C_{16}H_{12}O_5$; it contained one methoxyl group and gave a ferric reaction similar to carajurin (**IX**) and carajuretin (**VII** and **XXXI**). It was, therefore, considered to be a monomethyl ether of carajuretin. Though the pigment available

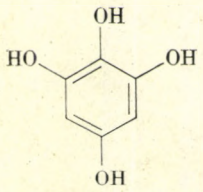
was too little for a detailed study, the perceptible odour of *p*-acetylanisole obtained by boiling carajurone with aqueous potash led to the suggestion that it was closely related to carajurin and that the methoxyl group was situated in the side phenyl nucleus, and thus the structure was **XXIV** [4].

Attempts were first made to synthesize carajurone using the method of Bulow, since earlier Chapman, Perkin and Robinson [4] had successfully condensed iretol with anisoylacetaldehyde and obtained 5,7-dihydroxy-6,4′-dimethoxyflavylium chloride (**VIII**). But the condensation of 1,2,3,5-tetrahydroxybenzene (**XXV**) with the same aldehyde unexpectedly gave a product which was different from carajurone hydrochloride and did not exhibit its characteristic properties [10]. On the other hand, its properties were very similar to those of isocarajuretin hydrochloride and 7,8-dihydroxy-4′-methoxyflavylium chloride and it should, therefore, be 5,7,8-trihydroxy-4′-methoxyflavylium chloride (**XXVI**).

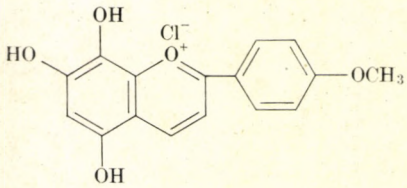
Hence it was necessary to use a suitable derivative of the tetrahydroxybenzene in which the ring closure would take place only in the required



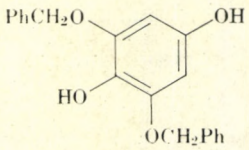
XXIV



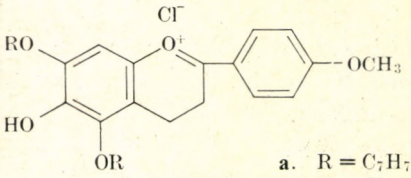
XXV



XXVI



XXVII



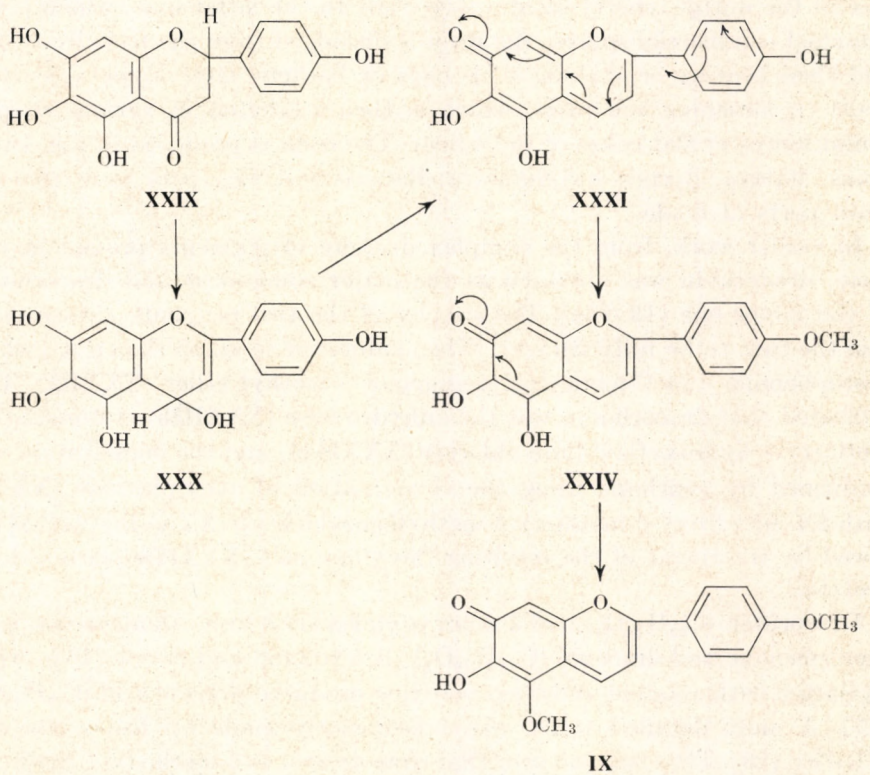
XXVIII **a.** R = C_7H_7 **b.** R = H

XXIV

direction. One such compound readily available was 2,6-dibenzyloxyquinol (**XXVII**), but there was a possibility of complications arising from debenzylation during the condensation. Earlier reports were not uniform about this debenzylation; different results were reported in different experiments and probably the solvent was important. In anhydrous ether the dibenzyloxyquinol condensed with anisoylacetaldehyde and yielded a dibenzyloxy compound (**XXVIIIa**) which underwent debenzylation to give 5,6,7-trihydroxy-4′-methoxyflavylium chloride (**XXVIIIb**). There was no doubt about the direction of ring closure as it happened without debenzylation; further, the properties of the final product agreed with those of 5,6,7-hydroxy compounds. On treatment with sodium acetate, the flavylium salt yielded the colour base carajurone (**XXIV**) (Ponniah and Seshadri [10]).

(*ii*) *Biogenesis*

Arora *et al.* [2] made a proposal for the possible biogenesis of the pigmentr of carajura. This was based on their study of the stability of anhydro-colous



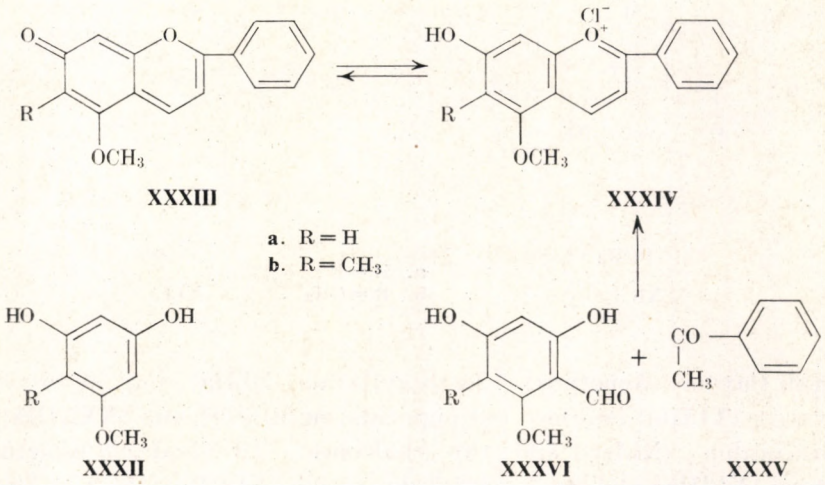
bases and on the findings of Krishnamurty *et al.* [11] that flavanones can undergo base-catalyzed isomeric change into pseudo-bases and colour bases of the corresponding flavylium salts. They suggested that the flavanone carthamidin (**XXIX**) could give rise to carajuretin (**XXXI**) which could undergo step-wise methylation first of the acidic hydroxyl group at the 4′-position to give carajurone (**XXIV**) and then also of the C-5 hydroxyl to form carajurin (**IX**).

IV. DRAGON'S BLOOD RESIN

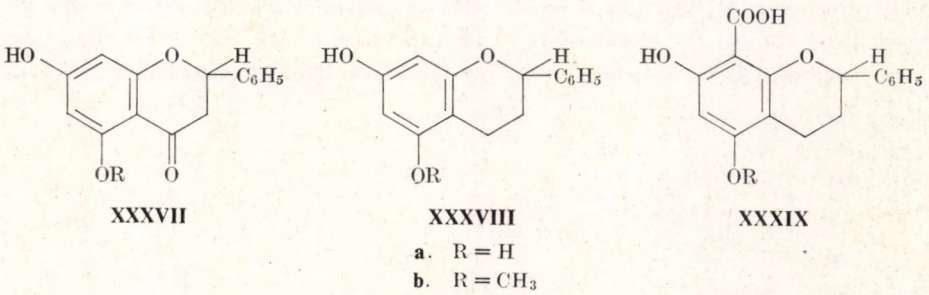
At about the same time when the constitution of carajurin was established, the first investigation on the dragon's blood resin was also reported by Frankel and David [12]. This coloured resin obtained from plants belonging to the genera *Dracaena* and *Daemonorops* has been used in medicine, in making zinc line engravings, and as a colouring matter for varnishes. The resin from *Dracaena* (trees) is collected from incisions made in the stem, but in the case of rattan palms *(Daemonorops)* it is gathered from the surface of the unripe, cherry-sized, scaly fruit. In the latter case, which is the principal commercial source, the resin is dislodged from the fruit by drying and then beating or shaking it in bags or baskets with coarse objects to assist in loosening the resin, which is then marketed in various shapes, round lumps or flat cakes of red colour. The resin is produced in the Andamans, Malaya, Borneo and Sumatra; Batavia and Singapore have been the main ports of trade.

In earlier work, from the complex mixture of pigments present in this resin, dracorubin was obtained as the major component and dracorhodin as the minor one [13, 13a]. The simpler of the two pigments, dracorhodin was the first to be fully studied. When subjected to alkali fission it yielded acetophenone and C-methylphloroglucinol β-methyl ether (**XXXIIb**). This indicated that dracorhodin was the anhydro-base (**XXXIIIb**) of 6-methyl-5-methoxy-7-hydroxyflavylium chloride (**XXXIVb**), and the constitution was confirmed by synthesis using the condensation of acetophenone (**XXXV**) with 2,4-dihydroxy-6-methoxy-5-methylbenzaldehyde (**XXXVIb**) [14, 15] followed by treatment of the resulting flavylium salt (**XXXIVb**) with sodium acetate.

Dracorubin ($C_{32}H_{24}O_5$) had a more complex structure. Alkali fission gave acetophenone and draconol ($C_{24}H_{20}O_6$) (Brockmann and Haase [16]), while oxidation with alkaline hydrogen peroxide produced dracoic acid ($C_{17}H_{16}O_5$) [17]. A more detailed study was subsequently made by Robertson and Whalley [18]. They have shown that dracoic acid is 5-methoxy-7-hydroxy-

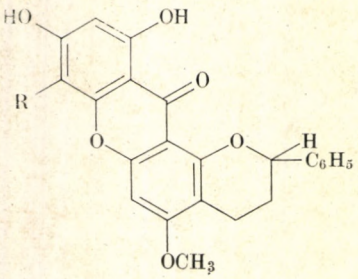


flavan-8-carboxylic acid (**XXXIXb**) undergoing decarboxylation to 5-methoxy-7-hydroxyflavan (**XXXVIIIb**), which can be obtained by the Clemmensen reduction of alpinetin (**XXXVIIb**). The acid **XXXIXb** has been synthesized from noralpinetin (**XXXVIIa**) through the intermediate stages **XXXVIIIa** and **XXXIXa**.



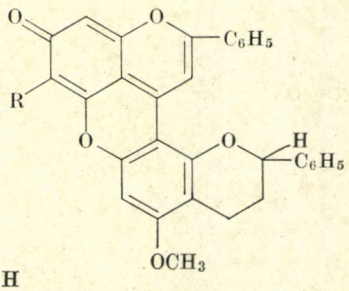
The phenol, draconol was considered to have the structure **XLb**, partly by analogy with dracorhodin and partly because a number of properties of its partial methyl ether were reminiscent of a 1-hydroxyxanthone structure. Since dracorubin yields draconol by the loss of acetophenone with the simultaneous formation of the carbonyl and 1-hydroxy groups of the xanthone residue, Robertson and Whalley [18] assigned structure **XLIb** to dracorubin.

Cardillo *et al.* [19] have recently isolated a number of other compounds from the extract of a commercial sample of dragon's blood resin imported from Singapore; careful chromatographic procedures yielded two new



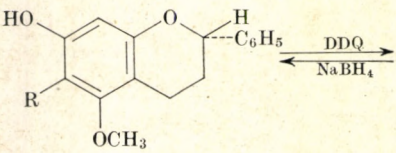
XL

a. R = H
b. R = CH_3

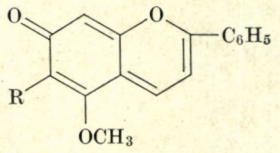


XLI

natural flavans: 5-methoxy-7-hydroxyflavan (**XLIIa**) and its 6-methyl derivative (**XLIIb**); two new red pigments: nordracorhodin (**XXXIIIa**) and nordracorubin (**XLIa**); and two chalcones: 2,4-dihydroxy-6-methoxychalcone (**XLIIIa**) and its 5-methyl derivative (**XLIIIb**). The two flavans were obtained from the less polar fractions in the chromatography of the acetone extract and their structures determined from their spectral data and ready conversion into nordracorhodin (**XXXIIIa**) and dracorhodin (**XXXIIIb**), respectively, which were also obtained in the later fractions of chromatography. Reduction of these pigments with $NaBH_4$ afforded the flavans and reoxidation could be effected with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). The 6-methylflavan **XLIIb** was oxidized even by air, indicating its greater oxidizability. The absolute configuration of the above two flavans was established as 2*S* by CD data. Nordracorhodin (**XXXIIIa**)

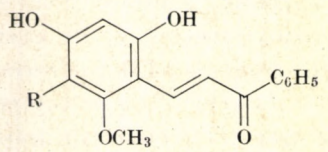


XLII

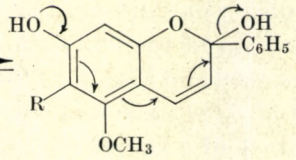


XXXIII

a. R = H
b. R = CH_3



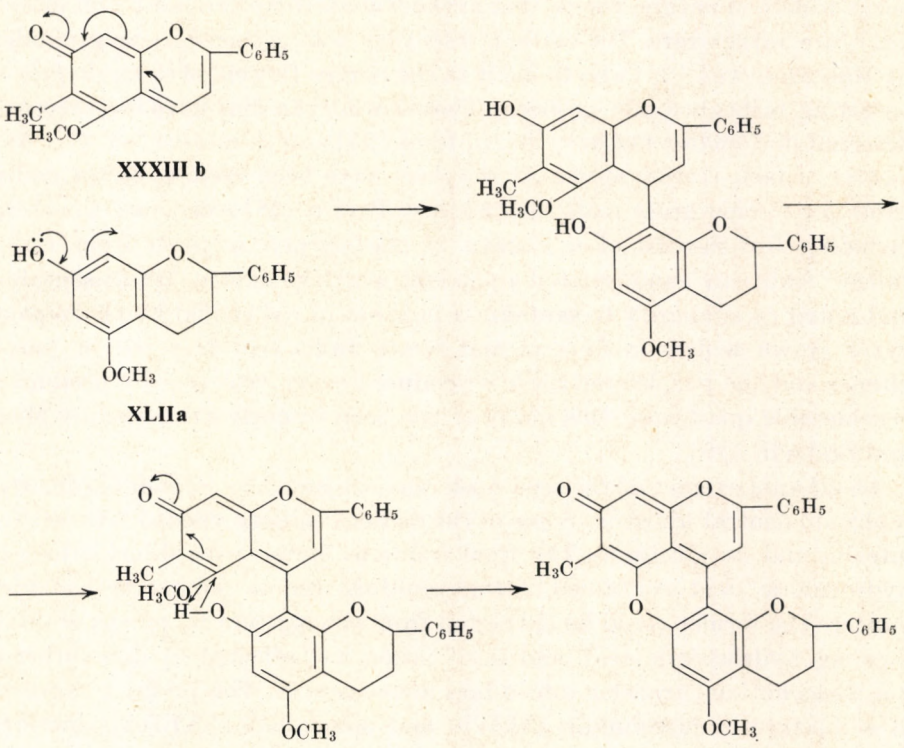
XLIII



was also synthesized by the condensation of acetophenone with 2,4-dihydroxy-6-methoxybenzaldehyde (**XXXVIa**).

The two chalcones (**XLIIIa** and **b**) were closely related to nordracorhodin and its homologue, and were formed when solutions of the latter were allowed to stand; the interconversion is expressed by formulas **XLIII** to **XXXIII**.

Dracorubin and nordracorubin were obtained as red pigments from the more polar fractions of chromatography. The structure originally proposed for dracorubin (**XLIb**) has also been supported by NMR and mass spectral data, and that of nordracorubin (**XLIa**) followed from its close similarity, except for the lack of the aromatic CH_3 group. It was observed as significant that nordracorubin was minor in amount and the dracorubin isomer with the methyl group in the flavan part was not detected. These along with the markedly higher oxidizability of the 6-methylflavan provided support for the biosynthesis of dracorubin as proposed by Whalley [20]. It involves as the first step the condensation of the appropriate colour base (**XXXIIIb**) with the required flavan (**XLIIa**), followed by oxidation. Similar conden-



sation has been found to take place between a flavylium salt and a catechin [21].

Whalley made use of demethoxydracorhodin as the colour base component. Such a type does not occur in the resin. Therefore a modification using dracorhodin itself is given here. The final stage of cyclization involving loss of methoxyl has been found to take place in several analogous cases of xanthone synthesis [21a].

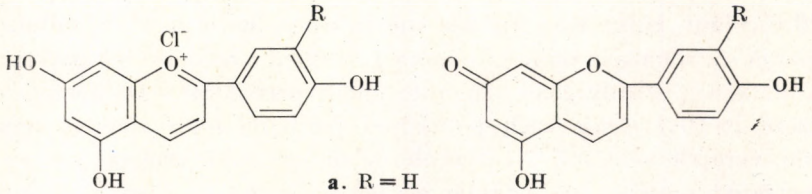
Dracorubin and nordracorubin are examples of biflavonoids made up of a colour base and a flavan. They have the 4,8-interlinking as is common in many proanthocyanidins, and have further a definitely established ether linkage involving the hydroxyls of the 5,7-positions which seems to be also present in some proanthocyanidins [22].

V. SORGHUM PIGMENTS: LUTEOLINIDIN AND APIGENINIDIN

Though simpler flavones and flavanones like apigenin and naringenin occur widely in nature, the related anthocyanidins, apigeninidin and luteolinidin are rather rare. The earliest discovery was gesneridin (apigeninidin) by Robinson *et al.* [23] who found it in the orange flowers of *Gesneria fulgens* occurring as its glucoside, gesnerin. These anthocyanidins have been recently detected in *Sorghum vulgare* [24], in ferns [25] and in mosses [26, 27]. As a matter of fact, certain species of *Sorghum* have been used in Egypt and in India for dyeing grass mats and leather. Perkin [28] examined the stems including leaf-sheaths of a variety of *Andropogon sorghum* from Egypt called "Sikhtyan", and isolated a pigment which he named "durra-santalin" and noted its similarity to santalin, a pigment of red sandal wood. *Sorghum durra* grown widely in Central and South India has deep red or purple glumes and the purple seed husk is produced every year as a by-product in considerable quantities; this material has been recently examined by Misra and Seshadri [29].

An ethanol extract of the seed husk was subjected to column chromatography on neutral alumina. Some of the earlier fractions yielded 7-O-methylluteolin and its glucoside. The final fractions eluted with dilute ethanolic hydrochloric acid contained anthocyanidins and a polymeric colouring matter. The former could be extracted from the mixture by means of dilute aqueous hydrochloric acid, and purification was effected by dissolution in amyl alcohol and transfer into dilute aqueous acid. The product consisted of a mixture of luteolinidin (**XLIVb**) and apigeninidin (**XLIVa**) chlorides.

Thus the husk seems to be a good source of these two anthocyanidins. Tests indicated that they are most probably present as flavylium salts; however, in the process of dyeing the stable colour bases (**XLV**) are involved.



XLIV **XLV**

The polymeric pigment obtained from the glumes had the properties of an anhydro-colour base and gave protocatechuic acid and phloroglucinol on alkali fission. When the methyl ether was oxidized with permanganate, it gave both veratric acid and anisic acid. These results indicated that the polymer contained both the above-mentioned anthocyanidin units.

VI. RED SANDAL PIGMENTS: SANTALIN-A AND SANTALIN-B

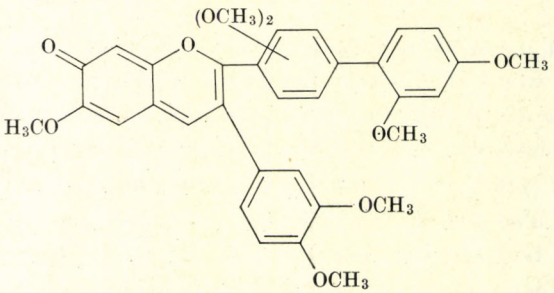
The important insoluble redwoods have already been mentioned. Among them red sandal-wood *(Pterocarpus santalinus)* has been most fully investigated. A number of colourless crystalline components have also been found in it and their constitutions established. Some of them belong to the group of isoflavanoids (pterocarpin, homopterocarpin and santal); later normal flavonoids were also found, liquiritigenin (flavanone) and isoliquiritigenin (chalcone). Thus both flavone and isoflavone derivatives occur in this wood besides pterostilbene which is also a characteristic component, and their co-occurrence may have some bearing on the structure of the red colouring matter.

A crude isolation of the colouring matter called santalin was first made by Pelletier as early as 1832 [30]. It was obtained in a crystalline state by Cain and Simonsen in 1912 [31], but this sample was not pure either, and had a lower melting point. Later Robertson and Whalley [32] obtained the substance in a purer state and made a detailed study. Though owing to complexities their molecular formula was not correct, very useful chemical work was accomplished and a tentative structure suggested. The compound had the properties of a quinonoid anhydro-colour base and formed a hydrochloride which was called santalylium chloride. The molecule had

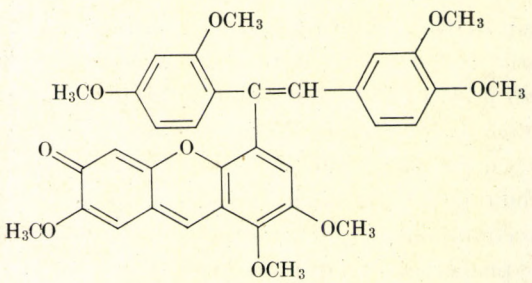
no C-methyl groups, but contained methoxyls and could be fully methylated with an excess of methyl sulfate and aqueous methanolic sodium hydroxide, or by the methyl iodide–potassium carbonate–acetone method, to obtain a complete methyl ether which retained the properties of the quinonoid anhydro-colour base. But further methylation with methyl sulfate and potassium carbonate in boiling benzene furnished a colourless 0-polymethylsantalol, which readily gave a picrate and a perchlorate. Oxidation of the complete methyl ether with potassium permanganate yielded veratraldehyde, veratric acid and 2,4-dimethoxybenzoic acid, whereas fission with concentrated aqueous methanolic potash gave 2,4-dihydroxy-5-methoxybenzaldehyde. Based on these results, structure **XLVI** was proposed for santalin methyl ether.

Santarubin, a related colouring matter obtained from camwood and barwood, formed an isomeric complete methyl ether which was very similar in its properties except for the formation of 2,4-dimethoxybenzaldehyde in the oxidation instead of veratraldehyde. It was therefore given the isomeric structure, in which the two side phenyl rings were interchanged.

Dean [33] observed that the above structure of 0-methylsantalin failed to explain many of its reactions and did not agree with the current ideas



XLVI



XLVII

of biogenesis. He therefore proposed a xanthone structure (**XLVII**) which, however, had no definite experimental support.

Ravindranath and Seshadri [34a, b] have very recently examined the heartwood in detail and found that it contains a number of red pigments, two of which, *viz.* santalin-A, $C_{30}H_{17}O_7(OCH_3)_3$ and santalin-B, $C_{30}H_{16}O_6$-$(OCH_3)_4$, are major. Since both compounds give the same complete methyl ether on methylation, they are partial methyl ethers of the same polyphenol, for which the name santalin should now be reserved.

Santalin permethyl ether, for which the new molecular formula $C_{38}H_{36}O_{10}$ has been proposed, has the characteristic property of melting first at 155–156°C, resolidifying, and melting again at 230–231°C. Its anhydrobenzopyranol character (**a**, Chart 1) has now been established by the preparation of a number of typical derivatives; the reactions are summarized in Chart I.

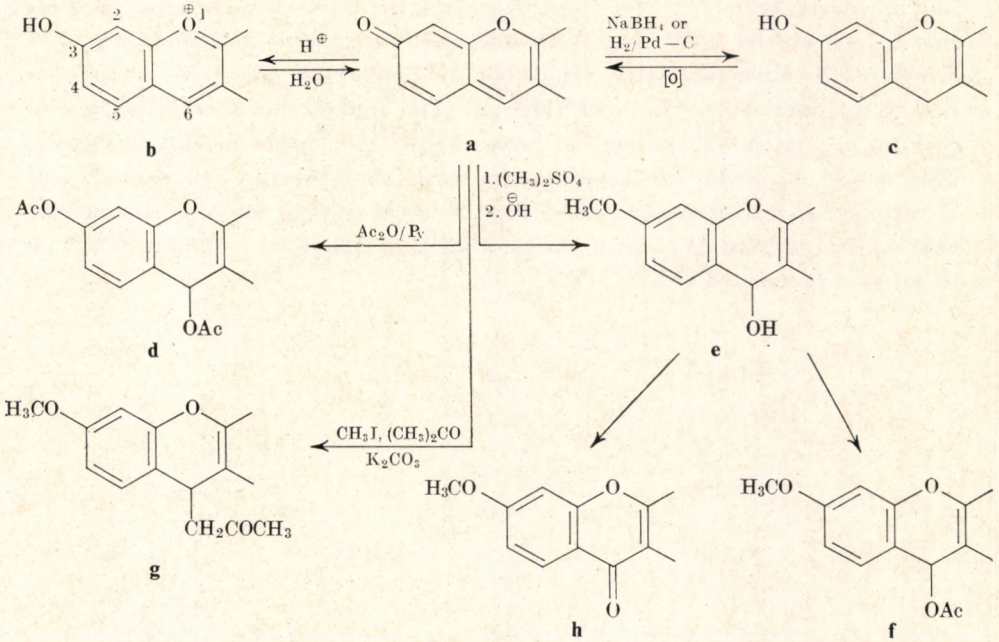
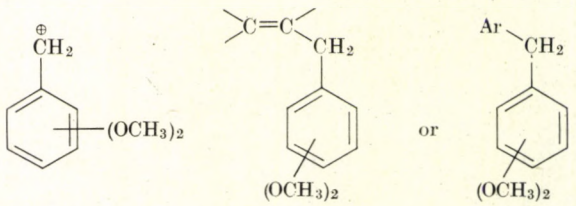


Chart I

Compound **g** (Chart I) was formed by an interesting participation of the solvent acetone during methylation; it was obtained as a colourless solid indicating aromatization of the quinonoid system. Its formulation as in **g** was based on its IR spectrum showing the aliphatic carbonyl band at 1710 cm^{-1}, and its mass spectrum showing peaks at m/e 724 (M^+),

709 ($M-CH_3$), 681 ($M-COCH_3$) and 667 ($M-CH_2COCH_3$), the last mentioned peak being the base peak; the structure was supported by observations using simpler models. The molecular formula of compound **g** was established as $C_{42}H_{44}O_{11}$ by the mass spectrum and this confirmed the above molecular formula of santalin permethyl ether also.

The NMR spectrum ($CDCl_3$) of the permethyl ether has been quite useful; it showed eight separate signals in the region δ 3.80–4.20 indicating the presence of eight methoxyls in the molecule. In addition, the spectrum showed a multiplet for eight aromatic protons at δ 6.60 to 6.80, a one-proton doublet at 7.20 ($J = 9$ Hz) and a one-proton singlet at 9.55 (C-6 H); these signals accounted for the presence of ten aromatic protons including those in the anhydro-base unit. Thus 34 of the 36 protons of santalin permethyl ether were accounted for. The nature of the remaining two protons was revealed by a careful examination of the mass spectra of santalin permethyl ether and its derivatives. All the spectra invariably showed an intense peak at m/e 151. Since the NMR spectrum had already indicated that the compound is essentially aromatic with a number of methoxyls, this peak should be due to a dimethoxybenzyl (**XLVIII**) ion. The signals due to the methylene protons in the NMR seemed to have merged with the methoxyl signals. They could, however, be located at δ 4.05 in the spectra of santalin-A and B recorded in trifluoroacetic acid. The chemical shift of the benzylic methylene indicated that this group was attached either to a double bond or to an aryl moiety (**XLIX**).



XLVIII **XLIX**

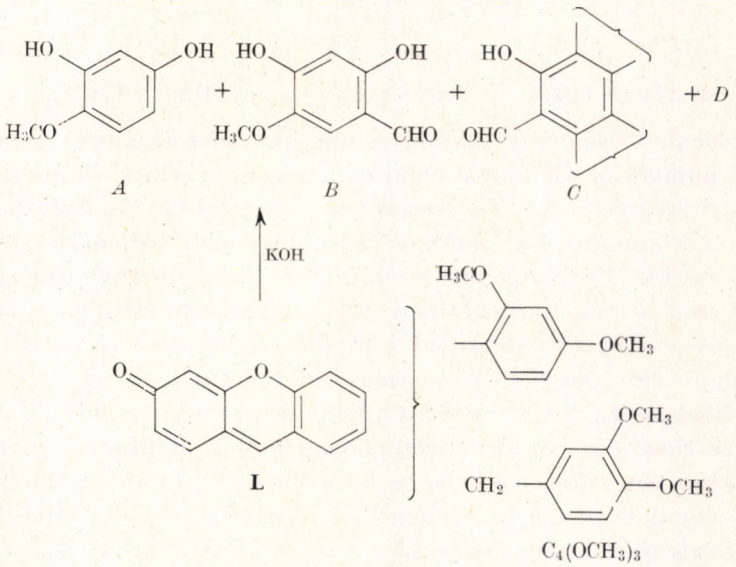
1. ALKALI DEGRADATION OF SANTALIN PERMETHYL ETHER

Alkali degradation of santalin permethyl ether gave a number of products of which four compounds, marked *A*, *B*, *C* and *D*, have been isolated in pure form and characterized. Compounds *A* and *B* could be identified as 4-methoxyresorcinol and 2,4-dihydroxy-5-methoxybenzaldehyde, respectively. Compounds *C* and *D* will be referred to later; but it should be mentioned here that compound *C*, $C_{31}H_{32}O_9$ (M^+ 548), could be considered to be an

o-hydroxy aromatic aldehyde based on its sparing solubility in aqueous alkali, positive ferric chloride colour and NMR spectrum. Such formation of two *o*-hydroxy aromatic aldehydes by treatment with alkali requires the presence of a fluorone unit (see **L**) in the molecule. This has been unequivocally established when santalin permethyl ether was obtained by the condensation of compound *C* with 4-methoxyresorcinol, using HCl in acetic acid (see **LVI** later).

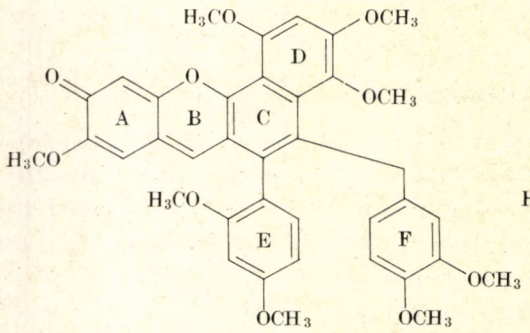
2. PERMANGANATE OXIDATION

Oxidation of the permethyl ether with neutral $KMnO_4$ yielded three acids, *E*, *F* and *G*. The first two have been identified as 2,4-dimethoxybenzoic acid and veratric acid, respectively. Acid *G* will be discussed later. The structure for the permethyl ether could now be written as (**L**), bearing in mind that veratraldehyde, isolated as one of the products of oxidation by Robertson and Whalley [32], could have come from a homoveratryl unit.

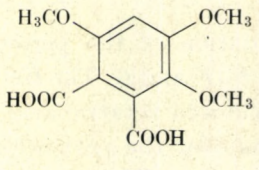


Substitution in rings A and B are defined by the isolation of 2,4-dihydroxy-5-methoxybenzaldehyde. Therefore, 2,4-dimethoxyphenyl, homoveratryl and $C_4(OCH_3)_3$ residues have to be attached to ring C. Of the various ways in which this can be done, structure (**LI**) is favoured in view of the observation that the NMR spectrum of octa-O-methylsantalanol diacetate (compound corresponding to **d**, Chart I) showed the alcoholic (C-6) acetoxyl

signal at δ 1.2, an unusually high field. This can only be explained as a result of the powerful shielding effect of an adjacent 2,4-dimethoxyphenyl ring. The homoveratryl unit is placed in the *ortho* position to the 2,4-dimethoxyphenyl unit on the basis of mass spectral fragmentation and biogenetic considerations discussed later.



LI



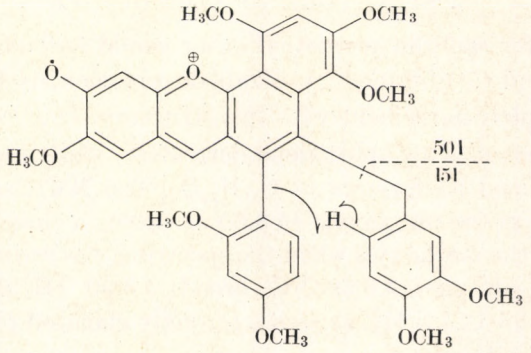
LII

3. STRUCTURE OF SANTALIN PERMETHYL ETHER

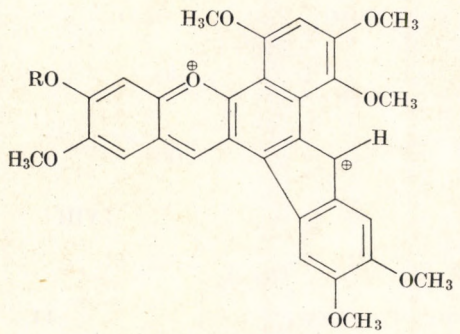
The molecular formula of santalin permethyl ether requires the presence of a total number of 21 double bond equivalents of which 18 are satisfied by the part-structure (**L**). The remaining three double bond equivalents, four carbon atoms and three methoxyls require another aromatic ring fused to ring C as (**LI**). Permanganate oxidation of the permethyl ether should, therefore, give a trimethoxyphthalic acid derivable from ring D. The acid *G* mentioned earlier was identified with this on the basis of its chromatographic mobilities and colour reactions. Of the two possible trimethoxyphthalic acids, *viz.*, 3,4,5- and 3,4,6-trimethoxyphthalic (**LII**) acids, the former was ruled out by direct comparison with a synthetic sample. For incorporating the latter, the choice between 9, 10, 12 and 9, 11, 12 substitutions could be made on biogenetic grounds (see later). Accordingly, the methoxyls were located as in **LI**.

4. MASS SPECTRUM OF THE PERMETHYL ETHER

All the chemical and spectral data on santalin permethyl ether are in complete agreement with structure (**LI**). Thus, the mass spectrum showed four intense peaks, m/e 652 (M^+ 100%), 501, 257 and 151. The peaks at m/e 501 and 151 obviously arise from a cleavage at the benzylic methylene



LIII

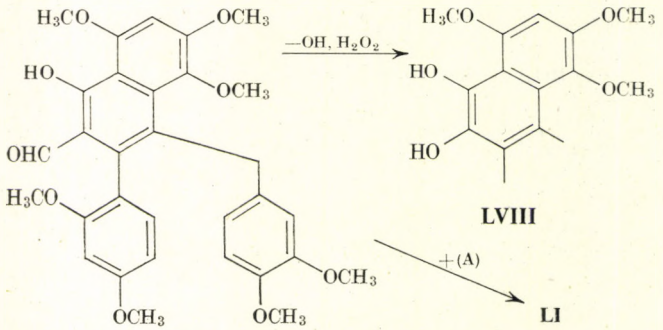


LIV R=H m/e 257
LV R=CH_3 m/e 264

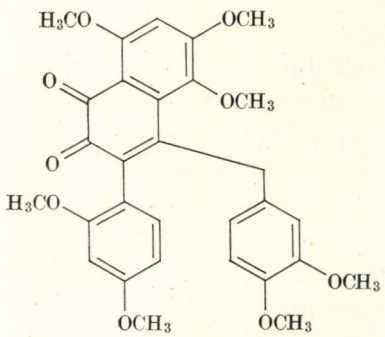
leading to complementary units. The peak at m/e 257 requires explanation. It would appear that ring E undergoes elimination along with a H atom from ring F, and the resulting dibenzocyclopentadiene acquires resonance stability with rearrangement involving the migration of a proton to the C=O group leading to the doubly charged aromatic ring structure **LIV**, which has a mass of 514 and gives a peak at 257. This is confirmed by the isotope mass peak at 257.5 and it represents a stable doubly charged ion with the charges localized in the pyrylium and cyclopentadienyl systems. The above explanation is also supported by the fact that the mass spectra of compounds corresponding to **e** and **g** (Chart 1) which bear a methyl on the C-3 oxygen show the corresponding peak at m/e 264 indicating a difference of 14 mass units (**LV**). Further, the observation of peaks due to the doubly charged ions **LIV** and **LV** would indicate that the 2,4-dimethoxyphenyl and homoveratryl units are in *ortho* positions attached to ring C of **LIII**.

5. STRUCTURE OF COMPOUND *C*

Structure **LI** for santalin permethyl ether would indicate that the structure of compound *C*, obtained by alkali degradation, is **LVI**. Its spectral data are in complete agreement with this structure. Thus, the UV spectrum has indicated that it is a naphthalene derivative, while the mass spectrum (M^+ 548) confirmed its formula as $C_{31}H_{32}O_9$. The NMR spectrum showed the presence of seven methoxyls (δ 3.50 to 4.00), a diarylmethane group (separated from the methoxyls when the spectrum was recorded in benzene), seven aromatic protons (δ 6.50, 6H, broad; δ 6.90 1H, d, J = 9 Hz), an aldehyde proton (δ 11.17, 1H, s) and a strongly chelated phenolic hydroxyl proton (δ 14.17, 1H, s).



LVI **LVIII** **LI**



LVII

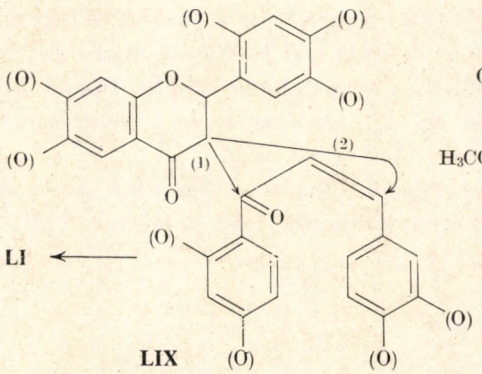
6. STRUCTURE OF COMPOUND *D*

Compound *D* (**LVII**), the fourth product of alkali degradation of santalin permethyl ether, was obtained as a red semi-solid, $C_{30}H_{30}O_9$ (M^+ 534, accompanied by a fairly intense $M^+ + 2$ peak). Its red colour, absorption spectrum

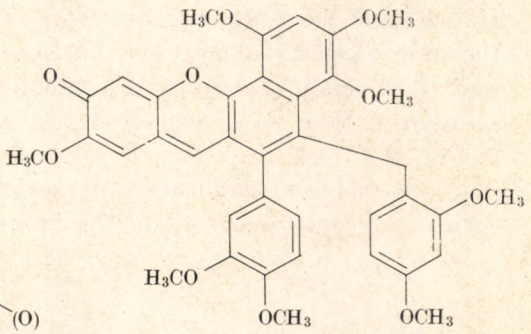
(λ_{max} 245 and 382 nm) and fragmentation pattern, particularly the appearance of peaks at m/e 506, 491 and 463, indicated that it is quinonoid in nature. Its NMR spectrum showed the presence of the diarylmethane group (δ 3.42, 2H, broad), seven methoxyls (δ 3.60–4.00, 21H), and seven aromatic protons (δ 6.00–7.00, 6H, m; 7.50, 1H, s). These data agree with the *o*-quinone structure **XLVII**, which is obviously formed by the oxidation of **LVI** during the alkali degradation *via* the intermediate **LVIII**. The structure of compound *D* has been confirmed by its preparation from compound *C* by Dakin's oxidation with alkaline hydrogen peroxide.

7. BIOGENESIS

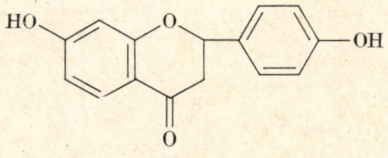
The proposed structure (**LI**) for santalin permethyl ether represents a new skeleton with many unique features. Thus, it is on one hand a quinone methide and on the other a naphthalene, fused together to form a benzofluorone; the molecule also has an extra phenyl group and a benzyl group, and eight methoxyls located in the various rings. Accordingly, it would be of interest to discuss the probable biogenetic origin of the compound. It appears to be derivable from two C_6-C_3-C_6 units (**LIX**) and can thus be a biflavonoid. In this connection the presence of liquiritigenin (**LX**, flavanone) and isoliquiritigenin (**LXI**, chalcone) in red sandal-wood appears to be significant.



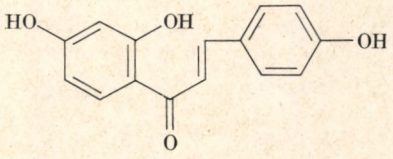
LIX



LXII



LX

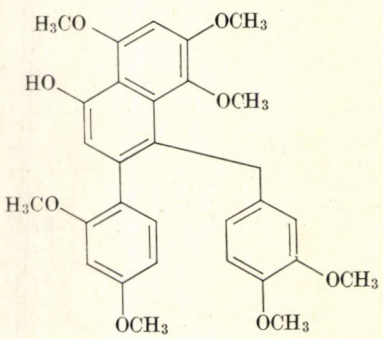


LXI

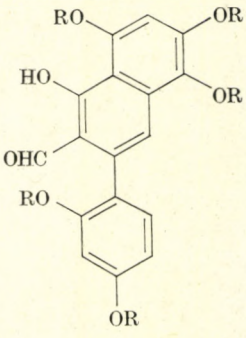
The reactive methylene of the flavanone part may be attached to the chalcone in two ways. Path (1), followed by a series of steps which can easily be envisaged, would lead to santalin, while path (2) would give rise to a structure of the type **LXII**. It has been mentioned above that Robertson and Whalley [32] found that the heartwood of *Baphia nitida* (camwood and barwood) contained, in addition to santalin, another pigment which they called santarubin; its methyl ether was isomeric with that of santalin and showed very similar properties. However, on oxidation santarubin methyl ether gave 2,4-dimethoxybenzaldehyde, while santalin methyl ether gave veratraldehyde. It is possible that structure **LXII** represents santarubin methyl ether.

Arnone, Merlini and Nasini [35] have made a more detailed study of santalin A, which is the trimethyl ether of santalin and is the major component of the wood pigments. They have also provided independent evidence to support the constitution of the permethyl ether. For example, in the alkali fission of the permethyl ether they have obtained compound $C_{30}H_{32}O_8$ (**LXIII**) which could be considered to be complementary to 2,4-dihydroxy-5-methoxybenzaldehyde in the fission of the fluorone structure. From santalin-A they have also obtained another fission product $C_{20}H_{18}O_7$ (**LXIV**) which arises as the result of not only the break-down of the fluorone system, but also of the loss of the homoveratryl (F) unit; this provided further evidence for the location of this unit in ring C *ortho* to the dimethoxyphenyl substituent (E) and led to the suggestion that rings A and F carry three hydroxyls, the three methoxyls being distributed in rings D and E. Based on the isolation of a small amount of 2,4-dihydroxybenzaldehyde in the fission of santalin-A, they have suggested that all its three methoxyls are present in ring D.

In a more recent publication Mathieson *et al.* [36] have presented spectral data on the semicarbazone of the hydroxyaldehydic fission product (**LVI**)



LXIII

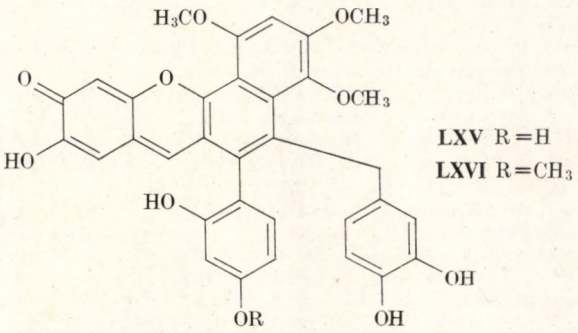


LXIV

R = H or CH_3

of the permethyl ether, providing additional confirmation of its structure. Further Gurudutt and Seshadri [37] have located unequivocally the position of methoxyl groups in the two santalin pigments. There are difficulties in the alkali fission as well as in the permanganate oxidation of flavonoid compounds having a number of free hydroxyls; usually the decomposition is complex and gives poor yields of the products. The method of ethylation and examination of the mixed methyl ethyl ethers is more straightforward, also affording higher yields of the products; it has therefore been adopted in these studies.

Santalin-A pentaethyl ether formed yellow needles from EtOAc–petroleum ether, m.p. 177–178°C. Its degradation with methanolic potash yielded four products. They were identified as (*i*) 2,4-dihydroxy-5-ethoxybenzaldehyde, (*ii*) 4-ethoxyresorcinol, (*iii*) a naphthaldehyde derivative and (*iv*) a 1,2-naphthoquinone derivative (*cf.* santalin permethyl ether) [34]. Products (*i*) and (*ii*) showed that ring A of santalin-A had a free hydroxyl at C-4. This was in agreement with its stability and its red colour. Compound (*iii*) was converted into compound (*iv*) by Dakin oxidation. Each contained 3 methoxyl and 4 ethoxyl groups. Permanganate oxidation of santalin-A pentaethyl ether gave three acids, *viz.*, 3,4-diethoxybenzoic acid, 2,4-diethoxybenzoic acid and 3,4,6-trimethoxyphthalic acid; their identity was confirmed by comparison with authentic samples. Based on these pieces of evidence santalin-A must have 3 methoxyls in ring D; thus the structure of 9,10,12-O-trimethylsantalin (**LXV**) was assigned to it. Arnone *et al.* [35] suggested the same structure based on the spectral properties of the alkali fission products of santalin-A itself and the isolation of a small amount of 2,4-dihydroxybenzaldehyde.



LXV R = H
LXVI R = CH_3

Santalin-B tetraethyl ether crystallized from EtOAc–petroleum ether in yellow needles, m.p. 182–183°C. Its degradation with methanolic potash

gave four products, (*i*) 2,4-dihydroxy-5-ethoxybenzaldehyde, (*ii*) 4-ethoxyresorcinol, (*iii*) a naphthaldehyde derivative and (*iv*) a 1,2-naphthoquinone derivative (*cf.* santalin permethyl ether [34]). Compound (*iii*) was convertible into compound (*iv*); each contained 4 methoxyl and 3 ethoxyl groups. Permanganate oxidation of santalin-B tetraethyl ether yielded 3,4-diethoxybenzoic acid, 2-ethoxy-4-methoxybenzoic acid and 3,4,6-trimethoxyphthalic acid. According to these results, santalin-B should contain three methoxyls in ring D and one methoxyl in ring E at the 4′-position. It has therefore been assigned the structure of 9,10,12,4′-O-tetramethyl santalin **LXVI**.

REFERENCES

1. Pratt, D.D., Robinson, R., *J. Chem. Soc., 1925*, 166.
2. Arora, S. K., Jain, A. C., Seshadri, T. R., *J. Indian Chem. Soc., 39*, 285 (1962).
3. Perkin, A. G., *Proc. Chem. Soc., 30*, 212 (1914).
4. Chapman, E., Perkin, A. G., Robinson, R., *J. Chem. Soc., 1927*, 3015.
5. Ponniah, L., Seshadri, T. R., *Proc. Indian Acad. Sci., 37A*, 548 (1953).
6. Robinson, R., Vasey, C., *J. Chem. Soc., 1941*, 660.
7. Ponniah, L., Seshadri, T. R., *Proc. Indian Acad. Sci., 38A*, 291 (1953).
8. Ponniah, L., Seshadri, T. R., *Proc. Indian Acad. Sci., 38A*, 288 (1953).
9. Ponniah, L., Seshadri, T. R., *Proc. Indian Acad. Sci., 38A*, 77 (1953).
10. Ponniah, L., Seshadri, T. R., *Proc. Indian Acad. Sci., 39A*, 45 (1954).
11. Krishnamurty, H. G., Seshadri, T. R., Venkataramani, B., *J. Sci. Ind. Research (India), 19B*, 115 (1960).
12. Frankel, S., David, E., *Biochem. J., 187*, 146 (1927).
13. Brockmann, H., Haase, R., *Ber., 69*, 1950 (1936).

13a. Hesse, G., Klingel, W., *Ann., 524*, 14 (1936).

14. Brockmann, H., Junge, H., *Ber., 76*, 751 (1943).
15. Robertson, A., Whalley, W. B., *J. Chem. Soc., 1950*, 1882.
16. Brockmann, H., Haase, R., *Ber., 70*, 1733 (1937).
17. Brockmann, H., Haase, R., *Ber., 77*, 279 (1944).
18. Robertson, A., Whalley, W. B., *J. Chem. Soc., 1950*, 1876 and 3117.
19. Cardillo, G., Merlini, L., Nasini, G., Salvadore, P., *J. Chem. Soc., 1971*, 3967.
20. Whalley, W. B., in "Chemistry of Natural Phenolic Compounds" (Ed., W. D. Ollis) pp. 26. Pergamon Press, Oxford, 1961.
21. Jurd, L., *Tetrahedron, 23*, 1057 (1967).

21a. Jain, A. C., Khanna, V. K., Seshadri, T. R., *Tetrahedron, 25*, 275 (1969).

22. Seshadri, T. R., Trikha, R. K., *Indian J. Chem. 9*, 928 (1971).
23. Robinson, G. M., Robinson, R., Todd, A. R., *J. Chem. Soc., 1934*, 809.
24. Stafford, H. A., *Plant. physiol., 40*, 130 1(965).
25. Harborne, J. B., *Nature, 207*, 984 (1965).
26. Bendz, G., Martensson, O., Terenius, L., *Acta Chem. Scand., 16*, 1183 (1962).
27. Nilson, E., *Acta Chem. Scand., 21*, 1942 (1967).
28. Perkin, A. G., *J. Chem. Soc., 97*, 220 (1910).
29. Misra, K., Seshadri, T. R., *Indian J. Chem., 5*, 409 (1957).
30. Pelletier, P. J., *Ann. Chim. Phys., 51*, 193 (1832).
31. Cain, J. C., Simonsen, J. L., *J. Chem. Soc., 101*, 1061 (1912).
32. Robertson, A., Whalley, W. B., *J. Chem. Soc., 1954*, 2794.
33. Dean, F. M., "Naturally Occurring Oxygen Ring Compounds" p. 414. Butterworths, London, 1963.

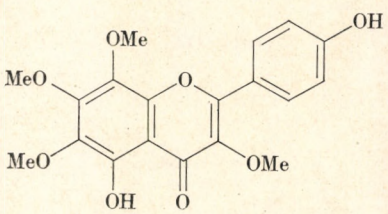
34a. Ravindranath, B., Seshadri, T. R., *Tetrahedron Letters*, *1972*, 1201.
34b. Ravindranath, B., Seshadri, T. R., Under publication.
35. Arnone, A., Merlini, L., Nasini, G., *Tetrahedron Letters*, *1972*, 3503.
36. Mathieson, D. W., Millard, B. J., Powell, G. W., Whalley, W. B., *J. Chem. Soc.*, *1973*, 184.
37. Gurudutt, K. N., Seshadri, T. R., Under publication.

K. VENKATARAMAN

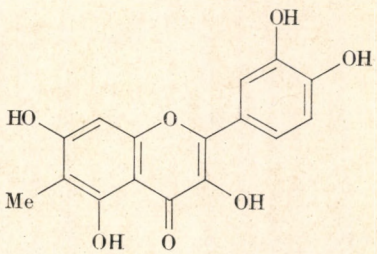
RECENT WORK ON SOME NATURAL PHENOLIC PIGMENTS

I. INTRODUCTION

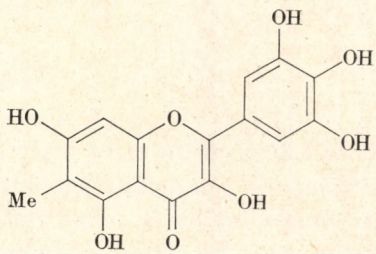
Spectroscopic methods of structure determination in relation to natural phenolic pigments were reviewed some years ago [1]. Examples were cited to illustrate both the scope and limitations of UV, IR, NMR and mass spectra. Thus the structures of calycopterin (**1**), pinoquercetin (**2**), and pinomyricetin (**3**), determined originally by tedious chemical methods, can be derived very simply from the NMR spectra.



Calycopterin (**1**)



Pinoquercetin (**2**)



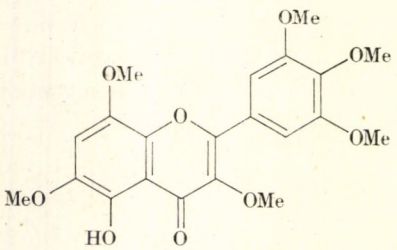
Pinomyricetin (**3**)

On the other hand, elucidation of the constitution of morellin in its entirety required X-ray crystallography, although many structural features were known from chemical and spectroscopic evidence. The present occasion

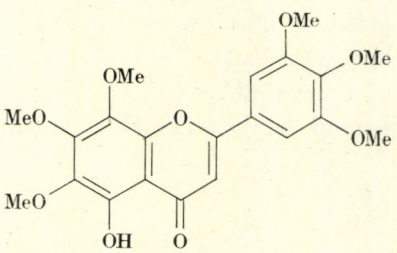
gives me the opportunity of elaborating further on this theme in the light of subsequent work on some Indian plant and insect pigments.

II. GARDENIN

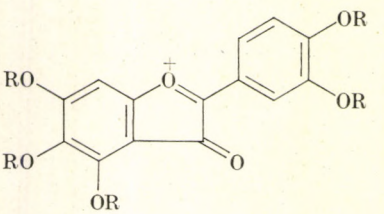
"Dikamali" gum, an Ayurvedic drug, is the mixed resinous exudate of *Gardenia lucida* and *G. gummifera*. In 1938 Bose [2] isolated the main pigment, gardenin A, and on the basis of colour reactions and alkaline degradation assigned it the structure **4**. NMR and mass spectral data have now shown that gardenin has the structure **5** [3]. The NMR spectrum* confirms six methoxyl groups (one at 5.87 and five at 6.0), a chelated hydroxyl (−2.78), and the 2′,6′-protons (two-proton singlet at 2.78) of the B-ring in **4** or **5**.



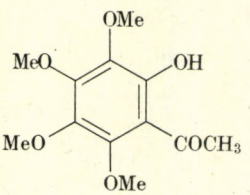
(4)



(5)



(7)



(8)

A single-proton singlet at **3.35** is in the right place for 3-H in **5**; its being the signal for 7-H in **4** cannot be completely excluded, although it should be expected to appear at somewhat lower field; 7-H in zapotin (5,6,2′,6′-tetramethoxyflavone) occurs at 2.72 and a second *o*-methoxyl group will effect a diagmagnetic shift of 0.5 ppm or less. The mass spectrum of gardenin (Chart 1) shows peaks at m/e 418, 403, 388, 373, 211, 193, 183 and 178.

* All NMR spectra were taken on a Varian A−60 or T−60 instrument. Solvent: $CDCl_3$, unless otherwise stated. Chemical shifts on the τ scale.

Although the molecular ion at 418 is abundant (80%), the base peak is at M—15 (403) indicating the facile loss of a methyl group to form the cation **6**, or the analogous *o*-quinonoid ion; this will not distinguish between **4** and **5**, because both contain methoxyl groups in the 6- and 8-positions. In quercetagetin hexamethyl ether (3,5,6,7,3′,4′-hexamethoxyflavone) [4] the base peak is at M—15. Another noteworthy fragmentation in quercetagetin derivatives is the loss of an acetyl radical from the molecular ion, probably from the 3-position, and the formation of an M—43 cation such as **7**.

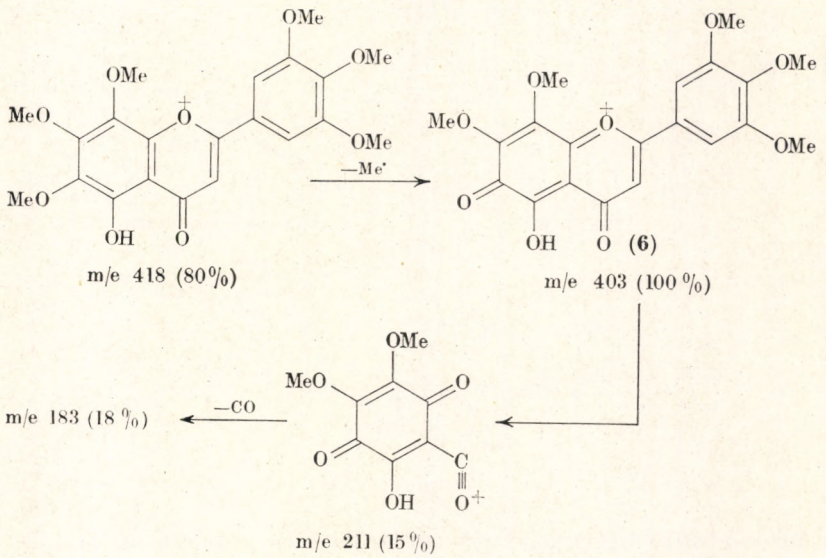


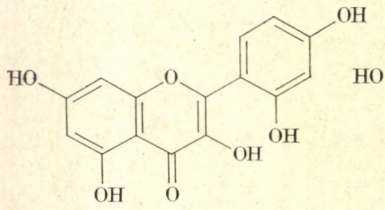
Chart 1. Mass spectral fragmentation of gardenin (**5**)

None of the peaks in the mass spectrum of gardenin can be explained by **4**, but the fragmentation shown in Chart 1 lends strong support to **5**. The fact that the peaks at m/e 211 and 183 are derived from the A ring of **5** was shown by (*a*) a shift of one mass unit by deuterium exchange, and (*b*) a shift of 14 mass units by methylation.

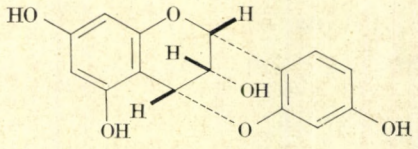
Conclusive evidence for **5** was obtained by hydrolysis of gardenin A to **8** and by synthesis [5]. Four pigments (gardenin B, C, D and E) isolated later in minute quantities from *G. lucida* gum, were found to be flavones with identical A-ring substitution. The variations in the B-ring were 4′-OMe; 3′-OH-4′,5′-$(OMe)_2$; 3′-OH-4′-OMe; and 3′,5′-$(OH)_2$-4′-OMe, respectively [6].

III. *ARTOCARPUS* AND *MORUS* FLAVONES

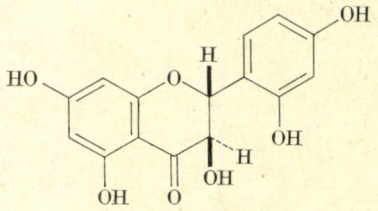
A request for a sample of morin, well known as a reagent for aluminium, led us to examine the heartwood of *A. heterophyllus* Lam. (*A. integrifolia* Linn.), from which Perkin and Cope isolated morin (**9**) and cyanomaclurin in 1895. In view of earlier work on cyanomaclurin and the chemical shifts of the protons in the 2, 3 and 4-positions in the NMR spectra of cyanomaclurin tetramethyl ether and its acetate, structure **10** was assigned to cyanomaclurin [7].



Morin (**9**)



Cyanomaclurin (**10**)

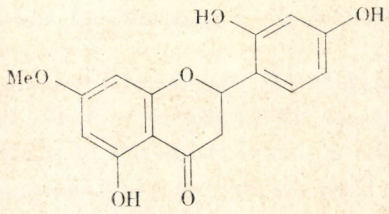


Dihydromorin (**11**)

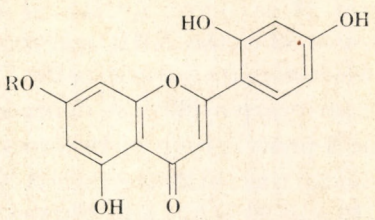
A. heterophyllus heartwood proved to be a rich source of flavones with two unique features: the β-resorcylic acid orientation of hydroxyl groups in the B-ring and the presence of one or more isoprenoid* groups in the 3-, 6- and 8-positions. In addition to the previously known dihydromorin (**11**), the flavanone artocarpanone (**12**) and eight flavones (**13** to **20**) were isolated [8]. The structure of artocarpin (**19**), which first became available because the compound crystallized readily from a hexane extract of some timber samples, was proved by chemical methods involving ozonization and ultimately by the synthesis of tetrahydroartocarpin dimethyl ether.

The second flavone isolated which carried isoprenoid substituents was cycloartocarpin (**20**), and degradative evidence led to the erroneous structure, 4′-prenyl ether of **15**. Subsequently the molecular ions at m/e 434 and 462 in the mass spectra of cycloartocarpin and its methyl ether indicated

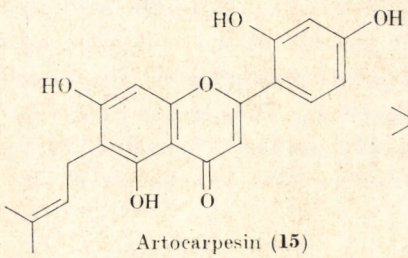
* In this review "prenyl" is used as equivalent to $\gamma\gamma$-dimethylallyl ($Me_2C{=}CH{-}{-}CH_2{-}$), and isoprenoid as a more general term for a group derived from isoprene.



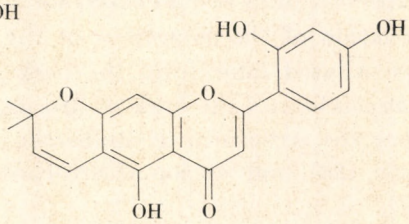
Artocarpanone (**12**)



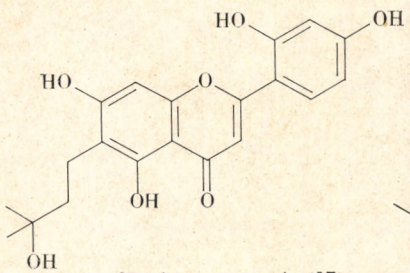
Norartocarpetin (**13**) R = H
Artocarpetin (**14**) R = Me



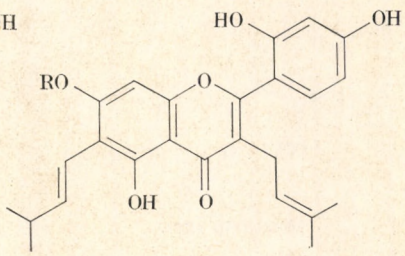
Artocarpesin (**15**)



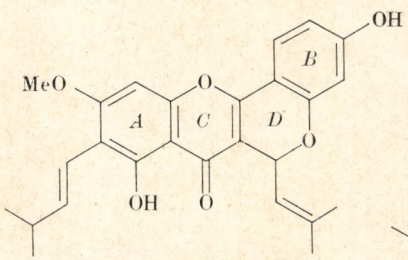
Cycloartocarpesin (**16**)



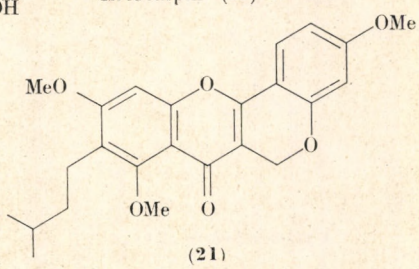
Oxydihydroartocarpesin (**17**)



Norartocarpin (**18**) R = H
Artocarpin (**19**) R = Me



Cycloartocarpin (**20**)



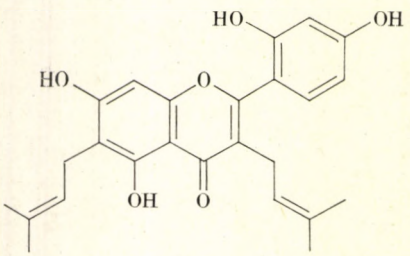
(**21**)

the presence of an additional ring and the correct structure **20**. A remarkable reaction of di- and tetrahydrocycloartocarpin, the mechanism of which is still obscure, is the fission of a C—C bond when they are heated with hydriodic acid and acetic anhydride; the product, isolated as the trimethyl

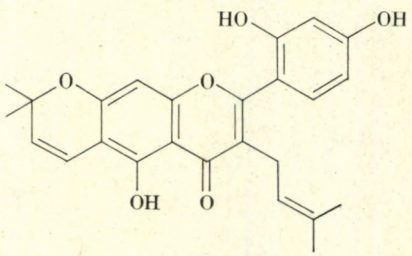
ether, was **21** as shown by M^+ at 410 and the appearance of the OCH_2 protons at 4.83 in the NMR spectrum.

Two characteristic features in the UV and NMR spectra of artocarpin (**19**) and cycloartocarpin (**20**) are dependent on the substituents in the 3-position. The 3-prenyl group in **19** forces the 2-phenyl group out of plane with the chromone ring; in **20** the rings A, C, D and B are coplanar. Consequently, λ_{max} of **19** and **20** is 324 and 370 nm, respectively; in the NMR spectra the 6′-proton of **20** appears in the normal position (about 2.2), but the corresponding proton of **19** undergoes an upfield shift to 2.92.

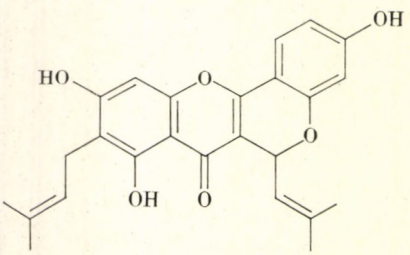
The spectral data accumulated for the flavones **13** to **20** facilitated the determination of the structures of the closely related and more complex flavones isolated later from the bark of *A. heterophyllus*, the heartwood of *A. chaplasha* and the bark of *Morus* species. Thus it was relatively easy to deduce the structures of mulberrin (**22**), mulberrochromene (**23**), cyclomulberrin (**24**) and cyclomulberrochromene (**25**), isolated from *M. alba* bark [9].



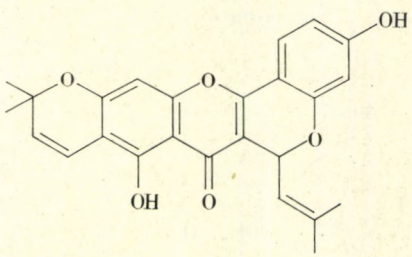
Mulberrin (**22**)



Mulberrochromene (**23**)



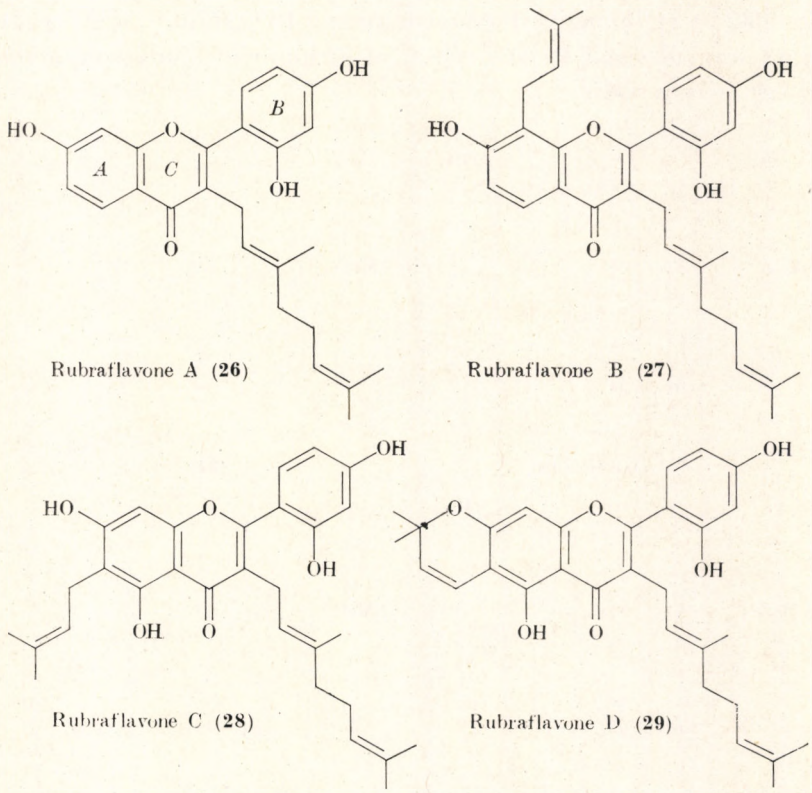
Cyclomulberrin (**24**)



Cyclomulberrochromene (**25**)

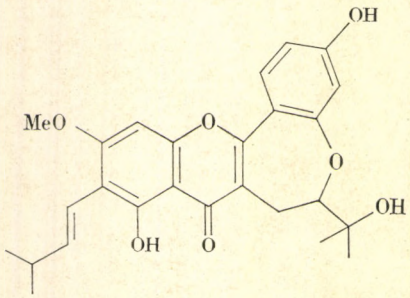
None of the four flavones of *M. alba* bark is present in the bark of *M. rubra*, an American species, from which, however, four new flavones with C_{10} side chains have been isolated: rubraflavones A (**26**), B (**27**), C (**28**), and D (**29**). The chromatographically homogeneous rubraflavones, isolable in mg-quantities from 1 kg of bark, are all amorphous; except for the trimethyl

ether of rubraflavone A, the ethers and acetates are also uncrystallizable, but NMR and MS data clearly showed the structures (**26–29**). The MS fragmentation of all the four flavones was characteristic of a nuclear $C_{10}H_{17}$ group: a base peak at M−69 (loss of C_5H_9) and prominent peak at M−123 (loss of C_9H_{15}). The UV and IR spectra showed that the rubraflavones were not flavonols, but substitution in the 3-position was indicated by the absence of a singlet in the 3–3.5 region of the NMR spectrum, which also had the expected signals for a $C_{10}H_{17}$ side chain formed by head-to-tail addition of two isoprene units, and for the other protons in structures **26–29** [10].

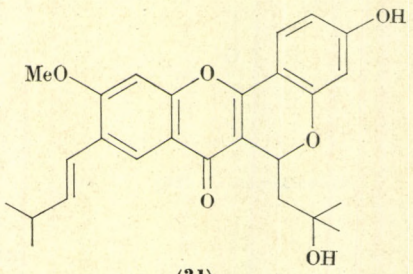


Chaplashin (**30**), isolated from the heartwood of *A. chaplasha*, contains an oxepine ring [11]. The NMR spectrum in DMSO and pyridine shows, in addition to the expected signals for structure **30**, a single-proton quartet at 5.7 and a two-proton multiplet in the region 7.25–7.6. Together with their splitting pattern they suggest that they are on adjacent carbon atoms, and one is probably part of a C_5-unit attached to the 3-position of a flavone

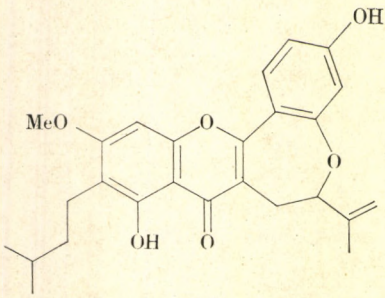
molecule. Two structures (**30** and **31**) can be considered, and structure **31** is excluded by the following facts: (*a*) the UV spectrum of chaplashin (**30**) resembles that of artocarpin (**19**) rather than cycloartocarpin (**20**); (*b*) the methine hydrogen of ring D appears in tetrahydrocycloartocarpin dimethyl ether at 4.42, while the corresponding hydrogen in chaplashin and its dihydro derivative is at about 5.7; and (*c*) the methylene protons adjacent to the asymmetric carbon atom in tetrahydrocycloartocarpin dimethyl ether are seen at about 8.4, while in chaplashin they have undergone a marked downfield shift (multiplet around 7.4). Final proof for the structure of chaplashin (**30**) was obtained by the synthesis of racemic dihydrochaplashin from **32** by refluxing in dioxan containing 10% sulfuric acid for several hours; **32** was obtained by the action of dichlorodicyanobenzoquinone on dihydroartocarpin (**33**).



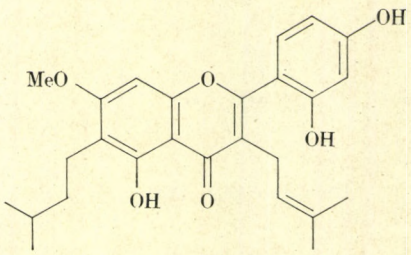
Chaplashin (**30**)



(**31**)

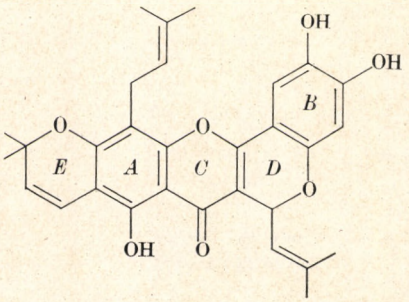


(**32**)

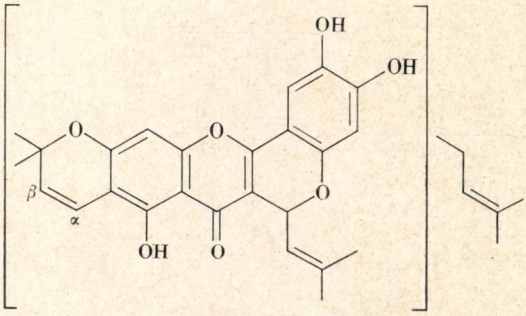


(**33**)

From the bark of *A. heterophyllus*, cycloheterophyllin (**34**) was isolated in mg-quantities [12]. The partial structure **35** was first deduced. The angular orientation of ring E was ruled out by a consideration of the changes in the chemical shift of the CH=CH protons when the dimethyl ether and its acetate were compared [12]. The fact that the third prenyl group is in



Cycloheterophyllin (**34**)



(**35**)

the 8-position in **35** was shown by the absence of coupling between H_α and 8-H, which is to be expected if the 8-position is unoccupied [13].

In the mass spectra of compounds having a prenyl chain adjacent to a hydroxyl group, fragmentation occurs with the loss of C_4H_8 (56 mass units) [14]. If the prenyl chain is adjacent to a methoxyl group there are additional rearrangements with a loss of C_3H_7 (43 mass units) [15]. These observations are in general agreement with the mass spectra of the *Artocarpus* and *Morus* flavones, details of which are discussed elsewhere [16]. For example, mulberrin (**26**) and its tetramethyl ether show fragmentations corresponding to the loss of 56 and 43 mass units, respectively. The mass spectra of cycloheterophyllin and its trimethyl ether have not shown any peaks corresponding to M—56 or M—43 ions respectively, thus supporting structure **34** for cycloheterophyllin. The main paths of fragmentation are outlined in Chart 2 (see p. 50). The two peaks at m/e 215 (**36**) and m/e 216 (**37**) indicate the substituents in rings A and B, respectively. The peak at m/e 215 is also seen in the dimethyl ether.

IV. MORELLOFLAVONE

Morelloflavone (**38**), a constituent of the heartwood of *Garcinia morella*, was the first 3-(8-) flavonylflavanone [16]. The biflavonoids known at the time were biflavonyls built from apigenin and its methyl ethers, the 3-position never being involved. Simultaneously with our work Scheinmann *et al.* reported the isolation of three new biflavanonyls (e.g. GB-1a, **39**) in which the flavanone units are linked in 3,8-positions [17]. Morelloflavone is identical with fukugetin [18], isolated several years ago from *G. spicata*. An analogue of morelloflavone (talbotaflavone [19] = volkensiflavone [20]) has been isolated more recently from other *Garcinia* species.

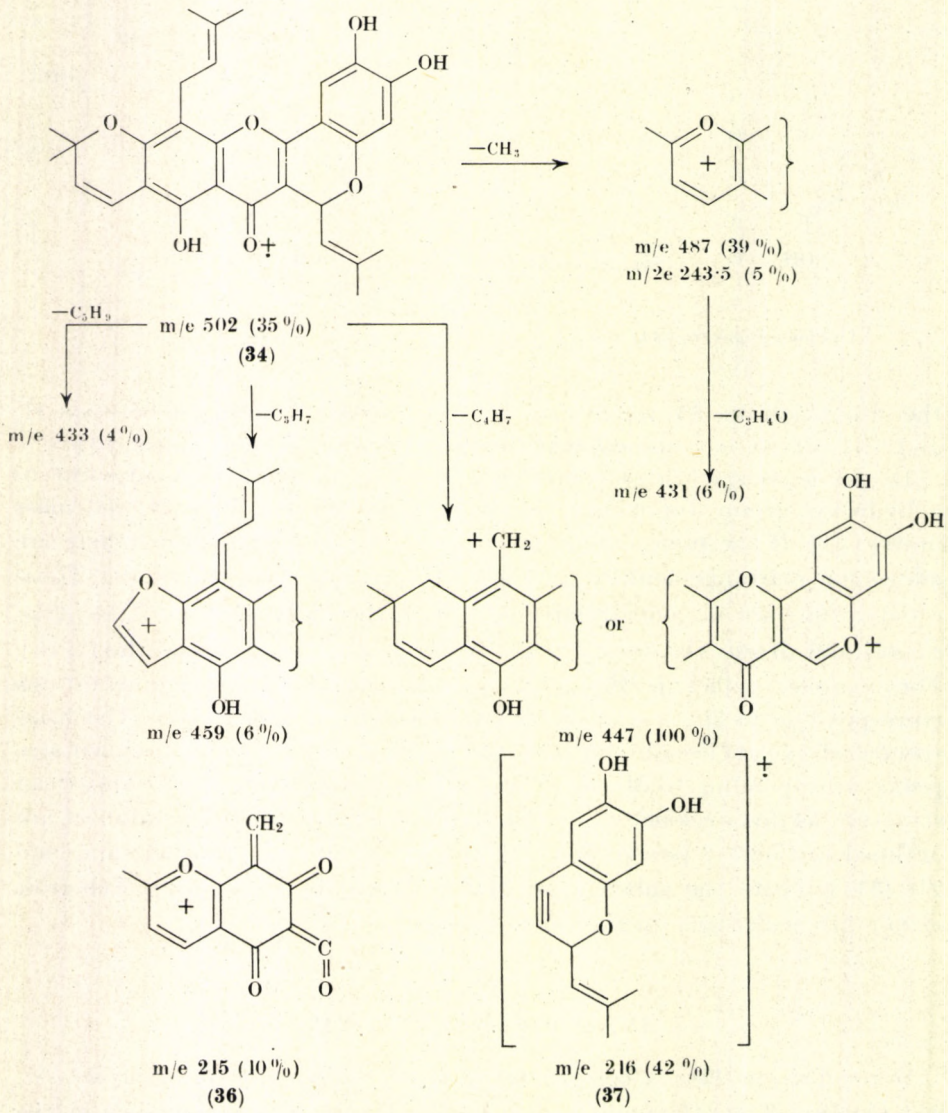
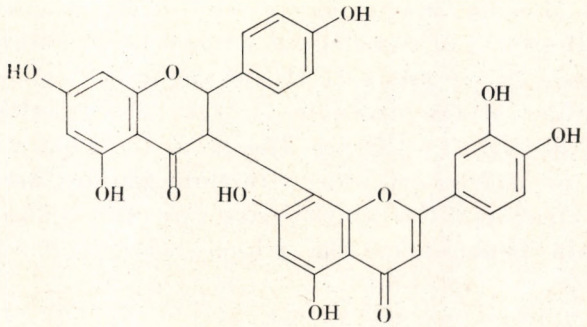
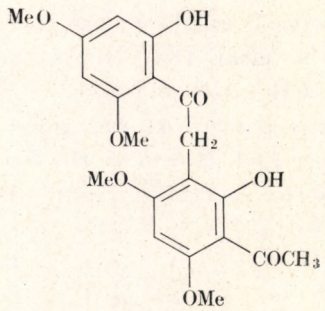


Chart 2. Mass spectral fragmentation of cycloheterophyllin **(34)**

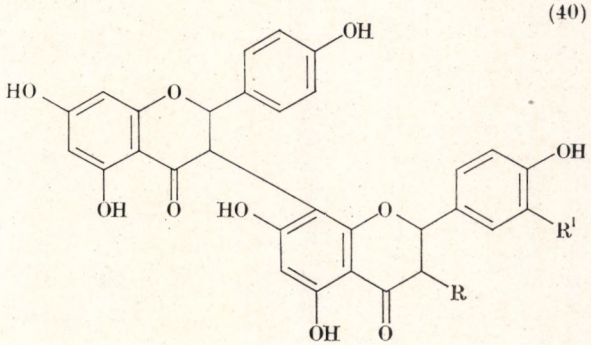
Morelloflavone, $C_{30}H_{20}O_{11}$, forms a heptamethyl ether. IR absorption at 1645 cm^{-1} (chelated C=O), resolving into two bands at 1670 and 1645 cm^{-1} on methylation, gave preliminary evidence of both 5-hydroxyflavone and 5-hydroxyflavanone units in morelloflavone. A pair of doublets at 4.14 and 5.09 (J = 12 Hz) in the NMR spectrum of the ether corresponded to



(38)



(40)



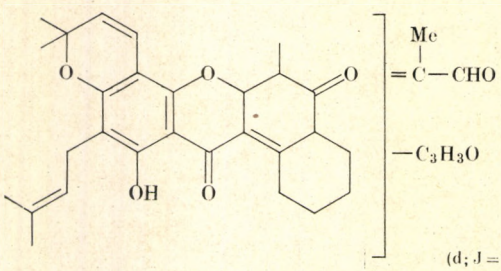
(39) GB—1a R=R¹=H

2- and 3-H in the flavanone, and a singlet at 3.0 to 3-H in the flavone half of **38**. Treatment of the ether with boiling 15% ethanolic potassium hydroxide yielded the deoxybenzoin **40**, M^+ 390, characterized by its NMR spectrum: 7.40 (CH_3CO); 6.10–6.18 (4 OMe); 5.75 (uncoupled CH_2CO); 3.9 region (3 aromatic H); −3.8 and −3.9 (two chelated OH).

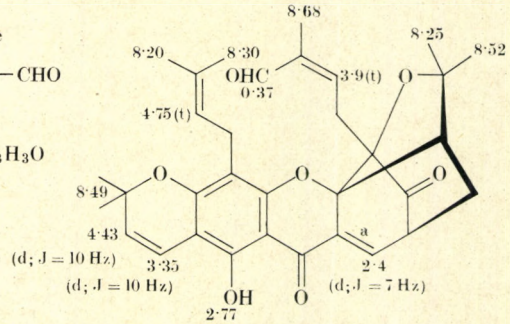
V. MORELLIN

Morellin, $C_{33}H_{36}O_7$, crystallized readily in large, beautiful orange-yellow needles from a hexane extract of the seeds of *G. morella*, but crystalline derivatives were difficult to prepare. Although it has powerful antibacterial properties, it is clinically valueless because of its toxicity and absence of activity in the presence of serum. Its structure **42** renders it a molecule of exceptional interest. Extensive chemical UV and IR investigations [21]

led to the part-structure **41**. Morellin formed a monomethyl ether, but gave a diacetate. When the NMR spectra of morellin (see **42**) and the diacetate became available, the presence of the group in **41** was confirmed, and it was clear that the formation of the diacetate involved the reaction $-CH_2-CH=C-CHO \quad -CH=CH-C=CHOAc$. The tetrahydronaphthalene part of **41** was ruled out and several other structural features were revealed. However, the formulation of the complete structure (**42**) required X-ray crystallographic data obtained on the *p*-bromobenzenesulfonyl ester [22].



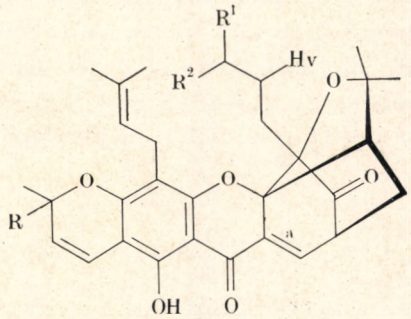
(**41**)



Morellin (**42**)

Morellin is isomerized to isomorellin under a variety of conditions; both morellin and isomorellin yield the same diacetate. The NMR spectra of morellin, isomorellin and the diacetate are in complete agreement with structure **42** for morellin and **43** for isomorellin [23]. Among the chemical shifts in the NMR spectrum of **42** attention may be drawn in particular to the aldehyde absorption at 0.37 and the triplet at 3.9. In isomorellin these appear at 0.73 and 3.52, respectively. By comparison with the chemical shifts of the vinyl proton H_v in other α,β-unsaturated *trans–cis* pairs, such as methyl tiglate and angelate, it was possible to distinguish between morellin and isomorellin. A consideration of the differences in the methyl spectra of the two isomers also permitted the assignment of the seven methyl groups. The doublet at 2.4 was useful in following the progress of the catalytic reduction of morellin and in determining the structure of dihydroisomorellin (one of the pigments accompanying isomorellin) [24] as **43** but with a single bond at *a*. The structures of desoxymorellin (**44**), which Bringi [21] isolated by chromatography of the residue after the removal of morellin, and morellinol (**45**) present in *G. morella* bark, were readily determined by the absence

of the aldehyde signal and the appearance of an additional methyl group or the CH_2 protons of a CH_2OH group (at 6.42) in the NMR spectra [24, 25].



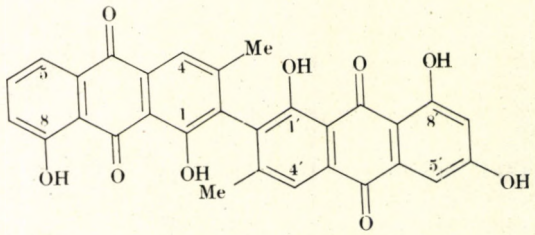
(42) Morellin R = R^1 = Me; R^2 = CHO
(43) Isomorellin R = R^2 = Me; R^1 = CHO
(44) Desoxymorellin R = R^1 = R^2 = Me
(45) Morellinol R = R^1 = Me; R^2 = CH_2OH
(46) Morellic acid R = R^1 = Me; R^2 = COOH
(47) Isomorellic acid R = R^2 = Me; R^1 = COOH
(48) Gambogic acid R = $CH_2 - CH_2 - CH = CMe_2$
R^1 or R^2 = Me or COOH

The occurrence of gambogic acid in the resinous exudate of *G. morella*, its molecular formula ($C_{38}H_{44}O_8$) [26] and the obvious similarities in chemical properties to morellin clearly showed that the replacement of the aldehyde group in **42** by carboxyl and the addition of a fifth prenyl substituent must represent the structure of gambogic acid. While we were unsuccessfully attempting to find gambogic acid in Indian *G. morella* gamboge, Yates *et al.* [27] and Ollis *et al.* [28] showed on the basis of our structure for morellin that gambogic acid (obtained from Thailand *G. hanburyi*) is constituted as **48**. Indian gamboge yielded morellic and isomorellic acids (**46** and **47**). Their stereochemistry helped us in correlating acetyl-α-gambogic acid with isomorellic acid [29].

The biosynthesis of morellin involves the interaction of 1,3,5,6-tetrahydroxyxanthone with four "active isoprene" units and subsequent cyclizations leading first to desoxymorellin, and then to morellinol, morellin and morellic acid.

VI. 2,2′-BIANTHRAQUINONYLS

Cassiamin A (**49**), a new pigment in the root bark of *Cassia siamea* Lam. (*C. florida* Vahl.), proved to be the first bianthraquinonyl in a plant and the first 2,2′-bianthraquinonyl from any source [30]. Sennosides A and B in *Cassia angustifolia* Vahl. are derivatives of 10,10′-bianthronyl [31]. Skyrin and its analogues isolated by Raistrick and Shibata as metabolites of *Penicillium* species are derivatives of 1,1′-bianthraquinonyl [31].



Cassiamin A (**49**)

Colour reactions and the electronic spectrum have shown that cassiamin A is an anthraquinone, and NMR data (5 methoxyls in the methyl ether, 2 C-methyls and 7 aromatic H) revealed its bimolecular character. In conjunction with IR carbonyl absorptions at 1670 and 1620 cm^{-1}, one β-hydroxyl and two pairs of hydroxyls in 1,8-positions were deduced. The NMR spectrum of the pentamethyl ether disclosed the *m*-coupled 5′-and 7′-protons, three α-protons (below 2.25) in addition to 5′-H at 2.67, and two β-methyl groups. The β,β'-linkage of the two anthraquinone moieties and the orientation of all the substituents as in **49** were thus determined. As a result of the hindered rotation about the 2,2′-bond, Cassiamin A is optically active. Cassiamin A is accompanied in the plant by two minor pigments, Cassiamin B and C, which are symmetrical analogues built from two molecules of emodin and of chrysophanol, respectively [32].

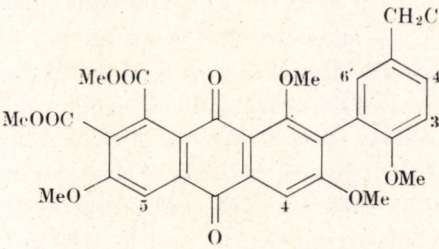
VII. INSECT PIGMENTS DERIVED FROM ANTHRAQUINONE

The lac insect (*Laccifer lacca* Kerr), which produces the well-known shellac resin, also elaborates a complex mixture of water-soluble and water-insoluble dyes. Till the end of the last century the water-soluble lac dye was commercially important, but it is now rejected (in quantities of about 100,000 kg) as a waste product during shellac manufacture. Simultaneously with his work on the closely related pigments of the kermes and

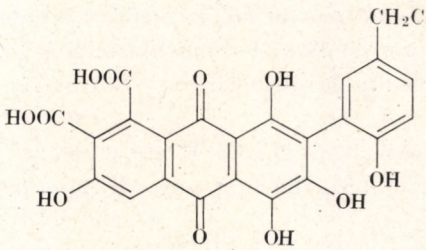
cochineal insects (*Kermococcus ilicis* L. and *Dactylopius coccus* Costa), Dimroth made an extensive study of "laccaic acid", which he regarded as the main constituent of lac dye [33].

Preliminary work showed that Dimroth's laccaic acid was a mixture of several pigments, at least two of which contained nitrogen. Five laccaic acids (A to E) were ultimately separated, but the products of methylation by dimethyl sulfate and potassium carbonate in boiling acetone were first found to be more readily separable and more suitable for mass spectral and NMR studies [34]. The laccaic acids, except D, are purpurin (1,2,4-trihydroxyanthraquinone) derivatives, and a useful line of attack was to dehydroxylate them to the corresponding xantholaccaic acids (1,3-dihydroxyanthraquinones), which were then examined as their methyl ethers, prepared by the action of methyl iodide and silver oxide in dimethylformamide.

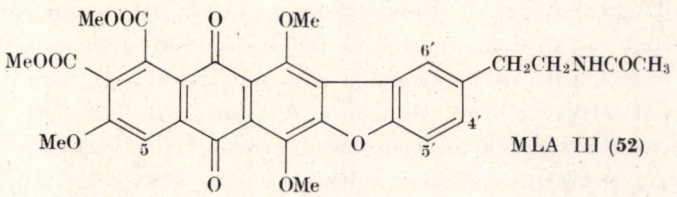
From the NMR and mass spectral data it was clear that the laccaic acids were 2-phenylanthraquinone derivatives, hitherto unknown as natural products. The NMR spectrum of one of the methylated xantholaccaic acids



MXLA III (**50**)



Laccaic acid A (**51**)



MLA III (**52**)

(MXLA III), $C_{32}H_{31}NO_{11}$, is in excellent agreement with structure **50**: in the aromatic region the signals of the 4- and 5-H of the anthraquinone ring appear as singlets at 2.38 and 2.25, and those of the 3′,4′,6′ protons of the benzene ring as an ABC pattern between 2.83 and 3.14; six OMe at 5.98 to 6.48; two triplets of the $ArCH_2CH_2$ group ($ArCH_2$ centered at 7.25 and CH_2NHAc at 6.57 merged with the upfield methoxyl group); Me of the $COCH_3$ group at 8.08; and a broad signal at about 4.2 representing the NH proton. The relation between xanthopurpurin and purpurin immediately leads to structure **51** for laccaic acid A, the corresponding N-containing lac dye constituent. Schofield *et al.* [35] have also arrived at structure **51** for laccaic acid A.

The methyl ether-ester (MLA III), which was apparently produced from laccaic acid A, had an anomalous molecular formula ($C_{31}H_{27}NO_{11}$ instead of $C_{33}H_{33}NO_{12}$) and an NMR spectrum explicable only on the basis of structure **52**. The sharp singlets at 2.22 (5-H) and 8.02 ($COCH_3$), broad NH absorption at 4.2, and the methylene triplets of $ArCH_2CH_2NHAc$ centered at 7.02 and 6.47 (the latter clearly separated from the methoxyl region in contrast with the corresponding signal in MXLA III) are not in conflict with the normal heptamethyl ether-ester of **51**. However, there are only five OMe groups at 5.68 to 6.07, the signal at 5.68 being too low for a normal COOMe or aromatic OMe. Secondly, the three protons of the benzene ring obviously correspond to those of MXLA III (**50**) as seen from their ABC splitting pattern, but in comparison with 3′,4′,6′-H of **50**, the former three protons have undergone a marked downfield shift. These departures from expectation can be explained by structure **52** containing a dibenzofuran system formed by cyclization involving the 3- and 2′-OH groups in **51**. An analogy was found in the behaviour of **53**, formed by alkaline fission of the brazanquinone (**54**); treatment of **53** with dimethyl sulfate and potassium carbonate in boiling acetone results in recyclization to **54** by the mechanism outlined in Chart 3 (see p. 57).

The structures of laccaic acids B, C, D and E are given in Chart 4. Of special interest is laccaic acid C (**55**), the first natural quinone bearing an amino acid side chain [31].

Several widely useful observations were made in the course of this work: (*a*) the prolonged action of dimethyl sulfate and potassium carbonate in boiling acetone on an alcohol such as laccaic acid B, an analogue of **51** in which the side-chain on the benzene ring is CH_2CH_2OH, can lead to the carbonate $CH_2CH_2—O—COOMe$. (*b*) Treatment with boron fluoride etherate and acetic anhydride at room temperature is a valuable method of distinguishing between aliphatic methoxyls (R—OMe), an α-methoxyl in

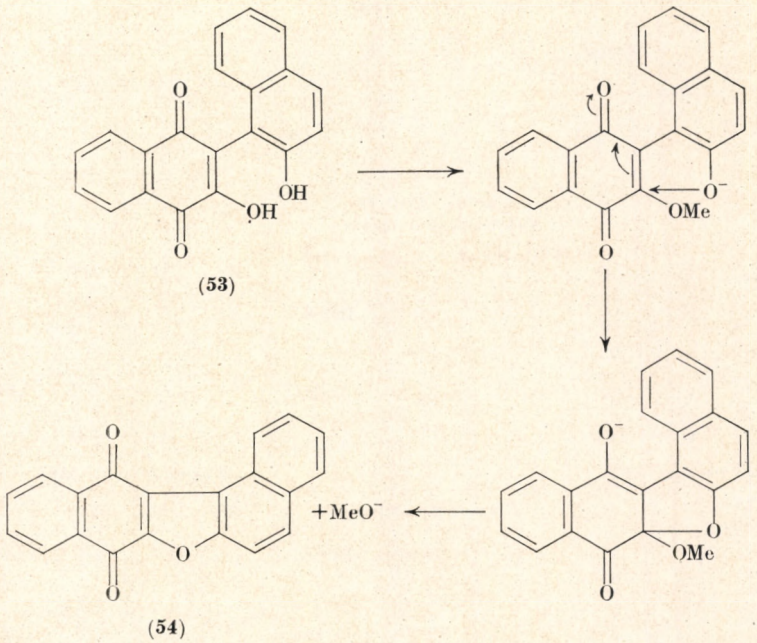


Chart 3. Cyclization of 3-hydroxy-2-(2′-hydroxy-1′-naphtyl)-1,4-naphthoquinone (**53**) to the brazanquinone (**54**)

an anthraquinone (or a 5-methoxyl in a chromone or chromanone) and other aromatic methoxyl groups (Ar—OMe); in general, R—OMe is converted to R—OAc and the Ar—OMe influenced by a CO group is demethylated. (*c*) A general method for distinguishing between certain isomeric α-hydroxyanthraquinones is based on the NMR spectra of anthraquinones and the corresponding anthrones [37].

The NMR spectrum of carminic acid showed that the only aromatic proton was an α-H in the anthraquinone nucleus; a minor structural revision was therefore necessary: a shift of the carboxyl group to the β-position as in **56** (see Chart 4) [38]. The structure of kermesic acid was likewise revised to **57** [39], and of ceroalbolinic acid to **58** [40].

Erythrolaccin (**59**), the main water-insoluble pigment [31] is accompanied by desoxyerythrolaccin (**60**) and isoerythrolaccin (**61**) [37]. In conjunction with colour reactions and IR spectra, the chemical shifts of α- and β-protons and of α- and β-methyl groups in the NMR spectra of anthraquinones were used in determining the structures of **59**, **60** and **61** [41, 37]. Basic NMR and mass spectral data on a series of anthraquinones, such as chrysophanol and emodin and their methyl ethers, were collected to provide the necessary background.

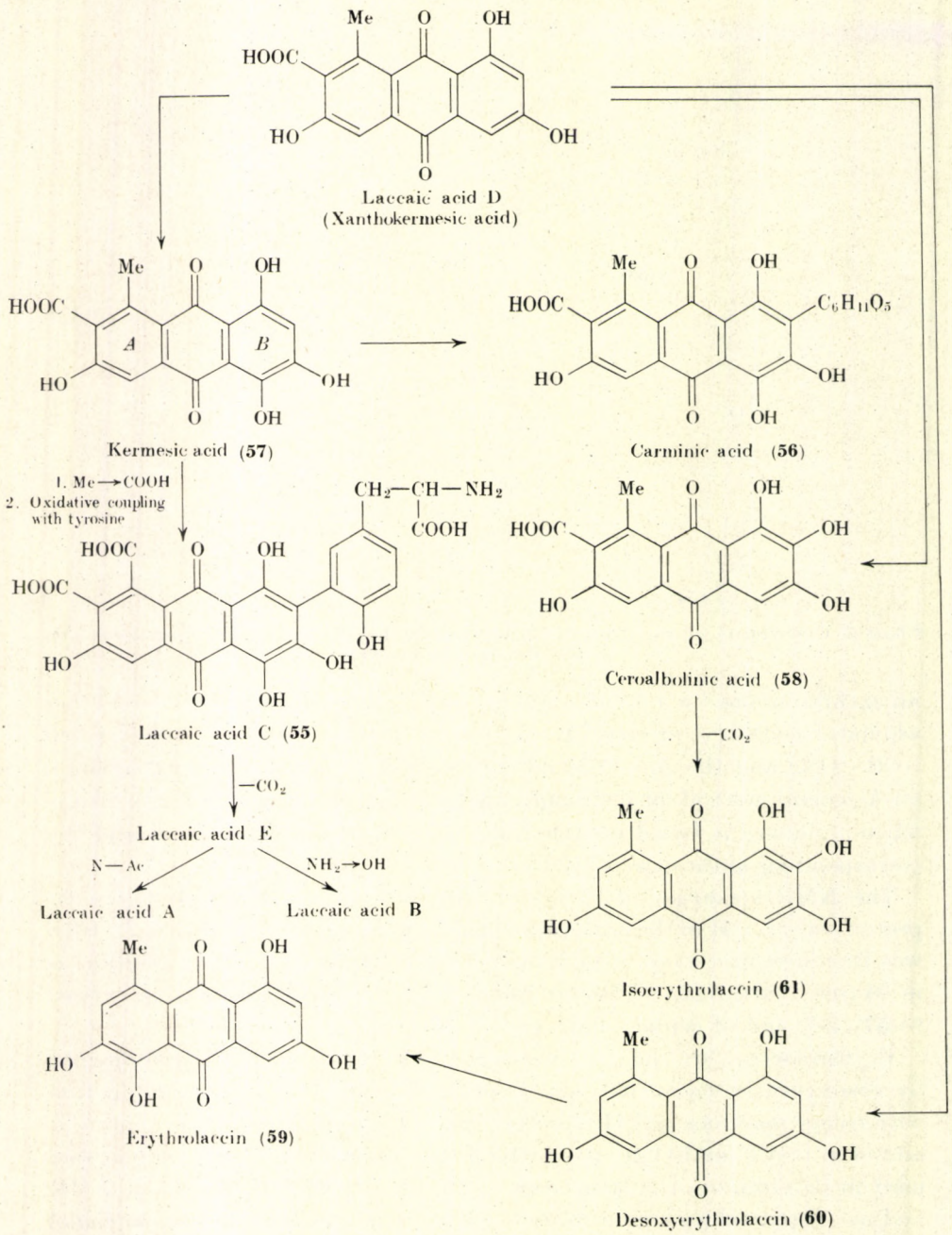


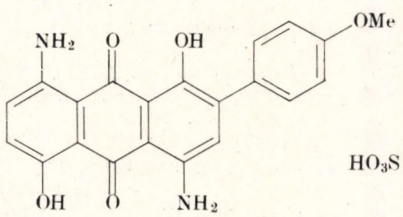
Chart 4. Biosynthesis of insect pigments derived from anthraquinone

VIII. BIOSYNTHESIS OF INSECT PIGMENTS DERIVED FROM ANTHRAQUINONE. 2-ARYLANTHRAQUINONES

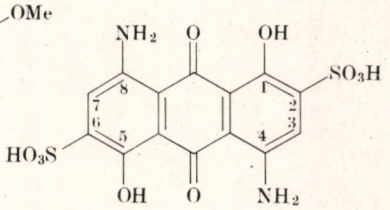
In the biosynthetic scheme outlined in Chart 4 xanthokermesic acid (laccaic acid D), built from acetate–malonate units, plays the central role.

During a search for synthetic methods applicable to the laccaic acids three old German patents were encountered [42] which described the preparation of **62** by a remarkable nuclear arylation: treatment of **63** in sulfuric acid and boric acid with anisole and desulfonation of the resulting monosulfonic acid. A 1963 patent [43] described a second arylation process in which **65** was obtained from **64** by treatment with sulfuric acid, boric acid and anisole, followed by reduction with sodium hydrogen sulfide.

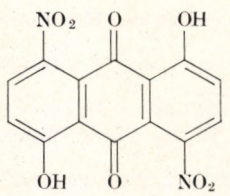
The NMR spectra of the products (**66** and **67**) obtained by deamination (*via* the diazonium salts) have shown that, contrary to the patents, the



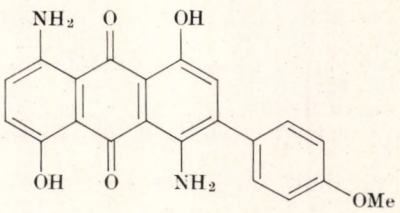
(**62**)



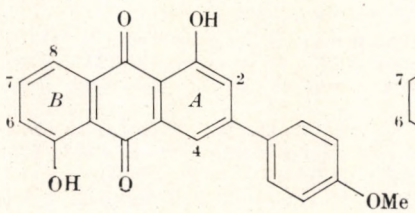
(**63**)



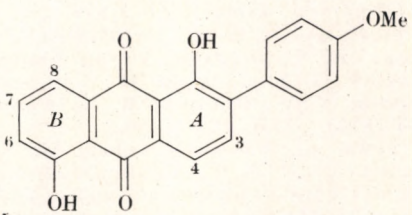
(**64**)



(**65**)



(**66**)



(**67**)

process from **63** yields **65**, and the process from **64** yields **62**. The *m*- and *o*-coupled protons of ring A in **66** and **67**, respectively, were adequate to characterize the two products.

*

I am deeply indebted to my collaborators whose names are mentioned in the references, and in particular to Dr. P. M. Nair and Dr. A. V. Rama Rao.

REFERENCES

1. Venkataraman, K., *J. Sci. Ind. Res.*, *25*, 97 (1966).
2. Bose, P. K., Nath, R., *J. Ind. Chem. Soc.*, *15*, 139 (1938); Bose, P. K., *J. Ind. Chem. Soc.*, *22*, 233 (1945). See also Balakrishna, K. J., Seshadri, T. R., *Proc. Ind. Acad. Sci.*, *27A*, 91, 260 (1948); Ahluwalia, V. K., Mukerjee, S. K., Seshadri, T. R., *J. Chem. Soc.*, *1954*, 3988.
3. Rama Rao, A. V., Venkataraman, K., *Ind. J. Chem.*, *6*, 677 (1968).
4. Bowie, J. H., Cameron, D. W., *Aust. J. Chem.*, *19*, 1627, (1966).
5. Kamalam, M., Rama Rao, A. V., *Ind. J. Chem.*, *8*, 573 (1970).
6. Rama Rao, A. V., Venkataraman, K., Chakrabarti, P., Sanyal, A. K., Bose, P. K., *Ind. J. Chem.*, *8*, 398 (1970).
7. Nair, P. M., Venkataraman, K., *Tetrahedron Letters*, *1963*, 317; Nair, P. M., Parthasarathy, P. C., Radhakrishnan, P. V., Venkataraman, K., *Tetrahedron Letters*, *1966*, 5357.
8. Venkataraman, K., *Phytochem*, *11*, 1571 (1972).
9. Deshpande, V. H., Parthasarathy, P. C., Venkataraman, K., *Tetrahedron Letters*, *1968*, 1715.
10. Deshpande, V. H., (Miss) Wakharkar, P., Rama Rao, A. V., Private communication.
11. Rama Rao, A. V., Rathi, S. S., Venkataraman, K., *Ind. J. Chem.*, *10*, 905 (1972).
12. Rama Rao, A. V., (Miss) Varadan, M., Venkataraman, K., *Ind. J. Chem.*, *9*, 7 (1971).
13. Arnone, A., Cardillo, G., Merlini, L., Mondelli, R., *Tetrahedron Letters*, *1967*, 4201.
14. Ritchie, E., Taylor, W. C., Shannon, J. C., *Tetrahedron Letters*, *1964*, 1437.
15. Stout, G. H., Krahn, M. M., Yates, P., Bhat, H. B., *Chem. Commun.*, *1968*, 211.
16. Rama Rao, A. V., Rathi, S. S., Venkataraman, K., *Ind. J. Chem.*, *10*, 989 (1972).
16a. Karanjgaokar, C. G., Radhakrishnan, P. V., Venkataraman, K., *Tetrahedron Letters 1967*, 3195.
17. Jackson, B., Locksley, H. D., Scheinmann, F., Wolstenholme, W. A., *Tetrahedron Letters*, *1967*, 878.
18. Konoshima, M. *et al.*, *Tetrahedron Letters 1969*, 121.
19. Joshi, B. S., Kamath, V. N., Viswanathan N., *Phytochem.*, *9*, 881 (1970).
20. Herbin, G. A., Jackson, B., Scheinmann, F., *Phytochem.*, *9*, 221 (1970).
21. Bringi, N. V., Padhye, M. R., Venkataraman, K., *J. Sci. Ind. Res.*, *14B*, 135 (1955); *15B*, 128 (1956).
22. Kartha, G., Ramanchandran, G. N., Bhat, H. B., Madhavan Nair, P., Raghavan, V. K. V., Venkataraman, K., *Tetrahedron Letters*, *1963*, 459.
23. Nair, P. M., Venkataraman, K., *Ind. J. Chem.*, *2*, 402 (1964).
24. Bhat, H. B., Nair, P. M., Venkataraman, K., *Ind. J. Chem.*, *2*, 405 (1964).
25. Yemul, S. S., Private communication.
26. Dyson, N. H., Rigby, W., *J. Chem. Soc.*, *1963*, 1858.

27. YATES, P., KARMAKAR, S. S., ROSENTHAL, D., STOUT, G. H., STOUT, V. F., *Tetrahedron Letters, 1963*, 1623.
28. OLLIS, W. D., RAMSAY, M. V. J., SUTHERLAND, I. O., *Tetrahedron, 21*, 1453 (1965).
29. KARANJGAOKAR, C. G., NAIR, P. M., VENKATARAMAN, K., *Tetrahedron Letters, 1966*, 687.
30. DUTTA, N. L., GHOSH, A. C., NAIR, P. M., VENKATARAMAN, K., *Ind. J. Chem., 8*, 109 (1964).
31. For references, see THOMSON, R. H., "Naturally Occurring Quinones", 2nd ed., Academic Press, New York, 1971.
32. PATIL, V.B., RAMA RAO, A. V., VENKATARAMAN, K., *Ind. J. Chem., 8*, 109 (1970).
33. For a review of O. Dimroth's work see THOMSON, R. H., "Naturally Occurring Quinones", 1st ed., Butterworths, London, 1957.
34. (*a*) BHIDE, B. S., JOSHI, B. S., PATWARDHAN, A. V., SRINIVASAN, R., VENKATARAMAN, K., *Bull. Nat. Inst. Sci. India, 1965*, (No. 28) 114; (*b*) BHIDE, N. S., SRINIVASAN, R., VENKATARAMAN, K., *Abstr. IUPAC 4th Int. Symp. Chem. Nat. Products*, Kyoto, 205 (1964); *(c)* PANDHARE, E. D., RAMA RAO, A. V., SRINIVASAN, R., VENKATARAMAN, K., *Tetrahedron*, Suppl. *8*, 229 (1966); *(d)* PANDHARE, E. D., RAMA RAO, A. V., SHAIKH, I. N., VENKATARAMAN, K., *Tetrahedron Letters, 1967*, 2437; *(e)* GADGIL, D. D., RAMA RAO, A. V. VENKATARAMAN, K., *Ind. J. Chem., 7*, 188 (1969); *(f)* PANDHARE, E. D., RAMA RAO, A. V., SHAIKH, I. N., *Ind. J. Chem., 7*, 977 (1969); *(g)* BHIDE, N. S., PANDHARE, E. D., RAMA RAO, A. V., SHAIKH, I. N., SRINIVASAN, R., *Ind. J. Chem., 7*, 987 (1969); *(h)* RAMA RAO, A. V., SHAIKH, I. N., VENKATARAMAN, K., *Ind. J. Chem., 7*, 188 (1969).
35. BURWOOD, R., READ, G., SCHOFIELD, K., WRIGHT, D. E., *J. Chem. Soc. (C), 1967*, 842.
36. *Cf.* NARAYANAN, C. R., IYER, K. N., *Tetrahedron Letters, 1964*, 759; YOUSSEFYEH, R. D., MAZUR, M., *Tetrahedron Letters, 1963*, 1287.
37. MEHANDALE, A. R., RAMA RAO, A. V., VENKATARAMAN, K., *Ind. J. Chem., 10*, 1041 (1972).
38. BHATIA, S. B., VENKATARAMAN, K., *Ind. J. Chem., 3*, 92 (1965). See also OVEREEM, J. C., VAN DER KERK, G. J. M., *Rec. Trav. Chim., 83*, 1023 (1964).
39. MEHENDALE, A. R., RAMA RAO, A. V., SHAIKH, I. N., VENKATARAMAN, K., *Tetrahedron Letters, 1968*, 2231.
40. GADGIL, D. D., RAMA RAO, A. V., VENKATARAMAN, K., *Tetrahedron Letters, 1968*, 2229. See also RIOS, T., *Tetrahedron, 22*, 1507 (1966).
41. BHIDE, N. S., RAMA RAO, A. V., VENKATARAMAN, K., *Tetrahedron Letters, 1965*, 33. See also YATES, P., MACKAY, A. C., PANDE, L. M., AMIN, M. *Chem. und Ind., 1964*, 1991; BHIDE, N. S., RAMA RAO, A. V., *Ind. J. Chem., 7*, 996 (1969).
42. I. G. Farbenindustrie, DRP. 445, 269; 446, 563; 456, 235.
43. Farbenfabriken Bayer, Belg. Pat. 627,010 (1963).

(Based on a lecture delivered under the auspices of the Hungarian Academy of Sciences at Budapest on May 29, 1971)

E. S. SEVERIN
AND
N. N. GULYAEV

STRUCTURE AND CHEMICAL TOPOGRAPHY OF THE ACTIVE SITE OF ASPARTATE AMINOTRANSFERASE

I. INTRODUCTION

The origin and fate of natural carbon compounds invariably involves the action of several enzymes. The specificity and efficiency of enzymes as catalysts have duly excited the attention of organic chemists and physicists assisting biochemists to unravel the submolecular basis of enzyme function. Knowledge of the three-dimensional structure of enzymes and enzyme–substrate (ES) complexes is indispensable for an understanding of the mechanism of enzyme action. X-ray diffraction analysis and amino acid sequencing have provided the richest source of information on the structure of proteins; however, the solution of several problems of enzyme catalysis depended for success on the application of modern spectroscopic techniques, e.g. measurement of proton magnetic resonance, on methods following the stationary and rapid kinetics, as well as on chemical modification [1—11]. Recent methods exploit the interaction of enzymes with specific inhibitors without dismissing the dynamic aspects of catalysis. The application of a synthetic inhibitor shaped chemically to possess a predictable effect on the enzyme offers one of the most powerful means for the elucidation of the structure and function of the active site. We have applied this approach to the study of the active site of aspartate aminotransferase enzyme (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1), which promotes the following reversible transamination reaction:

$$\text{L-aspartate} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{oxaloacetate} + \text{L-glutamate}.$$

The coenzyme is pyridoxal phosphate (PLP), a derivative of Vitamin B_6, which can reversibly form a Schiff's base with amines. There is evidence that when the aldehyde group of PLP is not occupied by an amino group arising from a substrate, it forms a Schiff's base with the ε-amino group of a specific lysine residue of the active site (internal aldimine). The incoming amino group of the substrate displaces the lysine amino group yielding the substrate aldimine. After release of the keto-acid produced, the amino group

can be transferred to the keto-substrate. The formation and dissociation of various complexes can be conveniently followed by measuring the changes in the absorption spectrum of the coenzyme.

Pyridoxal enzymes, in particular aspartate aminotransferase, which has been studied in detail, have separate zones for the binding of the substrate and the coenzyme in the active site. Accordingly, synthetic analogues of substrate and coenzyme may selectively block distinct parts of the active site. The fitting of the substrate into the active site has been investigated by using two different classes of substrate analogues. Inhibitors of rigid, substrate-like structure were helpful in the elucidation of the conformation of the substrate at the binding stage. Conformational inhibitors, on the other hand, were the tools for the demonstration of steric alterations in substrate-like, low-molecular weight compounds, brought about by the protein molecule. The chemical properties of complexes consisting of the apoenzyme and coenzyme analogues have been examined to elucidate the stereochemistry of the functioning coenzyme.

Covalent blocking of distinct functional groups of the enzyme protein has been accomplished by various techniques including affinity labeling and syncatalytic modification. The structural features of enzyme–inhibitor (EI) complexes are summarized to reach conclusions as regards the structure and function of the active site of aspartate aminotransferase.

II. MODIFICATION OF ASPARTATE AMINOTRANSFERASE BY SUBSTRATE ANALOGUES: AFFINITY LABELING

Substrate-like inhibitors bearing highly reactive groups may covalently block some of the functional groups of the active site after attachment to

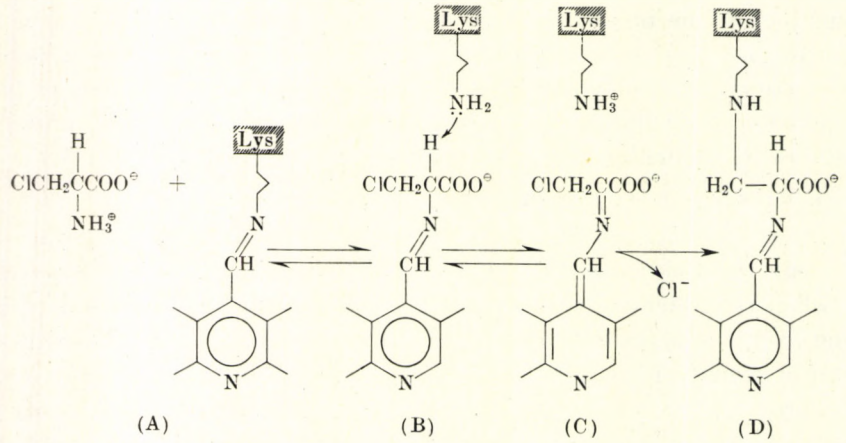


Fig. 1. Reaction of aspartate aminotransferase with β-chloro-L-alanine

the substrate zone. β-Chloro-L-alanine used by Morino and Okamoto [12] is an analogue of the amino acid substrate (Fig. 1A). Treating aspartate aminotransferase with this compound, first a pyridoxylidene inhibitor derivative is formed (B). Loss of a proton from the α-carbon atom yields a quinonoid structure (C). β-Elimination of chlorine is facilitated by the π-electron system of the complex, and a linkage is immediately formed between the nitrogen atom of the ε-amino group of the proximate lysine residue and the electrophilic β-carbon atom of the inhibitor (C → D). Incorporation of one molecule of ^{14}C-labeled inhibitor per enzyme subunit occurred in parallel with the inactivation of the enzyme. Subsequent reduction of the EI complex with $NaBH_4$, followed by carboxymethylation and

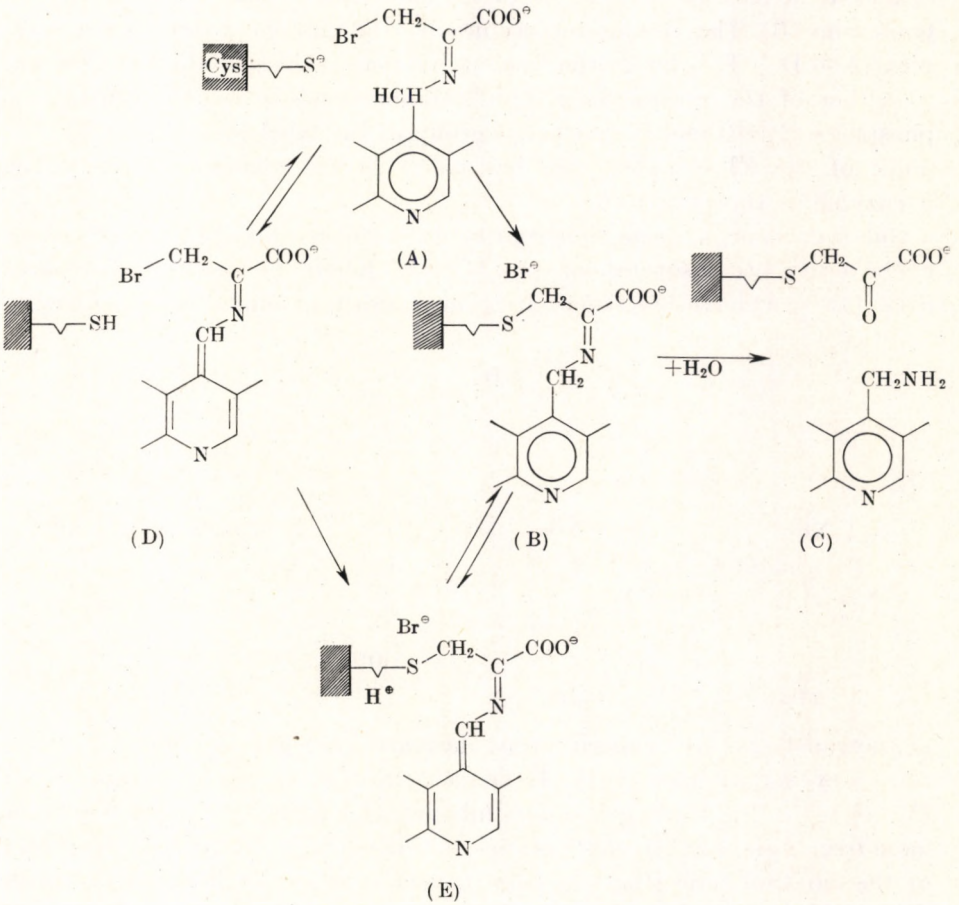
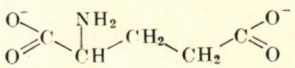


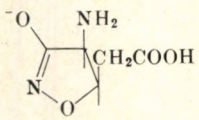
Fig. 2. Reaction of the pyridoxamine form of aspartate aminotransferase with bromopyruvate

trypsin digestion, yielded a radioactive peptide containing the phosphopyridoxyl residue. The amino acid composition of this peptide was identical with that of the peptide containing pyridoxyllysine obtained by trypsin digestion of the $NaBH_4$-treated native enzyme. Thus the selective alkylation of the functional lysine moiety of the active site, involved in the formation of the internal aldimine bond, has been achieved.

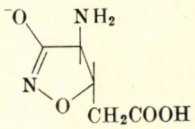
As an analogue of the keto-substrate, Okamoto and Morino [13] used bromo-2-[^{14}C]-pyruvate for the selective modification of a cysteine residue in the active site of the pyridoxamine form of aspartate aminotransferase. A ketimine derivative of the inhibitor is formed with pyridoxamine phosphate at the active site (Fig. 2A), which alkylates the nucleophilic SH group of a cysteine residue owing to the highly electrophilic character of the β-carbona tom (B). The alkylation may involve the quinonoid form of the complex (A → D → E → B). In the final stable inactivation product (C), formed by either of the routes, there is no ketimine bond between pyridoxamine phosphate (PMP) and the carboxyl group of the label, since $NaBH_4$ treatment of the EI complex does not result in the covalent binding of the coenzyme to the active site.

Substrate analogues with rigid conformation gained application in the study of the active site of several enzymes [14, 15]. Khomutov, Severin, Kovaleva *et al.* [15—19] have investigated the mechanism of inhibition of aspartate

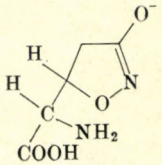




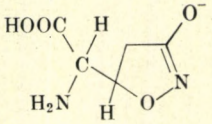
(I)



(II)



(III)



(IV)

aminotransferase by isomeric cyclic derivatives of glutamic acid (I: *threo-(cis)*-α-cycloglutamate (CG); II: *erythro(trans)*-α-CG; III: *erythro*-γ-CG; IV: *threo*-γ-CG). Both glutamate (above) and each of these compounds have free amino and carboxyl groups, whereas the hydroxyamide fragment of the isoxazolidone ring can be regarded as an equivalent of the other carboxyl group of glutamate. The fitting of at least one of these compounds to the substrate site, responsible for glutamate binding has been expected.

Besides, because of the high reactivity of the isoxazolidone-3 system, cycloglutamates are rather potent acylating agents, therefore they are promising compounds for the covalent blocking of some functional groups at the active site.

Kinetic studies suggested similar inhibition mechanisms for all isomeric cycloglutamates:

$$E + I \overset{K_I}{\rightleftharpoons} E \cdots I \overset{k_2}{\longrightarrow} EI.$$

The affinity constants of the inhibitors (K_I), the half-time of transformation at saturation concentration ($t_{1/2}$) and the modification rate constants (k_2) were determined from double reciprocal plots [19].

The greatest affinity for the substrate zone of the active site was exhibited by the compounds with *threo* configuration; in fact, the affinity of *threo*-α-CG was found to be higher than that of the substrate by one order of magnitude. *Threo*-α-CG can undergo normal enzymic transamination as well. Furthermore, a high modification rate was found with the latter compound ($k_2 = 0.5$ min^{-1}), while k_2 values for other isomers ranged between 0.08 and 0.12 min^{-1}.

The efficacy of *threo*-α-CG as an inhibitor prompted us to investigate the mechanism of its action in more detail [16, 17, 19]. It was found that *threo*-α-CG did not affect either the apoenzyme or the pyridoxamine form of aspartate transaminase; however, complete inhibition of the enzyme occurred when the molar ratio of inhibitor to enzyme was close to unity. Inhibition was accompanied by the disappearance of the absorption maximum at 340 nm, characteristic of bound pyridoxal; this maximum was replaced by a new peak of absorption at 330 nm typical for a ketimine. The position and heigth of the latter maximum was independent of pH throughout the range (3.8–8.2) examined. It has been concluded that the interaction of the inhibitor with the coenzyme at the active site of aspartate transaminase is a prerequisite step of the inactivation process.

The stereospecificity of the inhibition reaction was indicated by the fact that only the L-configuration of *threo*-α-CG was effective. In the presence of glutamic acid the rate of irreversible inactivation remained unchanged, but a marked decrease in the apparent affinity of *threo*-α-CG for the enzyme occurred, suggesting that the ionized carboxyl and pseudocarboxyl groups of the inhibitor, and the carboxyl groups of the natural dicarboxylate substrate react with identical sites of the substrate zone. Attempts at the reactivation of the enzyme by the addition of glutamate, α-ketoglutarate and pyridoxal phosphate, followed by gel filtration, remained unsuccessful. Complete hydrolysis of the EI complex in hydrochloric acid yielded pyridoxamine as the only Vitamin B_6 derivative.

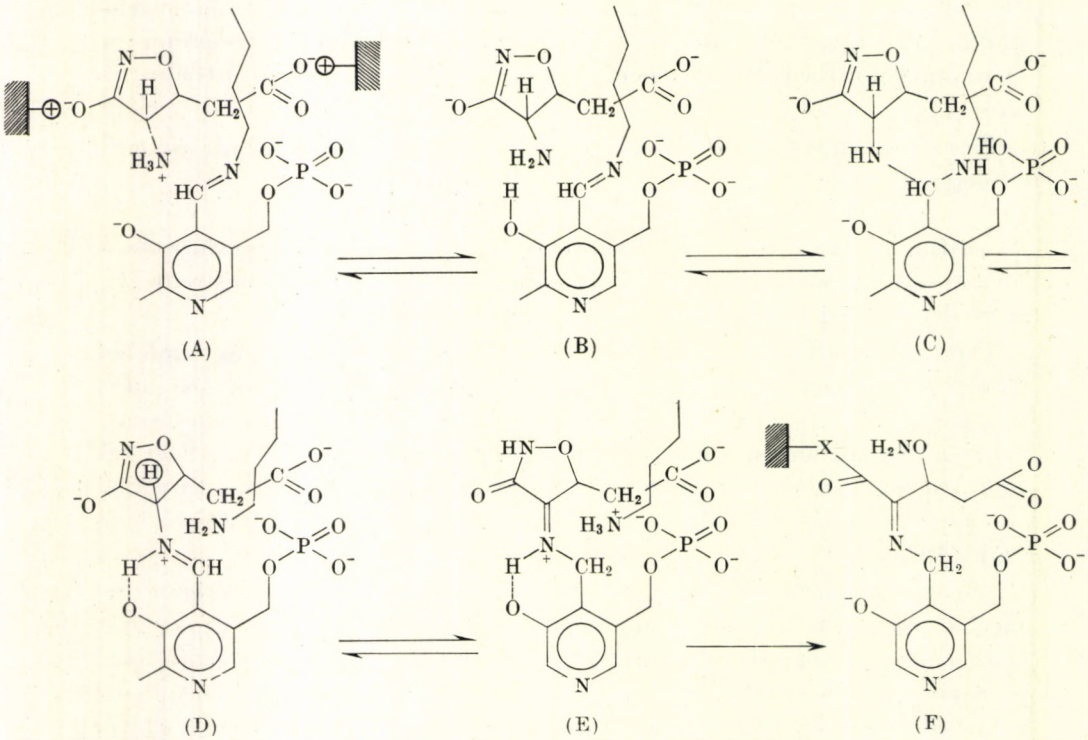


Fig. 3. Reaction of aspartate aminotransferase with *threo*-α-cycloglutamic acid

Comparing the spectral properties of the intermediate ES and EI complexes, and considering the presence of a double bond reducible with $NaBH_4$, the route of formation of the stable EI complex is postulated as shown in Fig. 3. The inhibitor fitting into the substrate zone forms an addition product with the coenzyme (C), which yields the aldimine (D), then the ketimine (E) complexes, the latter being a stabilized pyridoxylimino derivative of the inhibitor, containing the reducible double bond. The stability of the EI complex indicates that the prototropic rearrangement is accompanied by the covalent blocking of some group of the active site at the expense of opening the isoxazolidone ring of the inhibitor (F).

Easy cleavage of the covalent bond between the protein and inhibitor upon hydrolysis rendered improbable that acylation of a lysine amino group, or that of the hydroxyl group of a serine or threonine residue occurred. The linkage with a histidine imidazole moiety or with the phenolic hydroxyl of tyrosine seemed more likely. The formation of hydroxamate with hydroxylamine, an increase of the absorption at 250 nm in the course of the inac-

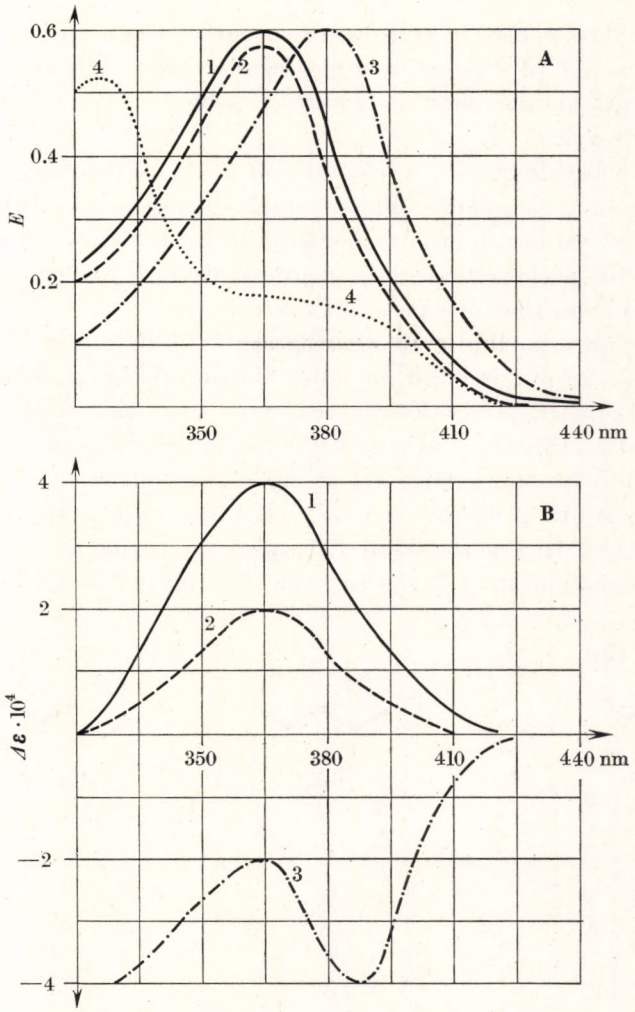


Fig. 4. Absorption (A) and circular dichroic (B) spectra of aspartate aminotransferase treated with *erythro*-γ-cycloglutamic acid. Curves in Parts A and B represent the corresponding spectra for the native enzyme (1), after treatment at pH 8.2 (2), which was followed by reduction with $NaBH_4$ (4), and after prolonged treatment at pH 6.3 (3)

tivation reaction, as well as the pH dependence of the reaction rate (S-shaped curve with a point of inflexion at pH 6.7) in agreement with some other findings have suggested the covalent blocking of an NH group of an imidazole ring at the catalytic site [18, 19]. Apparently, the interaction of *threo*-α-CG with aspartate aminotransferase results in the acylation of a

histidine imidazole ring by the modified carboxyl group of the inhibitor. On this basis, one of the histidine residues located in the active site of the enzyme (α-cationic site) may be held responsible for the binding of the substrate α-carboxyl.

A study of the effects of γ-cycloglutamic acids (bearing a modified γ-carboxyl group) has supplemented our results concerning substrate binding [18]. The *threo* isomer showed higher affinity for the enzyme, while *erythro*-γ-CG deserved special attention as it produced much higher rate of enzyme modification than that observed with *threo*-γ-CG.

Treatment of aspartate aminotransferase with an excess of *erythro*-γ-CG at pH 8.0 for 20 minutes did not alter the initial enzyme spectrum, but it decreased the positive circular dichroic band considerably (Fig. 4A and B, Curves 1 and 2); this effect might be due to the neutralization of a positively charged group by the α-carboxyl group of the inhibitor. Electrophoretic analysis of the low molecular weight products of hydrolysis of the EI complex with 0.1 N HCl demonstrated the presence of the oxime of PLP with β-aminohydroxyglutamate (proof of the opening of the isoxazolidone ring

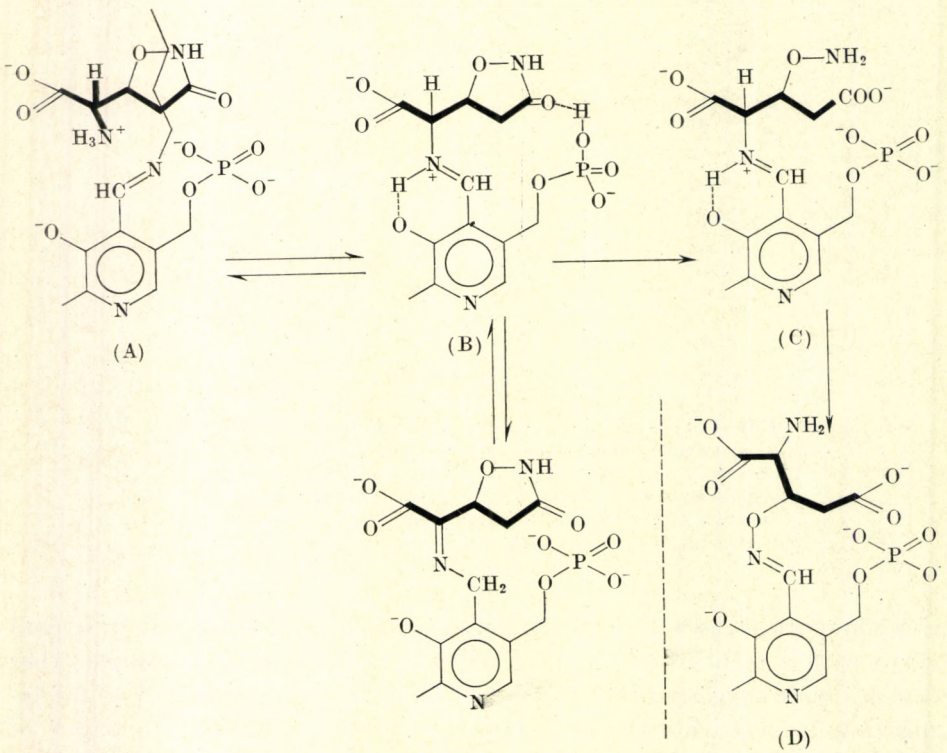


Fig. 5. Reaction of aspartate aminotransferase with *erythro*-γ-cycloglutamic acid

of *erythro*-γ-CG). After the reduction of the mixture of enzyme and inhibitor with $NaBH_4$, the absorption maximum at 360 nm was replaced by a new band at about 330 nm (Fig. 4A, Curve 4). Mild acid hydrolysis of the reduced EI complex did not yield PMP but, besides other derivatives of Vitamin B_6, the presence of N-pyridoxyl-γ-CG indicated the existence of an aldimine intermediate.

Prolonged treatment of the enzyme with *erythro*-γ-CG at pH 6 led to the formation of a single product showing an absorption maximum at 380 nm and exhibiting negative circular dichroism (Fig. 4A and B, Curve 3). This complex is the oxime of the enzyme.

From the foregoing we propose the following mechanism for the interaction of *erythro*-γ-CG with aspartate aminotransferase (Fig. 5). Similar to the substrate, *erythro*-γ-CG reacts at the active site with the coenzyme to produce a pyridoxylidene derivative (B), which may undergo normal prototropic rearrangement leading to a ketimine. The relatively low rate of transamination is apparently due to the engagement of the γ-pseudocarboxyl group in the ring and to the rigid structure of this substrate analogue. Parallel with the process of transamination involving the pyridoxylidene form, the opening of the isoxazolidone ring of the inhibitor occurs (C), resulting in the release of the aminohydroxy group. A subsequent rearrangement yields the oxime of aspartate aminotransferase with β-aminohydroxyglutamate (D).

III. INTERPRETATION OF THE EFFECTS OF SUBSTRATE ANALOGUES: CONFORMATION OF SUBSTRATES IN THE ES COMPLEX

The results obtained with various cycloglutamate isomers permit to draw some conclusions as regards the conformation of natural substrates fit for binding [19]. The synthetic, cyclic inhibitors can be regarded as "freezed" rotamers of glutamate with fixed conformation of four of the five carbon atoms. From a stereochemical point of view, investigations with *threo*-α-CG and *threo*-γ-CG complement each other, considering the stabilized conformation fixed by the isoxazolidone ring at the amino-end of *threo*-α-CG, matched by the rigid structure around the γ-pseudocarboxyl group of *threo*-γ-CG (Fig. 6A and B). In the ES complex the configuration of carbon atoms 1—4 of glutamate is most probably compatible with the three-dimensional structure of *threo*-α-CG, which has the greatest affinity for the active site of aspartate aminotransferase, and it can undergo enzymic

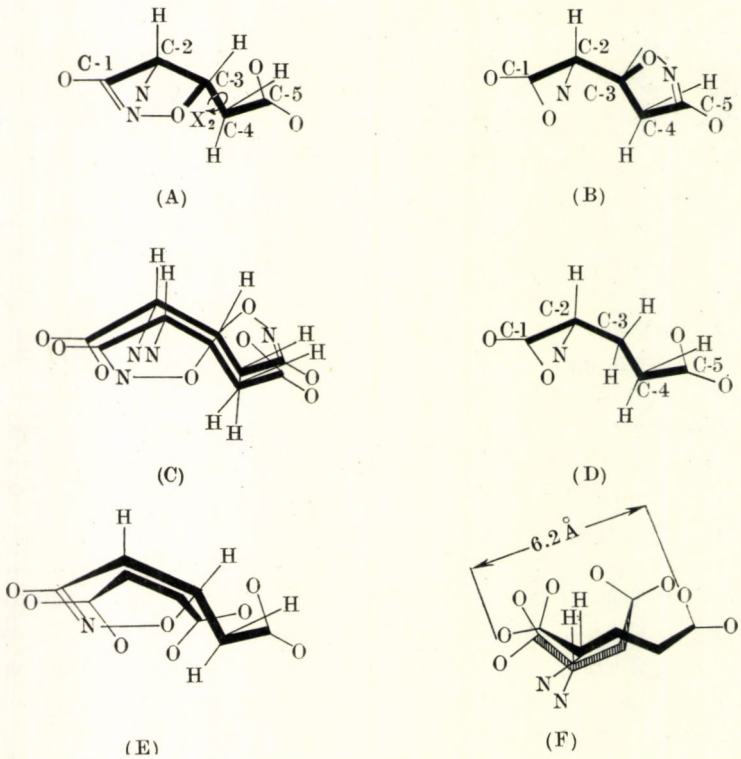


Fig. 6. Conformations of substrates and inhibitors bound at the active site of aspartate aminotransferase. *Threo*-α-CG (A); *threo*-γ-CG (B); *threo*-α-CG and *threo*-γ-CG (C); glutamate (D); *threo*-α-CG and maleate (E); glutamate and aspartate (F)

transamination, too. We assume that the negatively charged oxygen atom of the isoxazolidone ring of the inhibitor (as well as the ionized α-carboxyl group of glutamate) interact with a cationic site responsible for substrate binding, which is presumably a histidine residue. The α-amino group is apparently directed towards the aldimine linkage of the coenzyme with the ε-amino group of the lysine residue in the active site. The bond between the α-carbon atom and the hydrogen atom is perpendicular to the plane determined by carbon atoms 1 and 4, and the nitrogen atom of the amino group.

The positions of the γ-carboxyl group and of the hydrogen atoms joining carbon atom 4 are still left to be determined, since free rotation is allowed about the bond between carbon atoms 3 and 4 of *threo*-α-CG. However, considering the structure of γ-cycloglutamates, in particular that of *threo*-γ-CG which has higher affinity for the enzyme than the *erythro* isomer, one

can arrive at rather definite conclusions. The relative positions of carbon atoms 3, 4 and 5 are fixed in *threo*-γ-CG. By proper rotation of the isoxazolidone ring about the axis formed by the bond between C-2 and C-3, the carbon atoms 1 to 4 of *threo*-γ-CG will coincide with those of *threo*-α-CG. The quested location of carbon atom 5 of *threo*-α-CG is spotted by the corresponding carbon atom of *threo*-γ-CG (Fig. 6C). For the angle between carbon atoms 2, 3 and 5 of *threo*-α-CG a value of about 180° is obtained, which is in agreement with the parameters resulting from the theoretical calculations by Tumanyan; the completely "unfolded" conformation of *threo*-α-CG appears to be the most favourable energetically [20]. The coincidence of all relevant atoms of *threo*-α-CG with those of *threo*-γ-CG in the conformations shown in Fig. 6C suggests a similar conformation for glutamate apt to bind to the enzyme (Fig. 6D).

The "unfolded" conformation of glutamate at binding offers satisfactory explanation of the data accumulated up to the present on the interactions of dicarboxylic acids and substrate analogues with aspartate aminotransferase [19]. Thus an interpretation of the competitive inhibitory effect of maleate, a tetracarbon dicarboxylic acid with fixed conformation is feasible, whereas fumarate is not inhibitory. Assuming that the ionized carboxyl groups of maleate interact with the same cationic sites of the protein which are involved in the binding of pentacarbon substrates, then the structures of maleate and *threo*-α-CG can be superposed as shown in Fig. 6E. For aspartate, the position of carbon atom 4 is also determined unequivocally, and the distance between the negatively charged oxygen atoms which bind to the α- and ω-cationic sites of the substrate zone can be estimated as 6.2 Å (Fig. 6F).

A further characteristic of the complex of pentacarbon substrates with the enzyme is the proximity of the free γ-carboxyl group to the phosphate moiety of the coenzyme. The implications of this spatial relationship will be discussed later.

IV. SYNCATALYTIC MODIFICATION OF ASPARTATE AMINOTRANSFERASE

As regards the chemical agent applied, syncatalytic modification of the reactive groups of the enzyme protein is a rather unspecific manoeuvre. Its uniqueness is due to the circumstance that some peculiar residues of the protein are inaccessible to the reagent, but they may become prone to modification during the catalytic process performed by the enzyme.

The tyrosine residue in the active site of aspartate aminotransferase becomes accessible to nitration in the presence of the substrate pair only. Syncatalytic nitration leads to the inactivation of the enzyme. Polyanovsky *et al.* [21] isolated two similar peptides containing nitrotyrosine residues from the tryptic digest of the enzyme treated with tetranitromethane under syncatalytic conditions. A comparison of the amino acid compositions of these fragments with the primary structure of aspartate aminotransferase has shown that the modified residues correspond to Tyr-40 of the polypeptide chain:

$$\overset{40}{\text{Lys-Val-Asn-Leu-Gly-Val-Gly-Ala-Tyr}}(NO_2)\text{-Arg}$$

and

$$\text{Val-Asn-Leu-Gly-Val-Gly-Ala-Tyr}(NO_2)\text{-Arg.}$$

It is known that two of the five cysteine residues present in each subunit of aspartate aminotransferase are located on the surface of the protein molecule, and they readily react with alkylating agents without affecting the enzymic activity. Two SH groups are buried in the depths of the protein globule; these are accessible to chemical modification after enzyme denaturation only. The fifth, functionally important cysteine residue is relatively non-reactive, but it is unmasked in the presence of the substrate pair. After alkylation of the two exposed thiol groups, selective modification of this cysteine residue is feasible with alkylating agents such as N-ethylmaleimide, which results in a considerable impairment of the enzyme function [22]. The location of the functionally important cysteine residue has been determined by Torchinsky, Severin *et al.* [23]. Aspartate aminotransferase was treated with N-ethyl-[^{14}C]-maleimide under syncatalytic conditions, thereafter radioactivity was detected in the nonapeptide tryptic fragment containing Cys-390:

$$\overset{390}{\text{Ile-Asn-Met-}}\textit{Cys}\text{-Gly-Leu-Thr-Thr-Lys.}$$

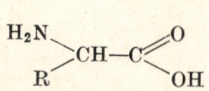
It should be noted that the residues Tyr-40 and Cys-390 are apparently not directly involved in the catalytic process, yet selective modification of these residues impairs the function of the active site.

V. INTERACTION OF ASPARTATE AMINOTRANSFERASE WITH CONFORMATIONAL INHIBITORS

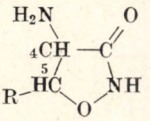
Change of the conformation of the substrate molecule before enzymic transformation may be an extremely important feature of enzymic catalysis.

Some earlier reports have suggested that the reactivity of certain compounds which are weakly active in model reactions with low molecular weight substances, may be enhanced on interaction with the active site of an enzyme, owing to an appropriate orientation brought about by the protein [24, 25]. However, the investigation of changes in the substrate are generally hampered by the rapidity at which the conversion of the substrate into the product proceeds. This problem might be overcome by the application of suitable inhibitory substrate analogues.

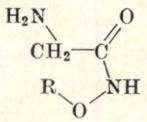
Starting with this concept, Severin, Khomutov and co-workers [26–28] have prepared a series of inhibitors to PLP enzymes, the effect of which was dependent on conformational changes occurring on interaction with the enzyme. These compounds are isomeric cyclic derivatives of glycine hydroxamic acid (VII) containing each functional group characteristic of the substrate amino acid (V). They may be regarded as cyclic substrate analogues (VI) dissected at the bond between carbon atoms 4 and 5. In



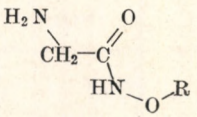
(V)



(VI)



(VIIa)



(VIIb)

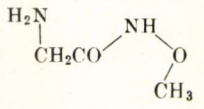
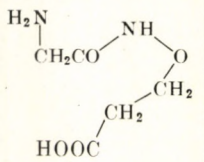
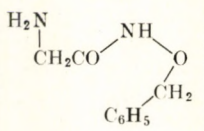
solution, the preferred conformation of the hydroxyamide group in these molecules is the *trans* form (VIIb).

Different esters of glycine hydroxamic acid analogous to alanine, glutamate and phenylalanine were tested with several PLP enzymes catalyzing the transformations of the respective amino acids. It has been found that each inhibitor affects mostly the "cognate" enzyme, whereas they are inactive with respect to other enzymes (Table I). The inhibitory properties of hydroxamic esters may be interpreted as to be due to a forced alteration of the conformation of the molecule at the enzyme active site, that is a transition from the *trans* form into a strained S-*cis* conformation (VIIa), which is fitting to the substrate zone of the enzyme. The time course of the enzyme inactivation caused by the inhibitors is consistent with the presumed mechanism. Similarly to the enzyme inactivated with cyclic substrate analogues, enzymes inhibited with glycine hydroxamic acid esters could not be reactivated by the addition of α-ketoglutarate, and by gel filtration.

The mechanism of the interaction of one of the conformational inhibitors, the β-carboxyethyl ester of glycine hydroxamic acid (VII, R = CH_2CH_2—COOH), with aspartate aminotransferase has been studied in detail. The

Table I

Effect of Glycine Hydroxymate Esters on Different PLP Enzymes
(Enzyme activity was measured after 10 minutes incubation with the inhibitor at the concentration indicated)

Substrate	Inhibitor	Concentration (M)	Inactivation of enzyme, %			
			Aminotransferase specific for			Glu-Decarboxylase
			Ala	Glu	Phe	
Ala		$1.0 \cdot 10^{-2}$	35	—	—	—
Glu		$2.0 \cdot 10^{-3}$	90	95	30	90
Phe		$1.0 \cdot 10^{-2}$	—	—	70	—

reaction involves the reversible formation of an EI complex ($K_I = 2.8 \cdot 10^-$ M); finally the oxime of the enzyme is produced. The changes observed in the ultraviolet and circular dichroic spectra during the reaction suggest the mechanism presented in Fig. 7. Considering the position of a pentacarbon substrate, glutamate in the substrate zone (Fig. 7A), only the propionyl residue of the inhibitor is able to occupy a corresponding position in the initial EI_1 complex (B). It is the transition of the conformation of the molecule into the strained S-*cis* form by which a tight fitting of the pseudocyclic inhibitor is achieved in EI_2 (C). The latter complex resembles the addition product of *threo*-α-CG with the enzyme (*cf.* Fig. 3C). The strained conformation of the inhibitor permits an interaction between the negatively charged oxygen atom of the hydroxyamide group and a cationic site of the protein. Subsequent addition of the amino group to the coenzyme becomes possible, producing a complex which exhibits absorption at 335 nm (D). The instability of the S-*cis* conformation, and the proximity of a nucleophilic group of the protein cause the cleavage of the C—N bond of the

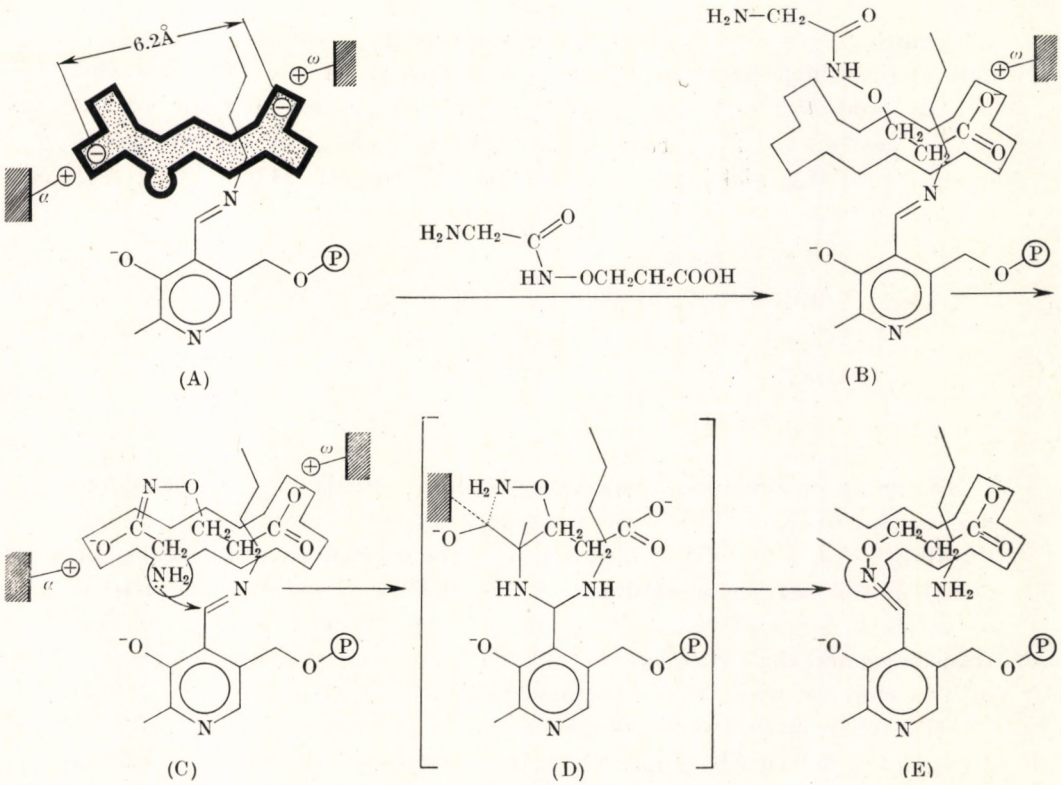


Fig. 7. Reaction of aspartate aminotransferase with the β-carboxyethyl ester of glycine hydroxamic acid (pseudo-cycloglutamate)

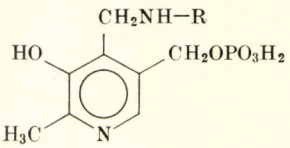
inhibitor, whereupon a free amino group appears. The final EI_3 complex is the stable oxime of the enzyme (E) with an absorption band at 380 nm.

Our results support the notion of a forced conformational alteration of a low molecular weight compound under the influence of a protein molecule. Thus an enzyme may induce specific changes in the three-dimensional structure of an inhibitor, fixing that conformation which has the greatest affinity for the active site. The multi-point binding at the substrate zone explains the ability of aspartate aminotransferase to exert such an effect.

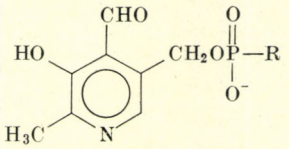
VI. INTERACTION OF ASPARTATE AMINOTRANSFERASE WITH COENZYME ANALOGUES

Phosphate esters of pyridoxal and pyridoxamine are known to exhibit high affinity for the active site of PLP enzymes. Generally they can be separated from the apoenzyme without damaging the enzyme structure.

Khomutov, Severin, Gulyaev and others [29–32] synthesized coenzyme derivatives which contained reactive R groups (VIII and IX). The compounds produced have been characterized with respect to their affinity for the active site of aspartate aminotransferase (K_I), the rate of enzyme modification (k_2), and inhibitory properties (Table II) [31].



(VIII)

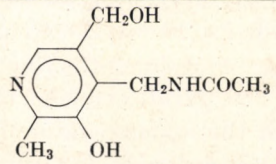
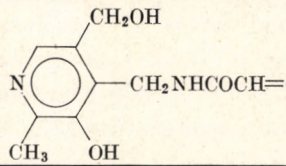
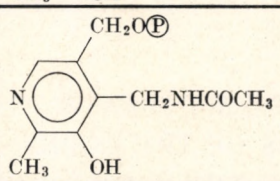
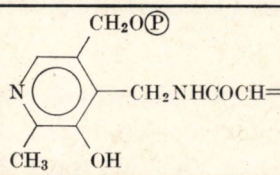
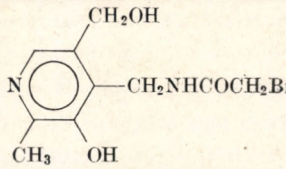
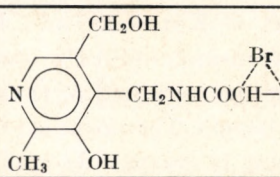
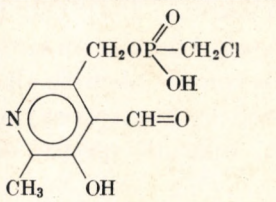


(IX)

No inhibitory effect was observed with compounds lacking the phosphate moiety (X, XI, XIV, XV) in spite of the presence of R groups with alkylating ability (XI, XIV, XV), which indicated the importance of the phosphate group in the enzyme mechanism. N-Acetyl-PMP (XII) was found to be an efficient competitive inhibitor. Marked inhibition of aspartate aminotransferase and the formation of stable EI complexes occurred on treatment of the enzyme with 5′-P-chloromethyl-PLP (XVI) and N-acrylyl-PMP (XIII). When present in equimolar amounts, the latter compound formed an optically active EI complex with the apoenzyme, the absorption maximum of which was at 335 nm. Reactivation of the enzyme was achieved by the addition of an excess of PLP. This suggested that no covalent blockage of any functional group of the active site had taken place [31].

Investigation of the action of 5′-P-chloromethyl-PLP has shown that this coenzyme analogue binds to the site responsible for PLP binding, and it forms a Schiff's base with a lysine residue in the enzyme active site. The EI complex exhibited a positive circular dichroism band at 365 nm at pH 8.0, and it did not dissociate upon gel filtration. The enzymic activity of the complex was about 10 times lower than that of the natural holoenzyme. The high affinity of the compound to the apoenzyme as well as the spectral properties indicating the formation of an aldimine bond have confirmed the suggestion that also PLP is bound as a monoanion to the coenzyme zone of the active site. Substitution of one of the hydroxyl groups of the phosphate moiety for the chloromethyl residue resulted, however, in the loss of the pH indicator properties of the complex: the spectral transition from 365 nm in the alkaline region to 430 nm in the acidic domain, a characteristic of the internal aldimine of the enzyme, was no longer observed with the EI complex.

Table II
Effect of Coenzyme Analogues on Aspartate Aminotransferase

No.	Compound	K_I (M)	k_2 (min^{-1})	$[I]=1\cdot10^{-2}$ M
X		—	—	No inhibition
XI		—	—	No inhibition
XII		$4.0\cdot10^{-4}$	—	
XIII		$8.5\cdot10^{-5}$	1.0	
XIV		—	—	No inhibition
XV		—	—	No inhibition
XVI		$4.8\cdot10^{-4}$	0.08	

The involvement of the lysine residue in the aldimine bond present in the EI complex of 5′-P-chloromethyl-PLP was demonstrated by the reduction of the double bond with $NaBH_4$, resulting in a tight linkage between the ε-amino group of lysine and the inhibitor. Carboxymethylation of the EI complex followed by tryptic digestion yielded a 19-membered peptide from the active site of aspartate aminotransferase, containing the fluorescent group of the inhibitor. A similar analysis of the pyridoxylidene form of aspartate aminotransferase, reconstituted from the apoenzyme and [^{32}P]-PLP and treated with $NaBH_4$, yielded 19-membered peptides containing the phosphorylated and the dephosphorylated forms of the coenzyme. Significant release of inorganic phosphate, observed in both cases, indicated that the phosphoester bond is rendered unstable in the complex. This circumstance bears importance as regards the stereochemistry of the internal aldimine bond present in the native enzyme.

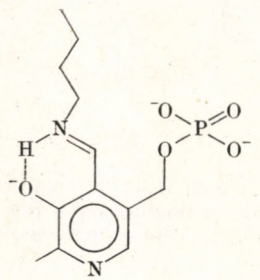
VII. MECHANISM OF ACTION OF ASPARTATE AMINOTRANSFERASE: INTERACTIONS OF THE COENZYME WITH THE APOENZYME AND WITH THE AMINO ACID SUBSTRATE

The interpretation of our results calls for a reconsideration of recent observations and ideas on the function of the enzyme in question. In the elegant experiments of Arigoni, [^{3}H]-$NaBH_4$ was used for the reduction of the double bond of the internal aldimine (formed by the ε-amino group of a lysine residue of the active site), and for the reduction of the substrate aldimine [35]. The tritium added was incorporated in the pro-R configuration in the case of the internal aldimine, whereas the position of tritium in the reduced substrate aldimine corresponded with the pro-S configuration. This seemed to confirm the earlier hypothesis of Karpeisky and Ivanov [33, 34], who have suggested that in course of transamination the coenzyme ring is revolving about an axis between carbon atoms 2 and 5, thus alternating exposure of either side of the coenzyme occurs. The proposed mechanism based on the rotation of the coenzyme ring is compatible with many characteristics of the enzymic transamination reaction [34]. Our results, however, while offering an explanation also of Arigoni's observations, suggest a different reaction mechanism, which is compatible with all known stereochemical features of the complexes formed by the apoenzyme with coenzyme, substrate and, last but not least, with all species of inhibitors.

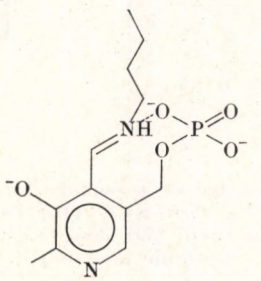
The properties of the coenzyme phosphoester bond provide a clue to be followed up. Although interactions between the phosphate group and some residues of the protein cannot be ruled out, several characteristics of EI complexes [17–19] have led us to the conclusion that the phosphate group of the coenzyme and the free γ-carboxyl group of glutamate are spatially close to each other. A pronounced activation of the phosphoester bond was observed in the complexes of aspartate aminotransferase with *threo*-α-CG and with N-pyridoxyl-5′-phosphate-L-glutamic acid (Fig. 8B and D). Labilization of the phosphoester bond was not manifest in EI complexes lacking a free carboxyl group in the vicinity of the phosphate moiety of the coenzyme (Fig. 8A and E) [18, 31]. On the other hand, the release of inorganic phosphate observed in experiments with the pyridoxylidene form of aspartate aminotransferase containing [^{32}P]-PLP, and with the complex of the apoenzyme with 5′-P-chloromethyl-PLP, indicates the labilization of the phosphoester bond in the free holoenzyme, too. The finding mentioned last has prompted us to reconcile our ideas concerning the stereochemistry of the internal aldimine of aspartate aminotransferase with the experimental results presented.

Recent theories have assumed a localization of the internal aldimine bond close to the phenolic hydroxyl group of PLP, permitting an interaction between the two (Fig. 9A and XVII). However, the available data enable one to propose a conformation where the aldimine bond is in the vicinity of the coenzyme phosphate group (Fig. 9B), and the translocation of a dissociable proton of the phosphate group ($pK_2 = 6.2$) to the nitrogen atom is possible (XVIII). This orientation of the aldimine bond permits the addition of the substrate amino acid in the direction indicated by the pencil in Fig. 9B without any rotation of the coenzyme pyridine ring.

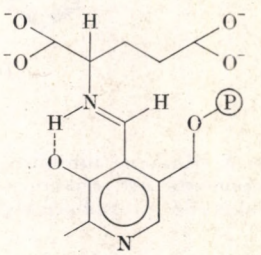
After the formation of an intermediate tetrahedral complex (Fig. 9C, *cf.* Fig. 3C), the ε-amino group of the lysine residue is released, and the



(XVII)



(XVIII)



(XIX)

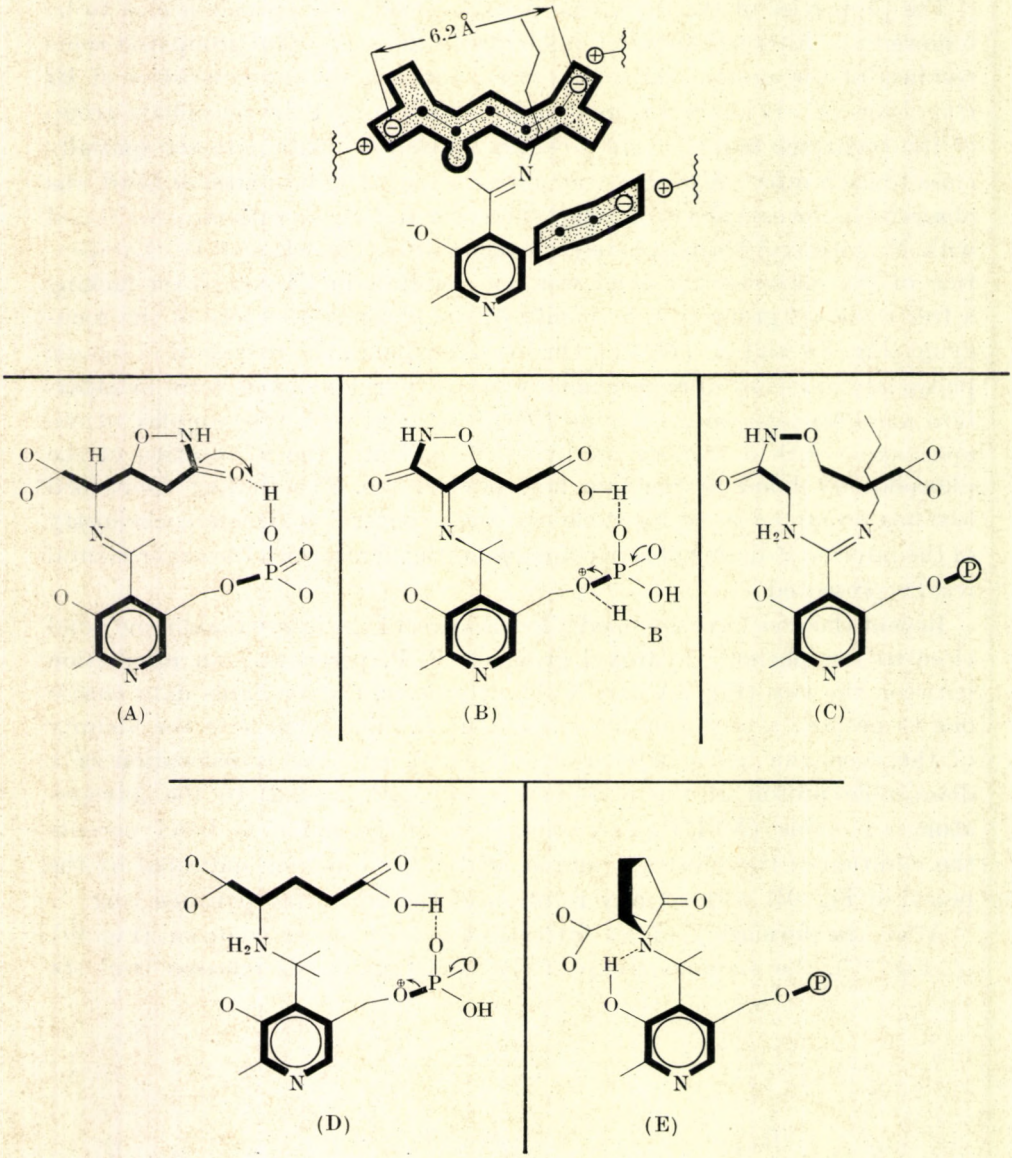


Fig. 8. Enzyme–inhibitor complexes of aspartate aminotransferase. The inhibitor components are *erythro*-γ-CG (A), *threo*-α-CG (B), pseudo-cycloglutamate (C), N-pyridoxyl-5′-phosphate-L-glutamic acid (D), and N-pyridoxyl-5′-phosphate L-pyroglutamic acid (E)

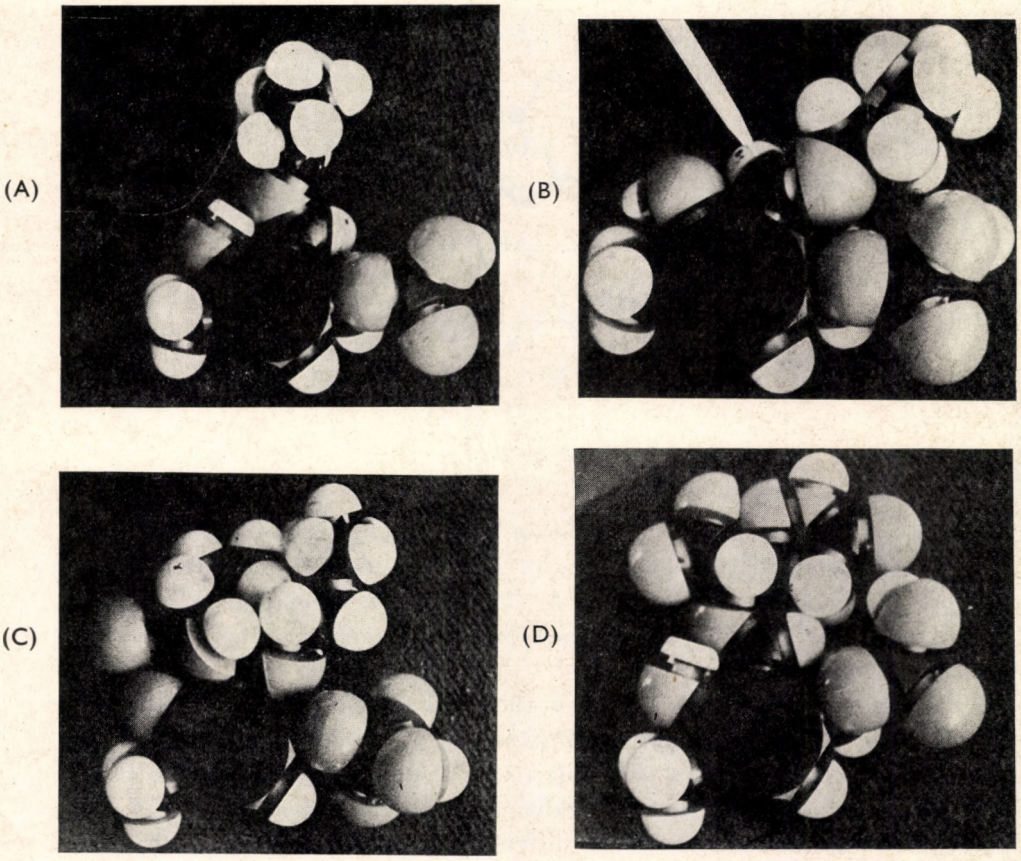


Fig. 9. Space filling models showing two possible conformations of the internal aldimine of aspartate aminotransferase (A and B), tetrahedral complex with the amino acid substrate (C), and the substrate aldimine (D)

substrate aldimine is formed. The steric conditions in the fixed substrate aldimine (Fig. 9D) may make possible the formation of an intramolecular hydrogen bond between the phenolic hydroxyl group and the nitrogen atom of the aldimine (XIX). At the same time the γ-carboxyl group of the pentacarbon substrate may move close to the phosphate moiety of the coenzyme (see also Fig. 8D).

Theoretical calculations by Tumanyan have shown that both the conformation proposed for the internal aldimine, and that suggested for the substrate aldimine are characterized by about the same values of potential energy minima. In the internal aldimine an inconsiderable declination of the aldimine bond from the plane of the pyridine ring appears energetically

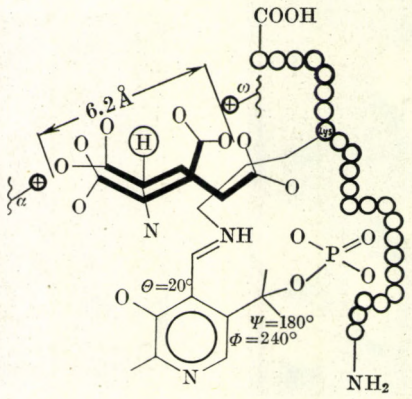


Fig. 10

favourable because of the orientation of the aldimine bond towards the phosphate group [36]. The angles of rotation about the bonds between carbon atoms 4 and 4′ (θ), 5 and 5′ (Φ) and between carbon atom 5′ and the oxygen atom (ψ) have also been arrived at by theoretical conformational analysis (Fig. 10).

There remained to be shown how the results of Arigoni could be reconciled with our structural models. Provided that the orientation of the aldimine bond of the internal aldimine (XVIII) is different from that in the substrate aldimine (XIX), the addition of tritium upon treatment with [^{3}H]-$NaBH_4$ yields evidently two different products as regards the position of the incorporated isotope; namely pro-R configuration will result with the internal aldimine, and pro-S configuration in the case of substrate aldimine. Thus the stereochemistry of tritium addition can readily be interpreted without assuming the rotation of the coenzyme ring in course of the transamination reaction.

The chemical topography of the active site of aspartate aminotransferase may be represented as shown in Fig. 10. Overlapping substrate zones for aspartate and glutamate lie between the α-cationic site (presumably a histidine residue) and the ω-cationic site. The distance between the negatively charged oxygen atoms involved in the binding to the cationic sites is estimated as 6.2 Å.

Various EI complexes described in this study might be regarded as "frozen" stages of the transamination reaction, by means of which an insight into the mechanism of enzymic catalysis has been attempted. In the course of these investigations the importance of interactions involving the

coenzyme phosphate moiety has emerged, and the chemical topography of the internal aldimine and substrate aldimine of aspartate transaminase has been outlined. Research is in progress which may elucidate further details; nevertheless, for the present the essential features of the enzymic transamination reaction can be interpreted on the structural basis suggested in this work.

REFERENCES

1. Eisenberg, D. in "The Enzymes" (Ed. Boyer, P. D.) Vol. 1, Academic Press, New York, 1970. p. 1.
2. Shaw, E., *ibid.*, p. 91.
3. Cohen, L. A., *ibid.*, p. 148.
4. Cleland, W. W., in "The Enzymes" (Ed. Boyer, P. D.) Vol. 2, Academic Press, New York, 1970. p. 1.
5. Hammes, G. G., Shimmel, P. R., *ibid.*, p. 67.
6. Bruice, T. C., *ibid.*, p. 217.
7. Timasheff, S. T., *ibid.*, p. 371.
8. Braunstein, A. E., Karpeisky, M. Ya., *J. of the All-Union Mendeleev Chemical Society, 16* (No. 4), 362 (1971).
9. Bistrov, V. F., Sacharovsky, V. G., *ibid.*, p. 380.
10. Beresin, I. V., Martinek, K., *ibid.*, p. 411.
11. Loshadkin, N. A., *ibid.* p. 403.
12. Morino, Y., Okamoto, M., *Biochem. Biophys. Res. Commun., 50*, 1061 (1973).
13. Okamoto, M., Morino, Y., *J. Biol. Chem., 248*, 82 (1973).
14. Cass, J. D., Meister, A., *Biochemistry, 9*, 842 (1970).
15. Khomutov, R. M., Severin, E. S., Kovaleva, G. K., Gulyaev, N. N., Gnuchev, N. V., Sastchenko, L. P., in "Chemistry and Biology of the Pyridoxal Catalysis", Nauka, Moscow, 1968, p. 381.
16. Kovaleva, G. K., Severin, E. S., Fasella, P., Khomutov, R. M., *Biokhimiya, 38*, 370 (1973).
17. Kovaleva, G. K., Severin, E. S., *Biokhimiya, 37*, 478 (1972).
18. Kovaleva, G. K., Severin, E. S., *Biokhimiya, 37*, 1282 (1972).
19. Severin, E. S., Kovaleva, G. K., Sastchenko, L. P., *Biokhimiya, 37*, 469 (1972).
20. Severin, E. S., Kovaleva, G. K., Sastchenko, L. P., Tumanyan, V. G., Abstracts of the IX FEBS Meeting, Dublin, 1973.
21. Polyanovsky, O. L., Demidkina, T. V., Egorov, C. A., *FEBS Letters, 23*, 262 (1972).
22. Birchemier, W., Christen, P., *FEBS Letters, 18*, 209 (1971).
23. Torchinsky, Yu., M., Zyfarova, R. A., Agalarova, M. B., Severin, E. S., *FEBS Letters, 28*, 302 (1972).
24. French, T. C., David, I. B., Buchanan, J. M., *J. Biol. Chem., 238*, 2186 (1963).
25. Wilson, I. B. *Federat. Proc., 18*, 752 (1959).
26. Severin, E. S., Gnuchev, N. V., Khomutov, R. M., *Izvestia Acad. Sci. USSR*, (Chemistry), *1967*, 1386.
27. Khomutov, R. M., Severin, E. S., Kovaleva, G. K., Gnuchev, N. V., *Izvestija Acad. Sci. USSR*, (Chemistry), *1967*, 1823.
28. Severin, E. S., Gnuchev, N. V., Gulyaev, N. N., Khomutov, R. M., in "Chemistry and Biology of the Pyridoxal Catalysis", Nauka, Moscow, 1968, p. 392.
29. Khomutov, R. M., Severin, E. S., in "Modern Problems of the Chemistry of Peptides and Proteins" Nauka, Moscow, 1969, p. 134.
30. Severin, E. S., Gulyaev, N. N., Khurs, E. N., Abstracts of the V Internat. Symp. on the Chemistry of Natural Compounds, D. 9, p. 208.

31. Severin, E. S., Doctoral Thesis, Moscow, 1972.
32. Gulyaev, N. N., Agalarova, M. B., Gnuchev, N. V., Chukanov, V. V., Khomutov, R. M., Severin, E. S., *Biokhimiya*, In the press.
33. Karpeisky, M. Ya., Ivanov, V. I., *Nature*, *210*, 494 (1966).
34. Ivanov, V. I., Karpeisky, M. Ya., *Adv. in Enzymology*, *32*, 21 (1969).
35. Arigoni, D., Report on Stereochemical Aspects of Pyridoxal Phosphate Catalyzed Reactions, at the Robert A. Welch Foundation Conferences on Chemical Research, 1972.
36. Karpeisky, M., Severin, E., Ivanov, V., Mamaeva, O., Tumanyan, V., Abstracts, FEBS, 402, Amsterdam, 1972.

K. WEINGES
AND
B. STEMMLE

ASYMMETRIC SYNTHESES OF α-AMINO ACIDS

I. INTRODUCTION

The separation of peptide hydrolysates and synthetic racemates, or the enzymic synthesis of optically active amino acids present no problem today; yet the practical and theoretical interest attached to the asymmetric syntheses of amino acids has been maintained. Substantial quantities of optically active amino acids are used as drugs and as building units for peptides in medical science and biochemistry, furthermore, many problems of the asymmetric synthesis of amino acids are still unsolved. Thus, in most asymmetric syntheses of amino acids the stereochemical pathway leading to the formation of the new chiral centre is only conjectural. For this reason, a division of the different amino acid syntheses into enantioselective or diastereoselective syntheses, following a suggestion of Izumi [55], is not possible. These concepts can, in our opinion, only be used if the reaction leading to the formation of the new asymmetric carbon atom is clearly known. Similarly, there are some other stereochemical concepts which, during the recent years, have remained ill-defined as far as asymmetric synthesis is concerned; therefore, they will not be used in the present paper. Such a concept is "stereospecificity", understood by different stereochemists in different ways. For an asymmetric reaction we shall use the unequivocally applicable concept of "stereoselectivity", giving the optical purity of the enantiomers produced, and the chemical yield.

Some other stereochemical concepts are clearly defined but are often ambiguously reproduced in several textbooks. For example, chirality should not be used synonymously with dissymmetry as it appears in the books of Eliel [26], Mislow [66] and Bähr and Theobald [15]. Chirality, dissymmetry and asymmetry are different concepts. Asymmetric objects possess no elements of symmetry at all; dissymmetric objects possess a low grade of symmetry, that is, they lack either elements of symmetry of first order (rotational axes of optional number) or of second order (centre of symmetry, plane of sym-

metry, rotational mirror axes). The latter are chiral objects. Objects of the point groups C_n and D_n ($n > 1$) are e.g. chiral dissymmetric but not asymmetric, because they contain symmetry elements of first order. Objects of point groups C_s and C_i are achiral dissymmetric; they lack symmetry elements of first order, but they possess those of second order. Table I shows the difference in these concepts for the various point groups:

Table I

Chiral		Achiral	
asymmetric	dissymmetric	dissymmetric	symmetric
C_1	C_n, D_n, T, O ($n > 1$)	C_s C_i	C_{nv}, C_{nh}, D_{nv}, D_{nd} D_{nh}, T_d, O_h ($n > 1$)

Though the classification of most amino acids into the D- or L- series according to Fischer is unequivocal, the use of the R, S notation of Cahn, Ingold and Prelog [18] will be preferred in this paper, since the D- and L- designations are not always unambiguous for chiral asymmetric and chiral dissymmetric auxiliary compounds applied to asymmetric syntheses.

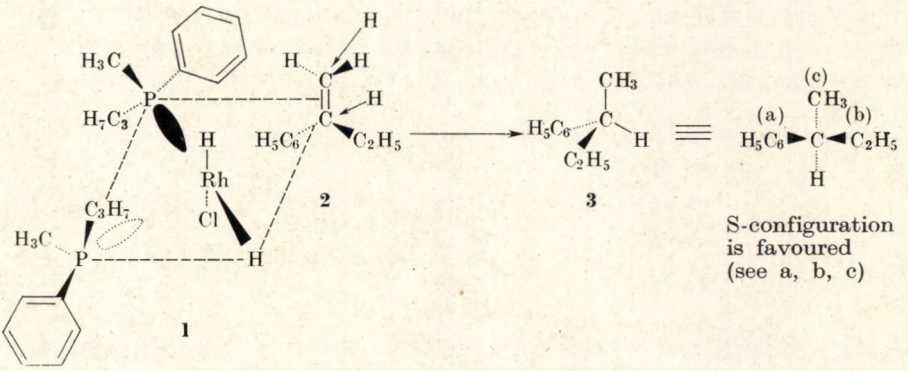
In the following we shall deal with recent researches (from 1969 till April 1974) on the asymmetric synthesis of amino acids. Earlier works will only be discussed when required for better understanding, or if they especially deserve mentioning. Otherwise this summary should be considered a supplement to the reviews by Morrison and Mosher [68] and Babievskii and Latov [14] covering theur literate up to 1969.

II. ASYMMETRIC HYDROGENATIONS

1. ASYMMETRIC HYDROGENATION OF THE C=C DOUBLE BOND IN ACHIRAL STARTING COMPOUNDS

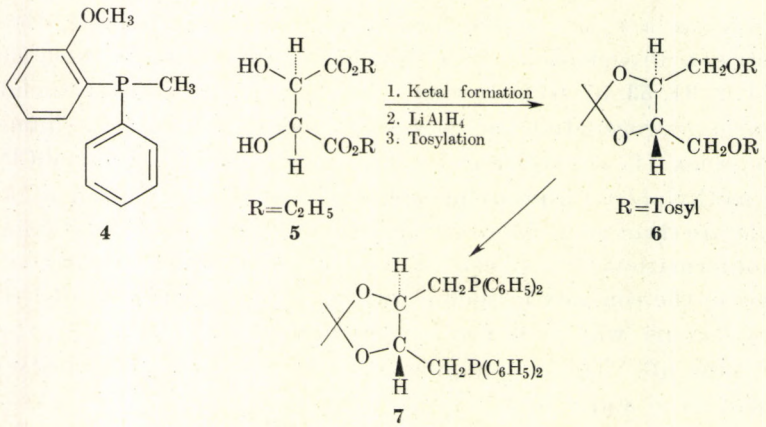
Asymmetric hydrogenation of C=C double bonds in achiral starting compounds, such as 4-benzylidene-2-methyl-5-oxazolone [5–7, 9, 54] or α-acetamidocinnamic acid [27, 28] for the synthesis of optically active phenylalanine, has been known for long. The heterogeneous catalysts mostly used are Pd on silk fibroin [9], Pd–charcoal–[S]-methionine [79], Pd–poly-[S]-leucine [16], or Raney nickel in the presence of alkaline glucose solution [72]. The stereoselectivity of these heterogeneous asymmetric hydrogenations may amount up to 70%, but it is usually less.

Recently, as a result of the development of suitable Rh(I) catalysts, asymmetric hydrogenations have become possible also in homogeneous phase [1, 2, 31, 53, 57, 67]. Horner, Siegel and Büthe [53] found that asymmetric hydrogenations are possible in benzene with a rhodium–phosphine complex (**1**) synthesized *in situ* from $[Rh(1,5\text{-hexadiene})Cl]_2$ and [S]-(+)-methylphenyl-n-propylphosphine. On the assumption of a complex structure (**1**) of an a-chloro-e,f-dihydrido-d-olefin-b,c-bisphosphine-rhodium, conformational consideration lead to the conclusion that the olefin **2** is fixed in the complex in such a way that the *cis* addition of hydrogen to α-ethylstyrene will give rise to predominant formation of [S]-(+)-2-phenylbutane (**3**). The S-configuration follows from the priority of the substituents a, b and c in **3**.

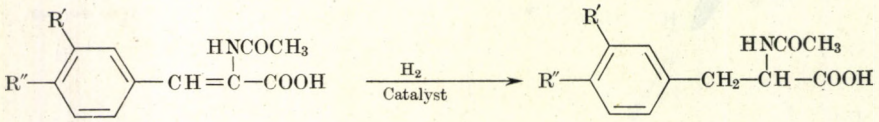


Configuration and conformation (CH_3 and C_3H_7 staggered) of the Rh-complex

The chiral rhodium complexes required for the synthesis of optically active amino acids are prepared [58] by the conversion of Rh^I-diene complexes, such as $[Rh(1,5\text{-hexadiene})Cl]_2$, with optically active *o*-anisylmethylphenylphosphine (**4**) [59, 73]. Complexes such as $[Rh^I(P{-}P)Cl]$ in which P—P represents a chiral diphosphine **7** are also used. They are produced *in situ* by adding 2 moles of **7** to a solution of $[Rh(cyclooctene)_2Cl]_2$ in benzene–ethanol. The chiral diphosphine **7** is synthesized from diethyl (+)-tartrate (**5**) through the ditosylate **6**. Introduction of the $P(C_6H_5)_2$ groups into **6** is brought about by sodium diphenylphosphide [19].

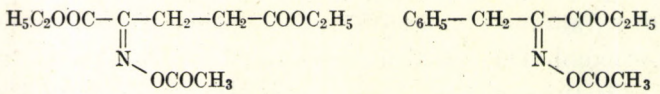


In the presence of these catalysts, N-acetylphenylalanine (**9**: R′, R″=H) and N-acetyl-3-methoxy-4-acetoxyphenylalanine (**9**: R′=OCH_3, R″=OAc) have been synthesized from substituted α-acetamidocinnamic acids (**8**) in 95% chemical yield and with stereoselectivities up to 90% [24].



2. ASYMMETRIC HYDROGENATION OF THE C=N DOUBLE BOND IN ACHIRAL STARTING COMPOUNDS

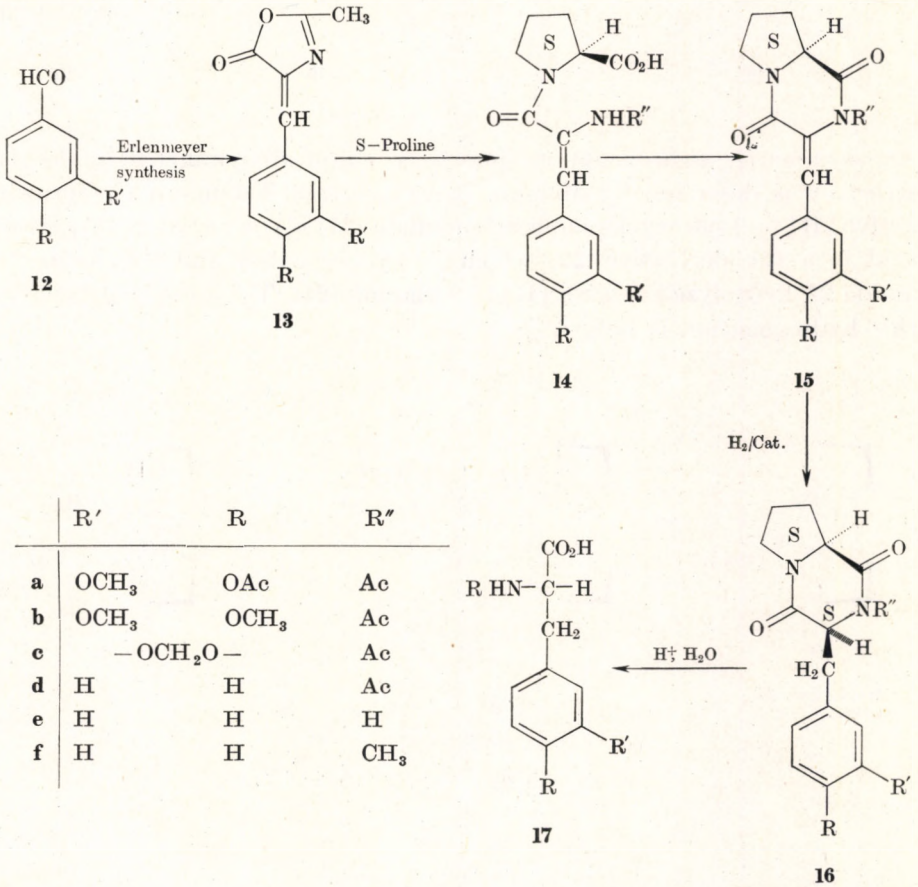
The first attempts of asymmetric hydrogenations of C=N double bonds of achiral starting compounds in the presence of chiral catalysts date back to Nakamura [71]. He succeeded in synthesizing α-phenylethylamine in 15–18% optical purity using a Pt–optically active tartaric acid catalyst. With Pd–silk fibroin catalysts also the oxime acetates **10** and **11** were successfully hydrogenated to yield optically active N-acetylglutamic acid [5, 9, 54] and optically active phenylalanine [7, 9]. The stereoselectivity of the hydrogenation was, however, very poor (8–30%). In recent years no further papers have been published on this line of research.



3. ASYMMETRIC HYDROGENATION OF THE C=C DOUBLE BOND IN CHIRAL STARTING COMPOUNDS

The heterogeneous asymmetric hydrogenation of arylidene derivatives of chiral diketopiperazines has been used for long in the synthesis of optically active phenylalanine [3] or tyrosine [61]. No precise data of the stereoselectivity of these reactions can be given, as the cleavage of the intermediate dipeptides takes place only in 55% chemical yield [4].

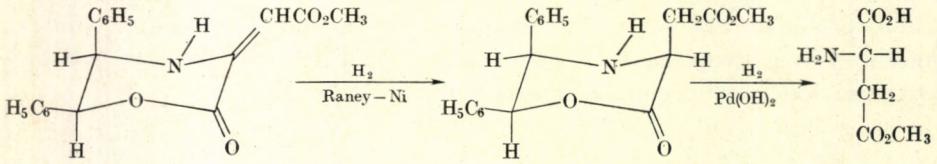
High asymmetric induction was observed by Poisel and Schmidt [77] during the catalytic hydrogenation of arylidene derivatives of glycyl-[S]-proline **15**, reported as early as 1944 by Bergmann and Tietzmann [17]. The saturated diketopiperazine **16** is obtained with at least 90% stereoselectivity by hydrogenation of **15**. The acid hydrolysis of the diketopiperazine then gives the optically active α-amino acids **17a–f** in 90% optical purity. The hydrogenation of alkylidene derivatives of glycyl-[S]-proline lactame does not occur asymmetrically.



	R′	R	R″
a	OCH_3	OAc	Ac
b	OCH_3	OCH_3	Ac
c	$-OCH_2O-$		Ac
d	H	H	Ac
e	H	H	H
f	H	H	CH_3

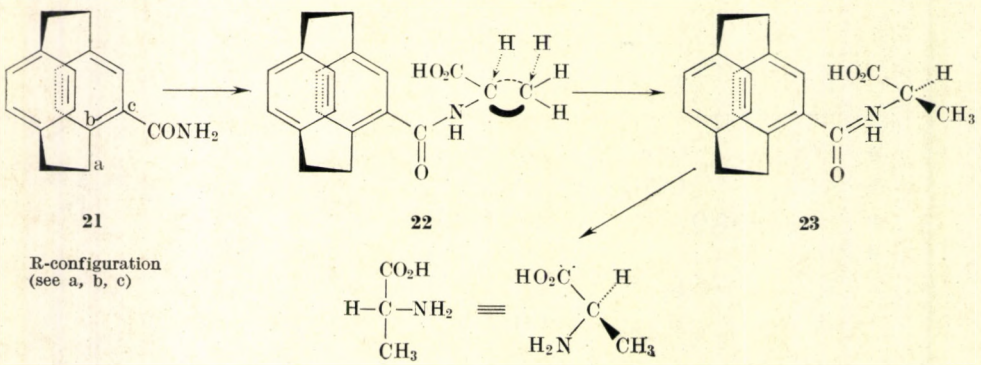
The glycyl-[S]-proline derivative **15** is prepared by Erlenmeyer synthesis with an aromatic aldehyde **12** and subsequent reaction of the product **13** with [S]-proline. Ring closure of the dipeptide **14** to **15** can be effected with cold acetic anhydride [17] or with dicyclohexylcarbodiimide and ketene [77].

Another interesting asymmetric hydrogenation starts with the oxazine derivative **18** prepared from (+)-1,2-diphenylethanolamine and dimethyl acetylenedicarboxylate. The hydrogenation of **18** in the presence of Raney nickel and then Pd catalyst leads to [S]-(+)-aspartic acid monomethyl ester (**20**) of 98% optical purity [89]. The chemical yields are almost quantitative.



18 **19** **20**

An asymmetric hydrogenation due to a chiral dissymmetric molecular structure is described by Matsuo, Kobayashi and Tatsuno [64]. Optically active [R]-(−)-paracyclophanecarbonamide (**21**) is converted with pyruvic acid into the derivative **22**, which is hydrogenated and the saturated amide **23** hydrolyzed to give [R]-(−)-alanine (**24**). The stereoselectivity of this hydrogenation is only 6%.



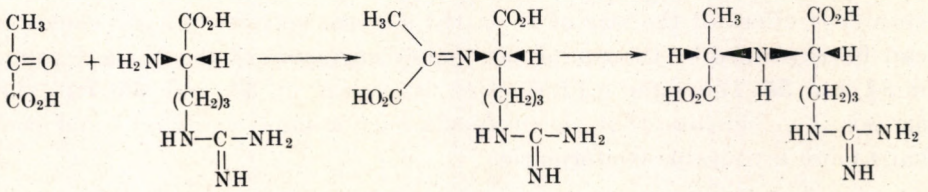
21 **22** **23**

R-configuration
(see a, b, c)

24

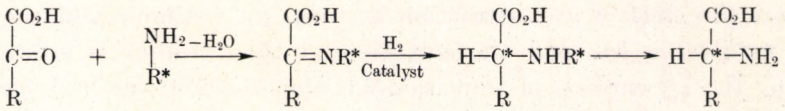
4. ASYMMETRIC HYDROGENATION OF THE C=N DOUBLE BOND IN CHIRAL STARTING COMPOUNDS

As early as 1939 Knoop and Martius [56] reported the synthesis of (+)-octopine. Natural (+)-octopine has R-configuration in the alanine part and S-configuration in the arginine part. The authors allowed to react [S]-(+)-arginine (**26**) with pyruvic acid (**25**) and obtained, on hydrogenation of the condensation product **27**, a substance which was assumed to be identical with natural (+)-octopine. Later it turned out, however, that it was actually [S,S]-(+)-octopine, (**28**; (+)-isooctopine) [10, 48], denoted today as [S]-(+)-allooctopine [32]. The hydrogenation of the condensation product **27** to [S]-(+)-allooctopine (**28**) is reported to occur with a stereoselectivity of about 70%.



25 **26** **27** **28**

Recently, asymmetric hydrogenations of chiral ketimines have been investigated mainly by the research groups of Harada and Hiskey. The results of the two groups are slightly different, as the optical purity of the endproducts was determined by different techniques of isolation and work-up. The chiral ketimines **31** are synthesized from α-ketoacids **29** with chiral amines **30**, and they are usually hydrogenated, without preceding purification, in the presence of Pd or Raney nickel catalyst, to the corresponding N-substituted amino acids **32**, or directly to the free amino acids **33**.

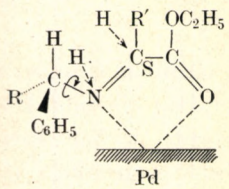


29 **30** **31** **32** **33**

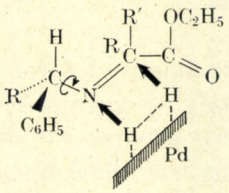
The removal of the auxiliary chiral centre in **32** to obtain **33** must be carried out in such a way that no racemization should take place on the newly formed chiral centre. Therefore, mostly the enantiomers of phenylglycine

[34, 35, 39], α-phenylethylamine [51, 52], α-phenylpropylamine [39, 63] and α-naphthylethylamine [40, 63] are used as chiral amine components, since in these cases the auxiliary chiral centre can be eliminated by hydrogenolysis in neutral medium. A more detailed discussion of the steric course of asymmetric hydrogenations in terms of configurations and conformations is given in the following.

The stereoselectivity of the asymmetric hydrogenation of the ketimines **31** depends on the size of the groups R and R′ in transition states **34** (pro-S) and **35** (pro-R), on the polarity of the solvent, and on the temperature. By variation of the group R using optically active α-phenylethyl-, α-phenylpropyl- and α-naphthylethylamines, it has been shown that the best results are obtained with the α-phenylethyl group (R=CH_3), i.e., the smaller R, the higher the optical purity. The same applies to R′, yet it has less influence. The effect of the size of R on the stereoselectivity of hydrogenation can be explained by the conformational arrangement of the N-alkyl group in **34** and **35**. The same conformation is shown in **34** and **35**, but the arrows (↷) indicate that the molecule may assume a different and perhaps more favourable conformation.



34 pro-S-transition



35 pro-R-transition

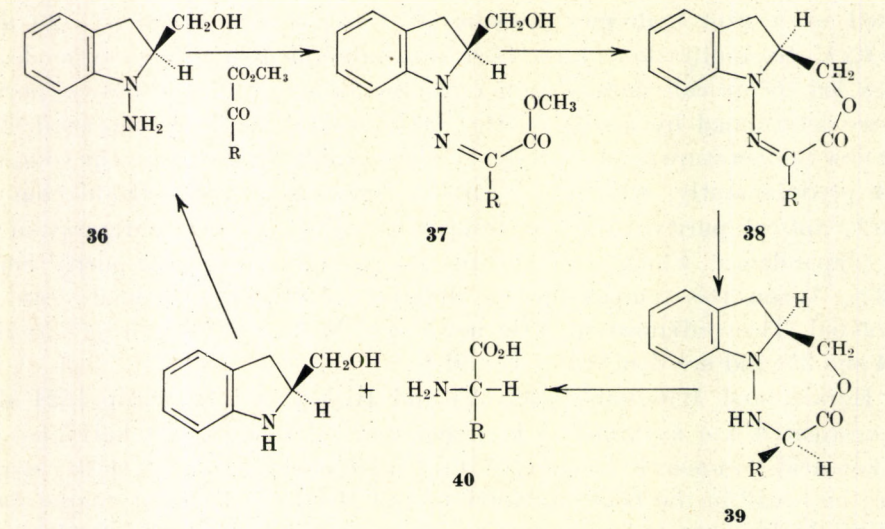
From the dependence of stereoselectivity on the polarity of the solvent it can be concluded that a complex [40, 44, 63] of ketimine and the palladium catalyst (see formula **34**) is formed only in non-polar solvents. In polar solvents the electrostatic attraction between the ketimine and the palladium catalyst is less and the solvation of the ketimine is stronger, favouring the appearance of transition **35**. The attack of the hydrogen may take place from both diastereotopic sides, thus stereoselectivity decreases. These considerations are confirmed by the experimental results according to which the enantiomeric purity of [S]-amino acids decreases when less polar solvents are used instead of more polar ones.

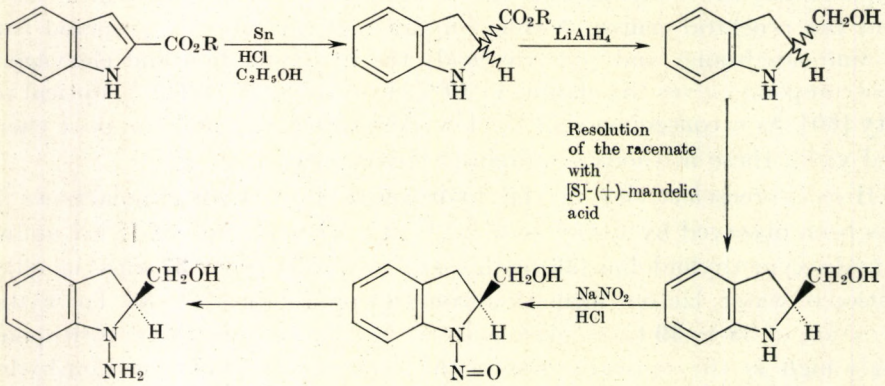
Lately Harada and Yoshida [37, 43, 45] investigated the temperature dependence of the asymmetric hydrogenation. The ketimines were hydrogen-

ated at 1 atm hydrogen pressure at temperatures between −20 and +65°C. Saponification of the ester and subsequent hydrogenolytic cleavage led to optically active alanine, whose configuration and optical purity were determined by means of the DNP derivative. It is suggested that at low temperature (−20°C) [S]-(−)-α-phenylethylamine gives the complex **34** predominantly, which, on further processing, yields [S]-alanine in 60% optical purity; at higher temperature (+17°C) the configuration **35** is preponderant, which gives rise to [R]-alanine, the optical purity being 43%. When the temperature is raised to 50°C, only racemate can be isolated. The configuration of the new chiral centre can be seen in formulas **34** and **35**, and are denoted as R and S.

Harada and Matsumuto [39, 62] and Hiskey and Northrop [52] also investigated the asymmetric hydrogenation of oximes and ketimines of α-ketoacid-(−)-menthyl esters. A tabulated presentation of these results can be found in the book of Morrison and Mosher [68]. In the same way, chiral hydrazones [8] prepared by the condensation of [R]-2-methyl-3-phenylpropionic acid hydrazide with an α-keto acid, could be hydrogenated asymmetrically; the products were [S]-alanine (8% optical purity) and [S]-phenylalanine (5% optical purity). The low stereoselectivity has been explained by the relatively large distance between the auxiliary chiral centre and the prochiral centre. A chiral hydrazone can also be prepared from N-aminoanabasine and pyruvic acid; the hydrogenation and cleavage of this compound gives [S]-alanine in 10% chemical yield and 40% optical purity [60]. As a consequence of the low stereoselectivity and the poor chemical yield, these methods acquired no importance.

High stereoselectivity in the hydrogenation of hydrazonolactones **38** has been observed by Corey *et al.* [21, 22]. The reaction of [S]-N-amino-2-hydroxymethylindoline (**36**) with α-ketoacid esters gives **37** with the elimination of water; lactone formation from this product leads to **38**. The hydrogenation of **38** to **39** takes place with 80–90% stereoselectivity; correspondingly high is the optical purity of the amino acids **40** obtained on hydrogenolytic splitting of the N—N linkage. [S]-2-Hydroxymethylindoline can be reconverted into **36** through nitrosation and reduction. A disadvantage of this asymmetric synthesis is the difficult preparation (*cf.* Scheme) of the starting compound **36**.



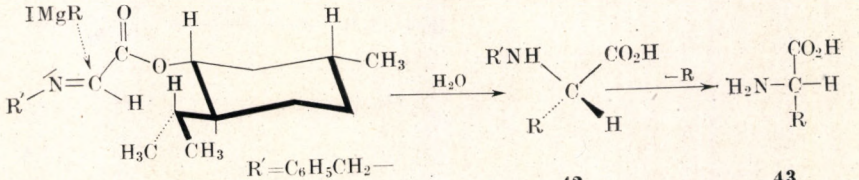


Scheme of the synthesis of [S]-N-amino-2-hydroxymethylindoline (**36**)

III. ASYMMETRIC ADDITIONS

1. ASYMMETRIC ADDITIONS OF GRIGNARD COMPOUNDS TO IMINES OF GLYOXYLIC ACID (−)-MENTHYL ESTERS

Fiaud and Kagan [29, 30] obtained optically active amino acids **43** by the addition of Grignard compounds (RMgI) to imines of glyoxylic acid (−)-menthyl ester (**41**) and by hydrogenolysis of the addition products. The chemical yield amounts only to 45%.

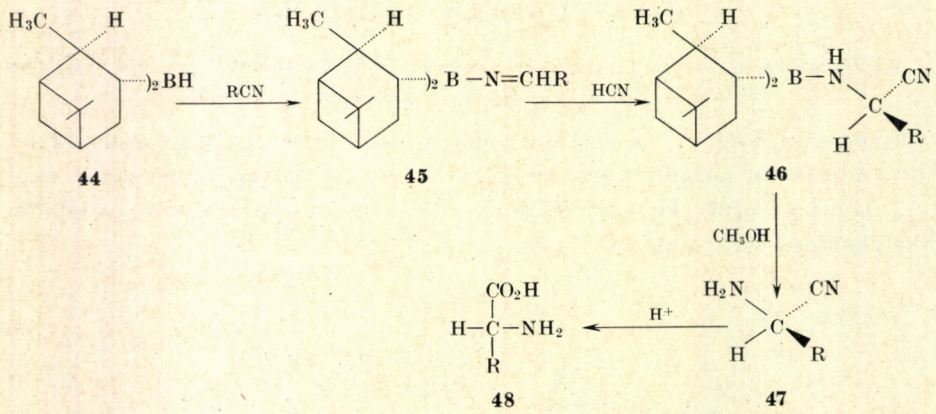


41 **42** **43**

The stereochemistry of this reaction is unequivocally determined by the conformation of the menthyl ester. The Grignard compound can associate itself to compound **41** only from the "rear side", since addition on the "front side" is prevented by the bulky isopropyl group. After hydrolysis of the ester and hydrogenolysis of the N-benzyl group, amino acids with S-configuration (see Formulas **42** and **43**) are obtained. By variation of the Grignard compound [S]-phenylalanine (**43**: R=$C_6H_5CH_2$), [S]-alanine (**43**: R=CH_3), [S]-norvaline (**43**: R=n-C_3H_7) and [S]-valine (**43**: R=i-C_3H_7) have been synthesized. The optical purity of the S-amino acids is between 38 and 63%.

2. ASYMMETRIC ADDITION OF HYDROGEN CYANIDE TO CHIRAL KETIMINE BORANES

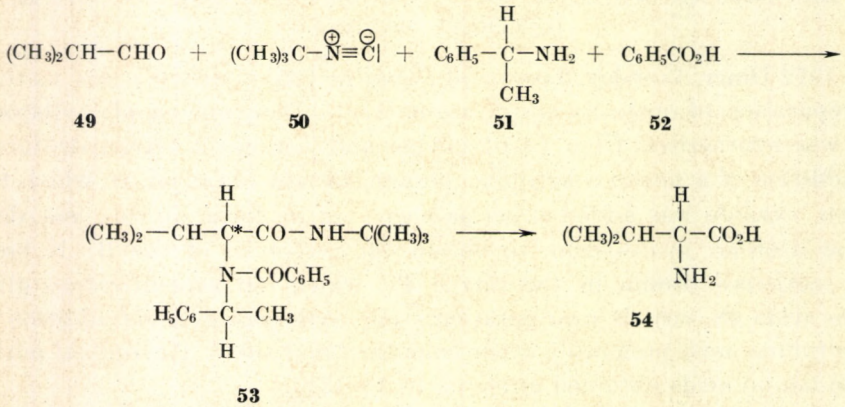
In 1972 Diner, Worsley, Lown and Forsythe [25] reported a new method of preparation of optically active amino acids. Diisopinocamphenylborane (**44**) was synthesized from (+)-2-pinene and borane in tetrahydrofuran. By addition of a nitrile a ketimine borane **45** was obtained, to which hydrogen cyanide was added either as a gas, or in form of the less dangerous acetone cyanhydrin, to obtain **46**. Solvolysis of the B—N bond with methanol results in the nitrile **47**, which, on saponification gives amino acids **48** with R configuration; thus hydrogen cyanide addition to the ketimine must preferably occur from the "rear side". The optical purity of the amino acids obtained amounts to 10–15%.



IV. ASYMMETRIC NAME REACTIONS

1. ASYMMETRIC UGI SYNTHESIS

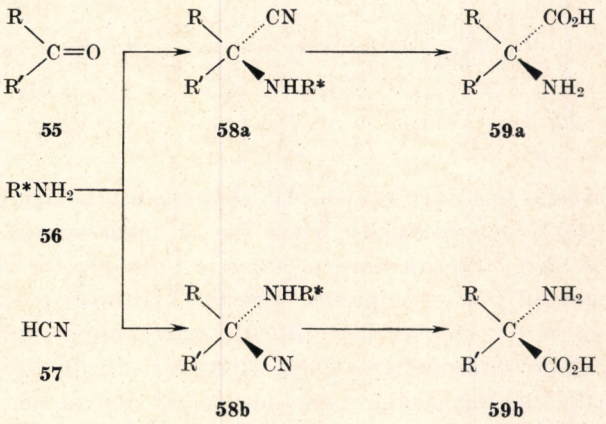
One of the theoretically most interesting amino acid syntheses is the four-component synthesis according to Ugi. Passerini [75] observed that in the presence of carboxylic acids, isonitriles and carbonyl compounds very readily give the amides of acyloxy acids. Ugi *et al.* [49, 50, 81, 82, 85, 88] carried out this reaction in the presence of a fourth component, a chiral amine. If e.g. isobutyraldehyde (**49**: 1st component), *t*-butylisocyanide (**50**: 2nd component), optically active α-phenylethylamine (**51**: 3rd component) and benzoic acid (**52**: 4th component) are allowed to react with one another, the optically active adduct **53** is obtained, whose hydrolysis and hydrogenolysis yields optically active valine (**54**).



The configuration of the newly formed chiral centre (*) depends on the chosen conditions of the reaction (concentration, solvent, and temperature) and on the configuration of the chiral amine **51**. Using a 1 M methanolic solution of all the four components **49–52** at −70°C and [R]-(+)-α-phenylethylamine (**51**), the end-product (**54**) has S configuration; using a 0.1 M solution at 0°C, it has R configuration. The optical purity of the resulting amino acids amounts to 70–75%. The stereochemical course of the reaction was investigated by Ugi *et al.* [83, 84, 86, 87].

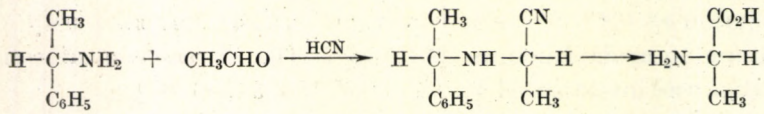
2. ASYMMETRIC STRECKER SYNTHESIS

The term asymmetric Strecker synthesis is used if during the conversion of a carbonyl compound **55** with an optically active amine **56** and hydrogen cyanide (**57**) the formation of one of the two possible diastereomeric aminonitriles **58a** or **58b** is preponderant. In the preparation of amino acids **59a** and **59b**, the aminonitriles **58a** and **58b** are saponified with concentrated acids, followed by cleavage of the N-alkyl group containing the axuliary chiral centre.



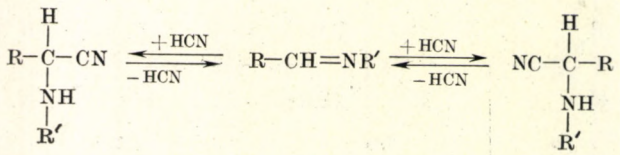
The chiral amino components **56** mainly used are again (*cf.* p. 98) the enantiomers of α-phenylethylamine, α-phenylpropylamine, α-naphthylethylamine, as well as optically active α-phenylglycine. Concerning the mechanism of the Strecker synthesis it cannot be stated with certainty today whether the carbonyl compound and hydrogen cyanide give first the enantiomeric cyanohydrins [36] which then react at different rates with the chiral amine, or the amine and the carbonyl compound afford ketimine to which hydrogen cyanide is added asymmetrically [76].

Harada [33] was the first to convert [S]-(−)-α-phenylethylamine (**60**) with acetaldehyde (**61**) in aqueous methanol to obtain the N-phenylethyl-α-aminonitrile **62**, which gave [S]-(+)-alanine (**63**) on hydrolysis and hydrogenolysis. The optical purity of [S]-(+)-alanine amounted to 68 %.



60 **61** **62** **63**

Very high optical purity (up to 100 %) of the amino acids was observed by Patel and Worsley [76] when the ketimine produced from the aldehyde and amine was first isolated and the addition of HCN to the aminonitrile was carried out afterwards. High stereoselectivity in the Strecker synthesis is due probably to a second order asymmetric transformation of the aminonitrile produced in the first reaction step, according to the following equilibrium:



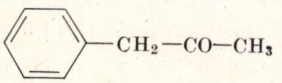
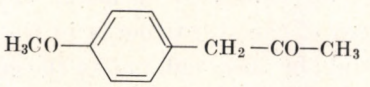
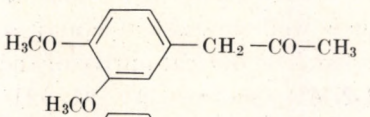
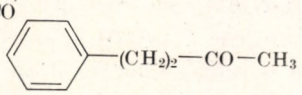
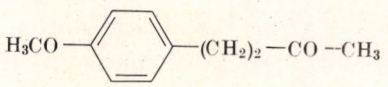
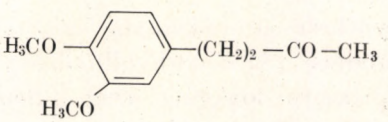
Recent investigations have shown [41] that the amino acids are obtained only in 20 to 53 % optical purity when the Strecker synthesis is effected in solution, i.e. if no intermediate product is isolated in crystalline form. It can be concluded that asymmetric Strecker synthesis gives amino acids of high optical purity only if the optically pure aminonitriles can be isolated as crystalline intermediates [90]. However, during the synthesis of the aminonitriles the formation of the new chiral centre also occurs with a certain stereoselectivity, before a second order asymmetric transformation gives rise to the crystallization of only one of the diastereomers. Therefore, also conformational effects, either during the addition of the hydrogen cyanide to the ketimine, or during the substitution of the hydroxyl group of the cyanohydrin by the amine, must be taken into account when explaining the course of the stereochemical reaction. Such conformational considerations were attempted by Harada [46] for the substitution by amine of the hydroxyl group of cyanohydrins, and by Weinges *et al.* [91] for the HCN addition to ketimine. As it is yet unknown whether a cyanohydrin or a ketimine is

the real intermediate in the Strecker synthesis, these considerations about the conformations will not be discussed here further.

Recent ^{1}H-NMR-spectroscopical investigations of the α-aminonitriles obtained in the first reaction step of the synthesis from methyl ketones, [S]-(—)-α-phenylethylamine and hydrogen cyanide, have shown [90] that the configuration of the newly formed chiral centre is influenced by the substitution in the aromatic part of the methyl ketones (*cf.* Table II). Furthermore, it can be proved by the ^{1}H-NMR-spectra of the crystalline aminonitriles that they are of 100% optical purity.

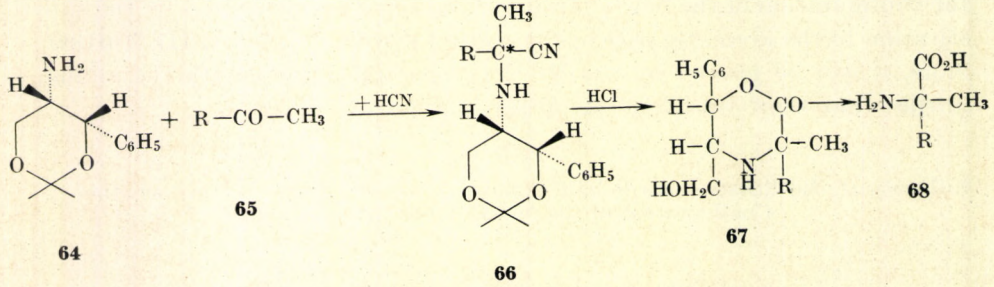
Table II

Influence of the Substituents in the Aromatic Part of Methyl Ketones on the Configuration of the Newly Formed Chiral Centre

Aminonitriles from [S]-(—)-α-phenylethylamine, hydrogen cyanide and:	Configuration of the newly formed chiral centre in the aminonitrile
	R
	S
	S
	R
	S
	S

Besides the optically active amines mentioned above, [4S,5S]-(+)-5-amino-2,2-dimethyl-4-phenyl-1,3-dioxan (**64**), a by-product of the chloramphenicol synthesis and available in large quantities, was introduced by Weinges, Graab, Nagel and Stemmle [91, 92] as the amine component in the Strecker synthesis. Compound **64**, aliphatic or aromatic methyl ketones

(**65**) and hydrogen cyanide react to give the corresponding aminonitriles **66**, whose saponification leads to the lactones **67**. The N-alkyl groups containing the auxiliary chiral centre is removed by treatment with Raney nickel and sodium hydroxide to obtain the optically pure amino acids **68** in 80–90% yield.

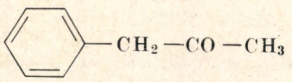
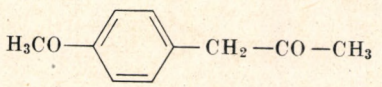
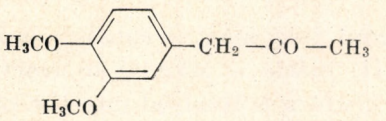
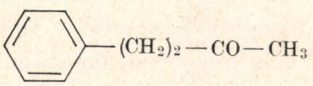


It has been found [92] that when alkyl methyl ketones **65** are used, different configurations arise on the newly formed asymmetric carbon, depending on the chain length of the alkyl residue. Alkyl methyl ketones with even-numbered carbon chains give amino acids of S configuration, whereas ketones with odd-numbered carbon chains yield acids of R configuration (see Table III). No influence of the substituents in the aromatic ring of phenylalkyl methyl ketones could be observed, unlike the cases mentioned above (Table II). It is to be stressed that optically pure amino acids are only obtained if the aminonitrile is well crystallizable and can be recrystallized until perfect purity is attained. The optical purity of the aminonitriles, which can be determined by ^{1}H-NMR-spectroscopy [91, 92], is very important, since the new chiral centre (see * in **66**) arises during the formation of the aminonitriles, and there is no further reaction on this carbon atom in the course of the later steps of the synthesis (saponification and cleavage of the auxiliary chiral centre). Therefore the optical purity of the aminonitrile is of decisive importance for the optical purity of the amino acid produced.

This asymmetric Strecker synthesis is used in the industry by the firm Boehringer, Mannheim, to produce [S]-α-methyldihydroxyphenylalanine ([S]-α-methyldopa), a blood-pressure lowering drug ("Sembrina"). The chemical yield of [S]-α-methyldopa amounts to about 90% and the end-product has 100% optical purity. To our knowledge, this is the only asymmetric synthesis carried out today on large industrial scale. As the cleavage of the auxiliary chiral centres is effected in alkaline solution at 120°C, so far only α-methyl-α-amino acids can be made according to this synthesis, i.e. only methyl ketones can be used. When aldehydes are employed as the

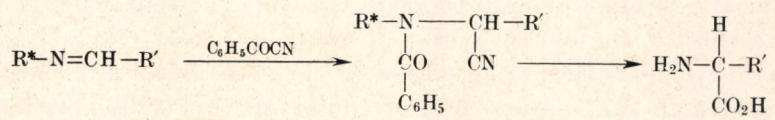
Table III

Influence of the Carbon Chain of Alkyl Methyl Ketones on the Configuration of the Newly Formed Chiral Centre of Aminonitriles

Aminonitriles from **63** hydrogen cyanide and:	Configuration of the newly formed chiral centre in the aminonitrile
$CH_3-CH_2-CO-CH_3$	S
$CH_3-(CH_2)_2-CO-CH_3$	R
$CH_3-(CH_2)_3-CO-CH_3$	S
$CH_3-(CH_2)_4-CO-CH_3$	R
	S
	S
	S
	R

starting compounds, racemization occurs. There are investigations in progress to develop a milder method of cleavage of the N-alkyl group [93].

A method of preparing amino acids analogous to the Strecker synthesis is the addition of benzoyl cyanide to chiral ketimines **69** obtained from aldehydes and chiral amines [42]. Saponification and hydrogenolysis lead to the corresponding amino acids **71**. The stereoselectivity of the reaction is lower (the optical purity of the end-product being 15–37 %) than in the case of the addition of hydrogen cyanide to the ketimine. From ketimines with S-configuration S-amino acids are obtained.

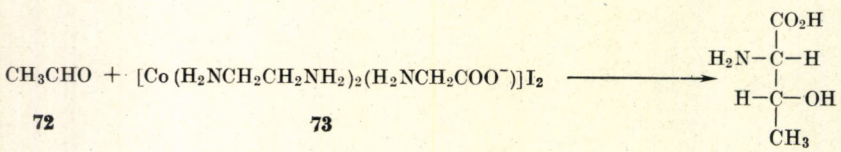


69 **70** **71**

V. ASYMMETRIC SYNTHESES OF AMINO ACIDS BY MEANS OF CHIRAL METAL COMPLEXES

1. ASYMMETRIC ALDOL ADDITION

The aldol addition product of acetaldehyde (**72**) to (−)-glycinato-bis-(ethylenediamine)–cobalt(III) iodide (**73**) gives, after hydrogenolysis and precipitation of the cobalt as sulfide, a mixture of glycine, allothreonine and threonine. In the course of this reaction two new chiral centres are formed. The isolated threonine (**74**) has an optical purity of about 8 %. The chiral dissymmetric cobalt complex (**73**) obviously gives rise to asymmetric aldol addition onto the pro-chiral centre of glycine. The stereoselectivity becomes lower if propylenediamine is used as the chelating ligand [65, 69, 70].

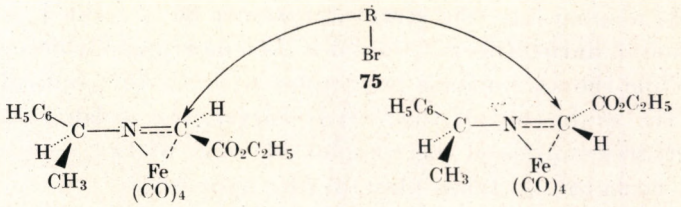


72 **73** **74**

For the preparation of the chiral dissymmetric cobalt complex **73**, diiodo-bis(ethylenediamine)–cobalt(III) iodide is allowed to react with glycine. The racemate of **73** is obtained, which is resolved into the enantiomers by means of bromocamphorsulfonic acid. The bromocamphorsulfonate of the levorotatory complex is considerably less soluble than the dextrorotatory compound and is obtained after a few crystallization processes in the optically pure state. Cleavage of the bromocamphorsulfonate gives the optically pure complex **73** [65].

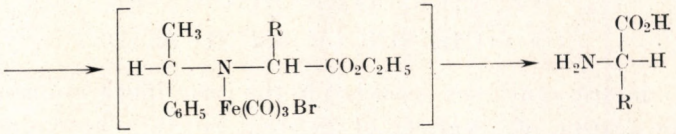
2. ASYMMETRIC ALKYL BROMIDE ADDITION

The reaction of alkyl bromides (**75**) with diastereomeric iron(0)- or nickel(0)-carbonyl complexes of [S]-α-phenylethyliminoglyoxylates (**76a** and **76b**) gives optically active amino acids **78** through the non-isolated intermediate compounds **77** [20]. The end-products contain an excess of the S-enantiomer. Since amino acids of S configuration can only arise by addition of the alkyl bromide to the diastereomers **76a**, the formation of the latter must be preferred during the reaction of $Fe_2(CO)_9$ with the chiral α-phenylethylimino-glyoxylic acid ester. The optical purity of the end-product (**78**) is highly temperature-dependent; thus amino acids **78** are obtained at 35 °C in 53 % chemical yield and 77 % optical purity, but at 80 °C only the racemate can be isolated.



75

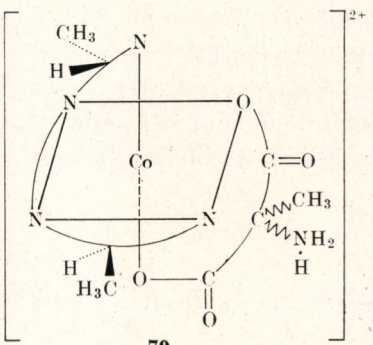
76a **76b**



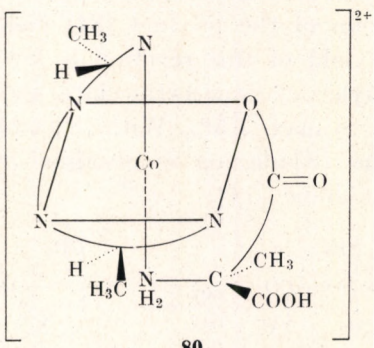
77 **78**

3. ASYMMETRIC DECARBOXYLATION

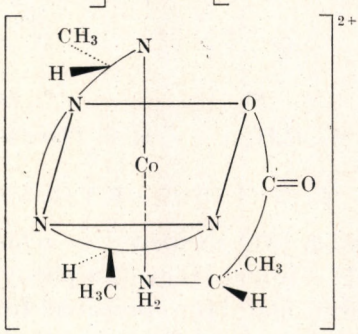
Asperger and Liu [11, 13] reported the synthesis of a chiral cobalt complex **79** from α-amino-α-alkylmalonic acid and (+)-*cis*-α-[Co(S,S-α,α′-dimethyltriethylenetetramine)Cl_2] [12]. Decarboxylation of **79** gives the complex **81**, which is hydrolyzed to amino acids of S-configuration. Morrison and



79



80



81

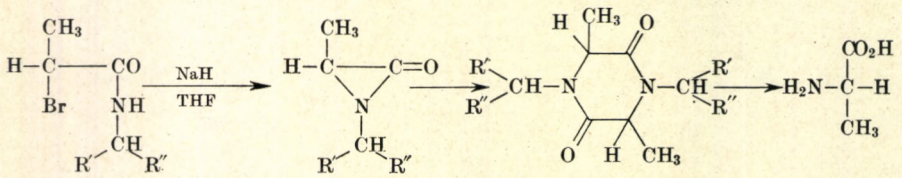
Mosher [68] assume that the structural isomer **80** is formed as an intermediate product during this reaction; it is then more quickly decarboxylated than **79**. While the amino acids in complex **81** have 75% optical purity as shown by the evaluation of their ORD curves, the isolated amino acids are, after hydrogenolysis of the complex **81**, only of 14% optical purity; i.e. partial racemization takes place during hydrolysis.

VI. ASYMMETRIC SYNTHESES OF SOME SINGLE AMINO ACIDS

The asymmetric syntheses discussed in the preceding sections are suitable for the preparation of a variety of different optically active amino acids; the methods described below have been used so far only for the synthesis of alanine, valine, and aspartic acid.

1. SYNTHESIS OF ALANINE

When chiral benzylalkylamines are acylated with α-bromopropionyl bromide, chiral α-halogenoacylamides of constitution **82** are obtained. These can be converted with sodium hydride in tetrahydrofuran, through the intermediate α-lactams **83**, into the N,N′-dibenzyldiketopiperazines **84**. Saponification of the product (**84**) yields optically active alanine **85**. The chemical yield of this reaction is low (11–36%). Using [S]-(−)- or [R]-(+)-α-phenylethylamine as the chiral amine component, the optical purity amounts only to max. 1.5%. With [S]-α-phenylethylamine and [S]-α-phenylpropylamine [S]-alanine is obtained, whereas [S]-α-naphthylethylamine yields [R]-alanine [74].



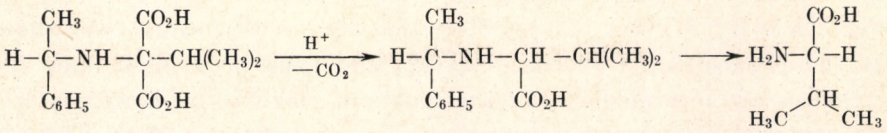
82 **83** **84** **85**

R′ = CH_3, C_2H_5 R″ = Phenyl, naphthyl

2. SYNTHESIS OF VALINE

Hayakawa and Shimizu [47] succeeded in achieving the stereoselective decarboxylation of [S]-(−)- and [R]-(+)-α-phenylethylamino-α-isopropylmalonic acid (**86**), which had been prepared from α-bromo-α-isopropylmalonic acid with the enantiomeric α-phenylethylamines.

The decarboxylated product **87** was converted by hydrogenolysis to valine **88**. The optical purity of the isolated valine depends on the temperature

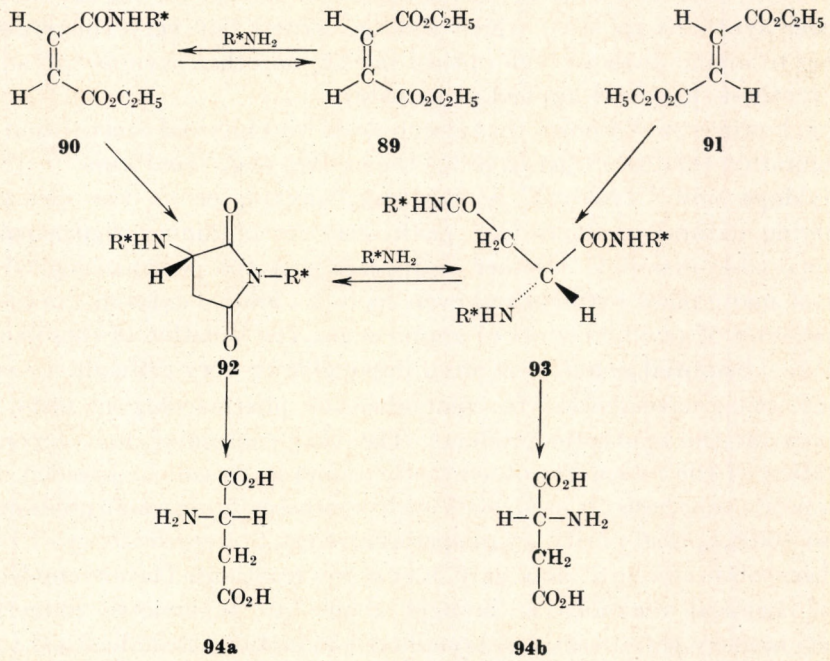


86 **87** **88**

of decarboxylation. At room temperature [S]-(−)-**86** gives [R]-valine of 20–26 % optical purity, and [R]-(+)-**86** yields [S]-valine. Higher temperatures reduce the optical purity of the end-products. It is supposed that at low temperatures there is stronger mutual sterical hindrance between the N-α-phenylethyl group and the two carboxyl groups, giving rise to a more stereoselective decarboxylation of **86**.

3. SYNTHESIS OF ASPARTIC ACID

The synthesis of optically active aspartic acid (**94**) is achieved by the conversion of diethyl maleate (**89**) with an excess of [S]-(−)-α-phenylethylamine; the product obtained (**90**) yields aspartic acid on hydrogenolysis



90 **89** **91**

92 **93**

94a **94b**

and hydrolysis. The formation of aspartic acid with S-configuration (**94a**) is favoured by 13–15% over the R-enantiomer (**94b**). If diethyl fumarate (**91**) is used, the S-enantiomer (**94a**) is again preferentially obtained, but stereoselectivity is lower (4.6%). More detailed investigations have shown that the N-substituted succinimide (**92**) and aspartic acid diamide (**93**) derivatives are intermediates of the reaction, yielding, on hydrogenolysis and saponification, the enantiomeric amino acids **94a** and **94b**. The configuration of the S-aspartic acid (**94a**) obtained as the end-product can be explained by conformational considerations which are analogous to the rules established by Cram [23] and Prelog [78].

The nucleophilic addition of chiral amines to C=C double bonds were achieved by Terent'ev, Gracheva and Dedenko [80] as early as 1965, when synthesizing (+)-α-aminobutyric acid (10% optical purity) from crotonic acid and [S]-(−)-α-phenylethylamine.

VII. PERSPECTIVES

The most often used natural amino acids can be readily isolated from peptide hydrolysates or obtained enzymatically, thus it appears that the development of asymmetric amino acid syntheses, owing to their low stereoselectivity, have only theoretical interest. Except for the asymmetric Strecker synthesis (p. 103) which can be carried out very conveniently leading to amino acids of high optical purity, no other asymmetric amino acid synthesis has been applied in industry.

It is, however, well known that amino acids with unusual constitution and configuration find use either as drugs themselves (e.g. "Sembrina" = [S]-α-Methyldopa and "Trolovol" = [R]-Penicillamine), or as the means of modifying natural products (e.g. penicillins) for obtaining pharmacologically valuable materials. Further it appears that the pharmacological actions of many plant extracts and even more so, animal extracts are due to the presence of small amounts of amino acids. The isolation of these amino acids in substantial amounts is often impossible or very difficult. In order to put them, nevertheless, to comprehensive pharmacological tests, one depends on the synthetic products. The time-consuming and expensive resolution of the racemates of synthetic amino acids can be avoided if an asymmetric synthesis is used, and such syntheses giving end-products of almost 100% optical purity are of special interest. Stereoselectivity of 100% has been observed until now only in enzymic reactions. The stereoselectivity of chemical reactions is brought about by "asymmetric induction" of the auxiliary chiral centre. "Asymmetric induction" is understood today

as the formation of a definite configuration of the newly formed asymmetric carbon atom. In this process decisive importance is attached to effects of the conformation and to the resulting differences in the free energy of the diastereomeric transition states. These differences, however, are not so great that a 100% stereoselectivity of the reaction should result. In most cases of asymmetric syntheses a stereoselectivity of 60—70% can be achieved. In reactions giving the end-product with very high (almost 100%) optical purity, asymmetric induction is followed by a second order asymmetric transformation, resulting in quantitative yields of the optically pure products.

It can be concluded that when planning an asymmetric synthesis — in the knowledge of the stereochemical course of the reaction — the reactants and reaction conditions should be chosen such that

1. the energy difference between the diastereomeric transition states should be great,

2. the reaction product may undergo a second order asymmetric transformation after the formation of the new chiral centre,

3. no reaction should take place on the newly formed asymmetric carbon atom in the course of the subsequent steps of the synthesis.

REFERENCES

1. Abley, P., McQuillin, F. J., *Chem. Commun.*, *1969*, 477.
2. Abley, P., McQuillin, F. J., *J. Chem. Soc. C*, *1971*, 844.
3. Akabori, S., Ikenaka, T., Matsumoto, K., *Nippon Kagaku Zasshi*, *73*, 112 (1952); *C. A.*, *47*, 9938g (1953).
4. Akabori, S., Ikenaka, T., Matsumoto, K., *Proc. Jap. Acad.*, *27*, 7 (1951); *C. A.*, *47*, 3795g (1953).
5. Akabori, S., Izumi, Y., Fujii, Y., *Nippon Kagaku Zasshi*, *78*, 886 (1957); *C. A.*, *54*, 9889e (1960).
6. Akabori, S., Izumi, Y., Fujii, Y., Sakurai, S., *Biochem. (Moscow)*, *22*, 154 (1957); *C. A.*, *51*, 11356g (1957).
7. Akabori, S., Izumi, Y., Fujii, Y., Sakurai, S., *Nippon Kagaku Zasshi*, *77*, 1374 (1956); *C. A.*, *53*, 5149 (1959).
8. Akabori, S., Sakurai, S., *Nippon Kagaku Zasshi*, *78*, 1629 (1957); *C. A.*, *53*, 21687b (1959).
9. Akabori, S., Sakurai, S., Izumi, Y., Fujii, Y., *Nature (London)*, *178*, 323 (1956).
10. Akasi, S. *J. Biochem. (Tokyo)*, *35*, 261, 281, 291 (1937).
11. Asperger, R. G., Univ. Microfilm (Ann Arbor, Mich.) Order No. 66-5034, 108 pp. *Dissertation Abstr.*, *26* (11), 6358 (1956); *C. A.*, *65*, 8314d (1966).
12. Asperger, R. G., Liu, C. F. *Inorg. Chem.*, *4*, 1395 (1965).
13. Asperger, R. G., Liu, C. F., *Inorg. Chem.*, *6*, 796 (1967).
14. Babievskii, K. K., Latov, V. K., *Russ. Chem. Reviews*, *38*, 456 (1969).
15. Bähr, W., Theobald, H. "Organische Stereochemie", p. 15, Springer-Verlag, Berlin, Heidelberg, New York, 1973.
16. Beamer, R. L., Fickling, C. S., Ewing, J. H. *J. Pharm. Sci.*, *56*, 1029 (1967).
17. Bergmann, M., Tietzmann, J. E., *J. Biol. Chem.*, *155*, 535 (1944).

18. Cahn, R. S., Ingold, C. K., Prelog, V., *Experientia, 12*, 81 (1956); *Angew. Chem., 78*, 413 (1966).
19. Carmack, M., Kelley, C. J., *J. Org. Chem., 1968*, 2171.
20. Chenard, J. Y., Commereuc, D., Chauvin, Y., *Chem. Commun. 1972*, 750.
21. Corey, E. J., McCaully, R. J., Sachdev, H. S., *J. Am. Chem. Soc., 92*, 2476 (1970).
22. Corey, E. J., Sachdev, H. S., Gougoutas, J. Z., Saenger, W., *J. Am. Chem. Soc., 92*, 2488 (1970).
23. Cram, D. J., Abd. Elhafez, F. A., *J. Am. Chem. Soc., 74*, 5828 (1952).
24. Dang, T. P., Kagan, H. B., *Chem. Commun., 1971*, 481.
25. Diner, U. E., Worsley, M., Lown, J. W., Forsythe, J.-A., *Tetrahedron Letters, 1972*, 3145.
26. Eliel, E. L., "Stereochemie der Kohlenstoffverbindungen, p. 14. Verlag Chemie, Weinheim., 1966.
27. Erlenmeyer, E., Jr., *Ann. 337*, 265 (1904).
28. Erlenmeyer, E., Jr., Früstück, E., *Ann., 284*, 47 (1895).
29. Fiaud, J.-C., Kagan, H. B., *Tetrahedron Letters, 1970*, 1813.
30. Fiaud, J.-C., Kagan, H. B., *Tetrahedron Letters, 1971*, 1019.
31. Green, M., Kuc, T. A., Taylor, S. H., *Chem. Commun., 1970*, 1553.
32. Greenstein, J. P., Winitz, M. "Chemistry of the Amino Acids", Vol. 1, p. 205. J. Wiley & Sons, New York, 1961.
33. Harada, K., *Nature (London), 200*, 1201 (1963).
34. Harada, K., *Nature (London) 212*, 1571 (1966).
35. Harada, K., *J. Org. Chem., 32*, 1790 (1967).
36. Harada, K. und Fox, S. W., *Naturwissenschaften, 51*, 106 (1964).
37. Harada, K., Iwasaki, T., Okawara, T., *Bull. Chem. Soc. Japan, 46*, 1901 (1973).
38. Harada, K., Matsumoto, J., *J. Org. Chem., 31*, 2985 (1966).
39. Harada, K., Matsumoto, J., *J. Org. Chem., 32*, 1794 (1967).
40. Harada, K., Matsumoto, J., *J. Org. Chem., 33*, 4467 (1968).
41. Harada, K., Okawara, T., *J. Org. Chem., 38*, 707 (1973).
42. Harada, K., Okawara, T., *Bull. Chem. Soc. Japan, 46*, 191 (1973).
43. Harada, K., Yoshida, T., *Chem. Commun., 1970*, 1071.
44. Harada, K., Yoshida, T., *Bull. Chem. Soc. Japan, 43*, 921 (1970).
45. Harada, K., Yoshida, T., *J. Org. Chem., 37*, 4366 (1973).
46. Harada, K., Okawara, T., Matsumoto, K., *Bull. Chem. Soc. Japan, 46*, 1865 (1973).
47. Hayakawa, T., Shimizu, K., *Bull. Chem. Soc. Japan, 46*, 1886 (1973).
48. Herbst, R. M., Swart, E. A., *J. Org. Chem., 11*, 368 (1946).
49. Herlinger, H., Kleimann, H., Offermann, K., Rücker, D., Ugi, J., *Ann., 692*, 94 (1966).
50. Herlinger, H., Rücker, D., Kleimann, H., *Angew. Chem., 76*, 757 (1954).
51. Hiskey, R. G., Northrop, R. C., *J. Am. Chem. Soc., 83*, 4798 (1961).
52. Hiskey, R. G., Northrop, R. C., *J. Am. Chem. Soc., 87*, 1753 (1965).
53. Horner, L., Siegel, H., Büthe, H., *Angew. Chem., 80*, 1034 (1968); *Angew. Chem. Int. Ed., 7*, 942 (1968).
54. Isoda, T., Ichikawa, A., Shimamoto, T., *Rikagaku Kenkyusho Hogoku, 34*, 134 (1958); *C. A., 54*, 287f (1960).
55. Izumi, Y., *Angew. Chem., 83*, 956 (1971).
56. Knoop, F., Martius, C., *Z. physiol. Chem., 258*, 238 (1939).
57. Knowles, W. S., Sabacky, M. J., *Chem. Commun., 1968*, 1445.
58. Knowles, W. S., Sabacky, M. J., Vineyard, B. D., *Chem. Commun., 1972*, 10.
59. Korpiun, O., Lewis, R. A., Chickos, J., Mislow, K., *J. Am. Chem. Soc., 90*, 4842 (1968).
60. Kost, A. N., Sagitullin, R. S., Yurovskaja, M. A., *Chem. and Ind., 1966*, 1496.
61. Maeda, G., *Nippon Kagaku Zasshi, 77*, 1011 (1956); *C. A., 53*, 5147h (1959).
62. Matsumoto, K., Harada, K., *J. Org. Chem., 31*, 1956 (1966).
63. Matsumoto, K., Harada, K., *J. Org. Chem., 33*, 4526 (1968).
64. Matsuo, H., Kobayashi, H., Tatsuno, T., *Chem. Pharm. Bull. 18*, 1693 (1970); *C. A., 73*, 110103a (1970).
65. Meisenheimer, J., Angermann, L., Holstein, H., *Ann., 438*, 261 (1924).

66. Mislow, K., "Einführung in die Stereochemie", p. 23. Verlag Chemie, Weinheim, 1967.
67. Morrison, J. D., Burnett, R. E., Anguiar, A. M., Morrow, C. J., Phillips, C., *J. Am. Chem. Soc.*, *93*, 1301 (1971).
68. Morrison, J. S., Mosher, H. S., "Asymmetric Organic Reactions", p. 297. Prentice-Hall, Englewood Cliffs, New Jersey, 1971.
69. Murakami, M., Itatani, H., Takahashi, K., Kang, J. W., Suzuki, K., *Mem. Inst. Sci. Ind. Res., Osaka Univ. 20*, 95 (1963); *C. A.*, *60*, 15978b (1964).
70. Murakami, M., Takahashi, K., *Bull. Chem. Soc. Japan*, *32*, 308 (19599).
71. Nakamura, Y., *Nippon Kagaku Zasshi*, *61*, 1051 (1950); *C. A.*, *37*, 377 (1943).
72. Nakazaki, M., *Nippon Kagaku Zasshi*, *75*, 831 (1954); *C. A.*, *49*, 13937f (1955).
73. Naumann, K., Zon, G., Mislow, K., *J. Am. Chem. Soc.*, *91*, 7012 (1969).
74. Okawara, T., Harada, K., *Bull. Chem. Soc. Japan*, *46*, 1869 (1973).
75. Passerini, M., *Gazz. Chim. Ital.*, *51*, 126 (1921); *C. A.*, *16*, 555 (1922).
76. Patel, M. S., Worsley, M., *Can. J. Chem.*, *48*, 1881 (1970).
77. Poisel, H., Schmidt, U., *Chem. Ber.*, *106*, 3408 (1973).
78. Prelog, V., *Helv. Chim. Acta*, *36*, 308 (1953).
79. Senoh, S., Ouchi, S., Tsunoda, K., Jap. Pat. 13.307 (1963); *C. A.*, *60*, 3092h (1964).
80. Terent'ev, A. P., Gracheva, R. A., Dedenko, T. F., *Dokl. Akad. Nauk SSSR*, *163*, 386 (1965); *C. A.*, *63*, 11344b (1965).
81. Ugi, J., *Angew. Chem.*, *75*, 9 (1962).
82. Ugi, J., in: "Neuere Methoden der präparativen organ. Chemie." Vol. *IV*, p. 1.
83. Ugi, J., *Chimia (Basel)*, *19*, 89 (1965).
84. Ugi, J., *Z. Naturforschung*, *20b*, 405 (1965).
85. Ugi, J., Offermann, K., *Angew. Chem.*, *75*, 917 (1963).
86. Ugi, J., Offermann, K., Herlinger, H., *Angew. Chem.*, *76*, 613 (1964).
87. Ugi, J., Offermann, K., Herlinger, H., *Chimia (Basel)*, *76*, 613 (1964).
88. Ugi, J., Offermann, K., Herlinger, H., Marquarding, D., *Ann.*, *709*, 1 (1967).
89. Vigneron, J. P., Kagan, H., Horeaum, A., *Tetrahedron Letters*, *1968*, 5681.
90. Weinges, K., Gries, K., Stemmle, B., *Chem. Ber.*, in press (1976).
91. Weinges, K., Graab, G., Nagel, D., Stemmle, B., *Chem. Ber.*, *104*, 3594 (1971).
92. Weinges, K. Stemmle, B., *Chem. Ber.*, *106*, 2291 (1973).
93. Weinges, K., Stemmle, B., Brune, G., unpublished results.

K. MEDZIHRADSZKY

SYNTHESIS AND BIOLOGICAL ACTIVITY OF ADRENOCORTICOTROPIC AND MELANOTROPIC HORMONES

I. INTRODUCTION

The pituitary gland, or hypophysis is the part of the organism richest in hormones, and hence the richest in varied functions which are all essential to normal life. All these hormones are polypeptides or proteins originating from anatomically different parts of the pituitary. Not only their biological activity is known, but also the complete or partial chemical structures of most of these polypeptides have been established, and some of them have even been synthesized. Their molecular weights and primary structures are usually very different, still some of them contain shorter or longer common amino acid sequences, resulting in more or less common biological activities. To this group of closely related polypeptide hormones belong the adrenocorticotropic hormone (ACTH) and the melanotropins (melanocyte-stimulating hormone, MSH), which were the first pituitary hormones discovered and most thoroughly investigated. The last known representative of this hormone group, lipotropin (LPH), was isolated only in recent years.

Perhaps the most remarkable and undoubtedly most interesting property of polypeptide hormones is their ability to elicit similar biological responses in spite of their different molecular weights and chemical structures, while at the same time in many cases their biological activity can easily be affected by slight modifications in structure or in configuration. Having established the chemical structure of polypeptide hormones by analytical means, efforts for their synthesis are justified for different reasons, such as synthetic proof of the chemical constitution, elucidation of relationships between the primary structure and biological activity, clearing up details of the mechanism of hormone action in connection with the established structure, and finally a practical aim, hormone production for therapeutical use.

Because of the points of view mentioned above, the most intensive research has been conducted, besides the hormones of the posterior lobe,

oxytocin and vasopressin, in the field of the adrenocorticotropic-melanotropic hormones. This review will attempt to demonstrate how efficient and successful peptide synthesis can be in the interpretation of relationships between chemical structure and biological activity. A possibly complete survey will also be presented on the synthesis of adrenocorticotropins, melanotropins, their fragments and analogues, and the biological activities of these substances will be discussed.

In searching the original literature and in the discussion of some of the more important results concerning structure–activity relationships, earlier reviews in the corticotropin–melanotropin field were of great assistance to the author. In this respect the following papers have been particularly useful: C. H. Li [200] (1956, adrenocorticotropic hormone); [203] (1962, synthesis of ACTH peptides); K. Hofmann, H. Yajima [141] (1962, synthesis of ACTH peptides); K. Hofmann [122] (1963, chemistry and function of corticotropins and melanotropins); K. Hofmann, P. G. Katsoyannis [126] (1963, synthesis and function); J. Ramachandran, C. H. Li [276] (1967, structure–activity relationships); C. H. Li, W. Oelofsen [215] (1967, chemistry and biology of ACTH peptides); S. Lande, A. B. Lerner [178] (1967, biochemistry of melanotropins); A. B. Lerner, T. H. Lee [196] (1962, melanocyte-stimulating hormones), and finally: G. T. Young [366] (1969–1972, structure, synthesis, structure–activity relationships).

II. ADRENOCORTICOTROPIC HORMONE (ACTH)*

1. STRUCTURE

Adrenocorticotropin, the polypeptide hormone of the anterior lobe of the pituitary (adenohypophysis) was named after its most significant biological activity; stimulation of the adrenal cortex resulting in the production of corticosteroids. To date it has been isolated in pure state from four species. The primary structure of porcine ACTH was established by Bell, Shepherd and their co-workers [13, 14, 316, 317] and by White and Landmann [345]. The amino acid sequence of the ovine and bovine corticotropins was determined by Li *et al.* [207, 208], that of the human hormone by Lee *et al.* [192]. Details of the structure investigations were reviewed by Li and Oelofsen [215]. It is sufficient to mention here that all the four

* As the isolation and structure investigation of adrenocorticotropic hormones were undertaken by several groups of investigators at the same time, there is some confusion in the nomenclature of these polypeptides. In this paper the widely accepted proposal of C. H. Li (*Science*, *129*, 969 [1959]) will be employed. Accordingly, the main adrenocorticotropic principle isolated from the pituitary of a given species will be called α-corticotropin (α-ACTH), with a small letter denoting the species of origin; e.g., human adrenocorticotropic hormone will be abbreviated as α_h-ACTH.

hormones are single polypeptide chains consisting of 39 amino acids. They have common sequences on the N-terminal and C-terminal parts (24 and 6 amino acids, respectively) differing only in the amino acid arrangement of the nonapeptide at positions 25–33, which thus represents the species-specific part of the molecule. Structures believed to be correct until recently, are shown in Fig. 1.

Structure elucidation of the species-specific part proved to be a very difficult task resulting in controversy among the different research groups.

```
                1
H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-
                                                24
-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-
                   25                           33
Human           -Asp-Ala-Gly-Glu-Asp-Gln-Asr-Ala-Glu-
Bovine          -Asp-Gly-Glu-Ala-Glu-Asp-Ser-Ala-Gln-
Ovine           -Ala-Gly-Glu-Asp-Asp-Glu-Ala-Ser-Gln-
Porcine         -Asp-Gly-Ala-Glu-Asp-Gln-Leu-Ala-Glu-
                   34                        39
                -Ala-Phe-Pro-Leu-Glu-Phe-OH
```

Fig. 1. Amino acid sequences of the various adrenocorticotropins. (Status 1971)

Thus, sequence 25–33 of the porcine corticotropin was found by White and Landmann [345] to be

```
25  26  27  28  29  30  31  32  33
-Gly-Ala-Glu-Asp-Asp-Glu-Leu-Ala-Glu-
```

while Shepherd *et al.* [317] have characterized this part of the molecule by the following amino acid arrangement:

```
25  26  27  28  29  30  31  32  33
-Asp-Gly-Ala-Glu-Asp-Gln-Leu-Ala-Glu-
```

Divergencies between the two structures are the absence of an amide group on the Glu^{30} residue in the White and Landmann formula and the position of aspartic acid as residue 25 according to Shepherd *et al.* [317] instead of 28, as given by the former authors, simultaneously replacing the sequence Gly-Ala-Glu from positions 25–27 into positions 26–28. The arrangement of Asp-Gly-Ala-Glu was confirmed convincingly by Shepherd *et al.* [317] and later by Harris [117] as well.

Localization of the amide group proved to be even more difficult. Shepherd *et al.* [316, 317] have already mentioned that the species-specific part of porcine corticotropin is surprisingly unstable and shows some anomalies in reactivity. Edman degradation gave no unequivocal results, and exposure

of the molecule to pH 9 sodium bicarbonate for 18–22 hours at room temperature resulted in deamidation. Since White and Landmann had used slightly alkaline solutions for the purification of their corticotropin (corticotropin-A), they may have isolated the deamidated form of the native hormone (β-corticotropin) studied by Shepherd *et al.* On the other hand, location of the amide group was apparently confirmed by Bell *et al.* [14] in such a way that among the 1–31, 1–30 and 1–28 fragments of α_p-ACTH, obtained by partial peptic hydrolysis, only the first two contained the labile amide group, whereas the third, shortest one did not. Finally, stepwise degradation of the 1–31 fragment by carboxypeptidase yielded glutamine in the second step, while it was the product of the first step from the 1–30 fragment.

Uncertainties were also found in the localization of the amide groups in the human ACTH. Because of the limited supply of material, Lee *et al.* [192] were unable to determine the correct position of this group. Indication for Gln^{30} was given only by the electrophoretic mobility of the tripeptide Gln-Ser-Ala (positions 30–32), obtained from partial enzymic degradation. On the other hand, Lee *et al.* noticed that peptides containing the aspartic acid unit in position 25 are not so acidic as it could have been expected, and tried to explain this observation with cyclic imide formation.

It is worthy of mention that during the isolation of ovine ACTH, in addition to the main component containing two amide groups per molecule, Li *et al.* [209] were able to obtain a compound with four amide groups, although only one of them could be assigned to Gln^{33} [194].

The easy deamidation of porcine ACTH observed by Shepherd *et al.* [317] very much resembles the unusually rapid deamidation of asparagine at position 6, situated next to a glycine in the sequence of bovine prolactin [96]. Re-examining the C-terminal amino acid sequence of α_p-ACTH, Gráf *et al.* [94, 95] found the amide group on the aspartic acid residue in position 25 instead of on the glutamic acid located at position 30; this led to the conclusion that the rapid deamidation might be again a consequence of an Asn-Gly peptide bond. Furthermore, the similar tendency for deamidation in native human ACTH points to the presence of the same structural element, although that would involve the interchange of the 26 and 27 amino acids.

At about the same time, comparing the chemical properties of natural human ACTH with those of some synthetic fragments, Riniker *et al.* [278] found that synthetic peptides corresponding to the C-terminal part of human and porcine corticotropins did not show deamidation of the type which was a property of the natural hormones and also their tryptic frag-

ments comprising the 22–39 amino acid sequence. There was apparently some discrepancy between the natural and synthetic products. Riniker *et al.* re-investigated the amino acid arrangement in these tryptic fragments by means of Edman degradation. Surprisingly enough, aspartic acid from position 25 was obtained as PTH-asparagine, followed by glycine in both hormones. Thus the correct sequences of the species-specific parts in both human and porcine corticotropins (Fig. 2) contain Asn-Gly-Ala in position 25–27, the only difference between the two hormones being the presence of leucine instead of serine in the position 31.

	25						31		33
Human	-Asn-	Gly-	Ala-	Glu-	Asp-	Glu-	Ser-	Ala-	Glu-
Bovine	-Asn-	Gly-	Ala-	Glu-	Asp-	Glu-	Ser-	Ala-	Gln-
Ovine	-Asp-	Gly-	Ala-	Glu-	Asp-	Glu-	Ser-	Ala-	Gln-
Porcine	-Asn-	Gly-	Ala-	Glu-	Asp-	Glu-	Leu-	Ala-	Glu-

Fig. 2. Revised amino acid sequences of the adrenocorticotropins at positions 25–33

A similar re-investigation of the corresponding sequences in bovine and sheep corticotropin was undertaken by Li [204]. The C-terminal peptides from the tryptic digests of both corticotropins were isolated and their structures determined. The sequences found are represented in Fig. 2. It is noteworthy that the revised corticotropin sequences are very similar, differing from each other only in positions 25, 31 and 33.

It remains to be established how the results of Bell *et al.* [14] can be explained in the light of these newer developments.

2. SYNTHESIS OF CORTICOTROPIN FRAGMENTS

During the structure investigation of porcine corticotropin, Bell *et al.* [14] observed that limited peptic hydrolysis led to three well characterized fragments with full biological activity, comprising the amino acid sequences 1–31, 1–30 and 1–28. It was also shown that the peptide bond between the amino acid residues 24 and 25 was unstable to acid; still, corticotropin did not lose its biological activity under acidic conditions, where this labile peptide bond must have been hydrolyzed. Although the corresponding 1–24 fragment has never been isolated in pure form from the enzymic digest, it could be postulated that this fragment also possessed full biological activity. Since smaller biologically active fragments could not be found, Shepherd *et al.* [317] arrived at the conclusion that in view of the large size of the molecule "a practical synthesis in competition with the natural product seems unlikely".

On the other hand, the fact that a polypeptide hormone after the loss of almost half of its amino acid content still retained biological action,

made the corticotropins a very challenging subject for investigations of the structure–biological activity relationship. Immediately after completing the structure elucidation of these molecules, a number of research groups started synthetic work for obtaining corticotropin fragments of different structure. As the biological activity was greatly impaired even by the slightest modification on the N-terminus and, on the other hand, it was not affected by extensive degradation on the carboxyl end, efforts were mainly directed towards the synthesis of N-terminal fragments.*'**

The first synthetic peptide having a moderate but definite corticotropic activity was prepared by Boissonnas *et al.* in 1956 [30]. Coupling of the larger peptide fragments was accomplished by the dicyclohexylcarbodiimide method, which is known to proceed with possible partial racemization. Since Boissonnas *et al.* did not check the optical purity of their product, according to our present knowledge the only moderate biological activity could be accounted for by uncontrolled racemization on critical points. Figure 3 shows the main steps of the synthesis.

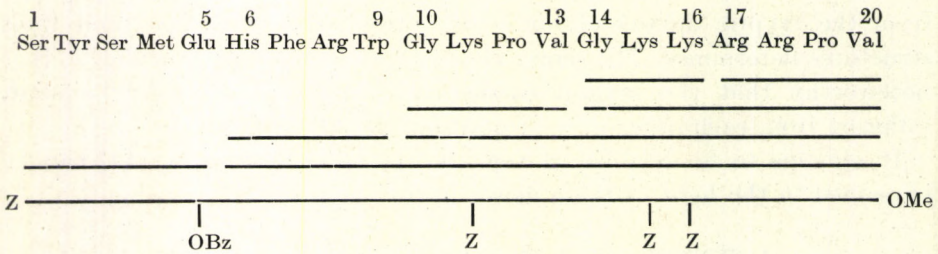


Fig. 3. Synthesis of the protected eicosapeptide according to Boissonnas *et al.* [30]

The protected eicosapeptide contained benzyloxycarbonyl protecting groups on the N-terminal and on the lysine ε-amino groups; the glutamic acid side chain was protected by benzyl ester, the terminal carboxyl with methyl ester group. Removal of the protecting groups was effected with hydrogen bromide in acetic acid, which left the C-terminal methyl ester

* As this review is not intended to be a methodological survey in synthetic peptide chemistry, details which would be valuable and useful only for practising chemists have been omitted. These aspects were reviewed up to 1965 in the excellent book of Schröder and Lübke [295]. It seems to be sufficient here to delineate schematically the synthetic routes leading to a particular peptide and to mention briefly a few characteristics or problems of some particular syntheses. Also, for better understanding, structures of the fully protected end products in abbreviated form will be given. For abbrevations the IUPAC-IUB Commission on Biochemical Nomenclature, Symbols for Amino Acid Derivatives and Peptides, Recommendations (1971), *J. Biol. Chem.*, *247*, 977 (1972) should be consulted.

** N-terminal corticotropin fragments synthesized thus far are summarized on pp. 145—146.

unchanged. Experimental details for the synthesis of the eicosapeptide are unknown; the preparation of the undecapeptide comprising the 10–20 sequence was published in 1961 [25].

For a study of the relationship between the structure and biological activity of α-melanotropin (p. 203) Guttmann and Boissonnas [105] synthesized the N-terminal tridecapeptide amide of corticotropins, using essentially the same methods as for the synthesis of the eicosapeptide. The last coupling was made again with dicyclohexylcarbodiimide at the glutamic acid residue, this time with a detectable amount of racemic product. The tridecapeptide had no corticotropic activity.

The same tridecapeptide amide was also prepared by Hofmann and Yajima at the same time [140], after they had reported, in a series of publications, the synthesis of several smaller peptides derived from the N-terminal part of corticotropins [124, 125, 127, 132, 134, 135, 138].

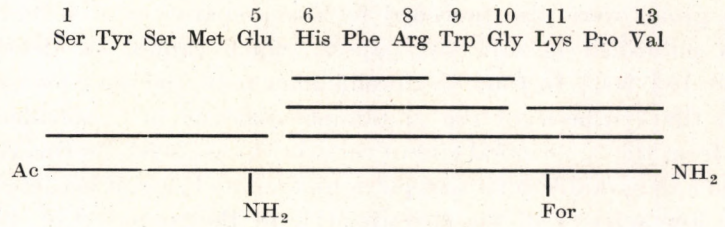


Fig. 4. Synthesis of the protected ACTH-(1–13)-tridecapeptide amide

To avoid racemization, the intermediary peptides containing sequences 1–5 and 6–13 were coupled by the azide technique according to the scheme given in Fig. 4.

It is noteworthy that in the course of preliminary experiments, when lysine ε-amino groups were protected by tosyl substituents, cleavage of this protecting group by reduction with sodium in liquid ammonia was accompanied by severe destruction of the peptide chain. For this reason, amino groups were preferably protected by formylation; the N-formyl, N-acetyl- and the ω-amide group of glutamic acid could be removed simultaneously by treatment with hot dilute hydrochloric acid. The tridecapeptide amide exhibited a small but reproducible corticotropic activity. During this synthetic work the N-terminal decapeptide was also prepared by deprotection of an N-acetyl intermediate containing glutamine residue in position 5.

In 1961 the synthesis of the N-terminal decapeptide was described by Schwyzer and Kappeler [301] too, using the combination of *t*-butyloxycarbonyl and *t*-butyl ester protecting groups, which were removable by

mild acidolysis. As it is shown in Fig. 5, the closing step in this experiment was coupling between the 1–4 and 5–10 fragments *via* the azide route. These intermediates served as widely used key products in a number of more recent syntheses.

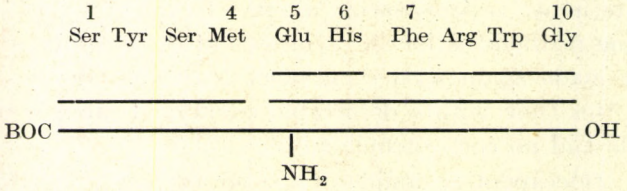


Fig. 5. Synthesis of the protected N-terminal decapeptide

Synthesis of biologically active corticotropin fragments by the Ciba research group were also preceded by the preparation of several smaller peptides published in different papers, which cannot be detailed here [157, 160, 162, 306]. In 1960, in a preliminary note, the same research group reported the synthesis of the glutamine analogue of a nonadecapeptide derived from the N-terminal end of corticotropins [308]. As this compound possessed a remarkable corticotropic activity of 30 U/mg, it became apparent that the low activity of the eicosapeptide of Boissonnas *et al.* [30] could not be attributed to the chain length. The synthetic scheme of the nonadecapeptide analogue ([Glu(NH_2)^5]-ACTH-(1–19)-nonadecapeptide) is outlined in Fig. 6. The N-terminal decapeptide was synthesized *via* the route indicated above (Fig. 5). Preparation of the C-terminal nonapeptide was published by Rittel [279].

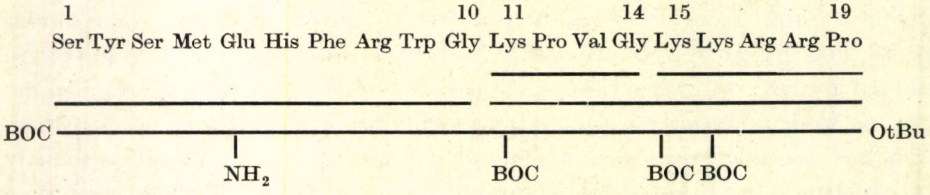


Fig. 6. Synthesis of the protected nonadecapeptide according to Schwyzer *et al.*[308]

The nonadecapeptide with free glutamic acid, corresponding to the sequence of the native corticotropins was also prepared by Schwyzer and co-workers, although details of this synthesis have appeared mostly in patents ([41, 300], see also [295], p. 211).

At about the same time a fourth research group entered the field of corticotropin synthesis: Li *et al.* [213, 214] also described the preparation of the nonadecapeptide with free carboxyl group on the glutamic acid residue using a similar sequence of coupling reactions (Fig. 7). In a footnote, they also announced the synthesis of the corresponding glutamine analogue.

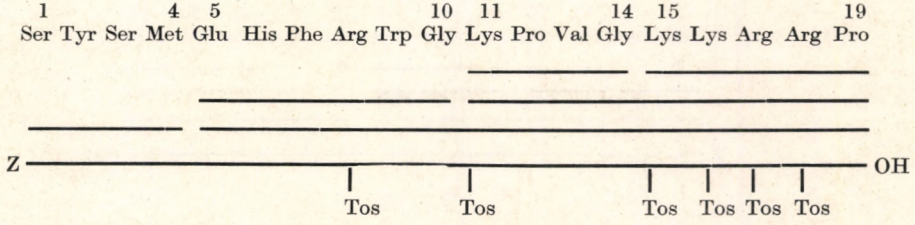


Fig. 7. Synthesis of the protected nonadecapeptide according to Li *et al.* [214]

Characteristic is the combination of the protecting groups (benzyloxycarbonyl at the N-terminus, and tosyl groups in the side chains of the lysine residues), which made again necessary deblocking by reduction with sodium in liquid ammonia, a process believed to be accompanied by significant destruction of certain peptide bonds [140, 308]. The peptide bond between fragments 11–14 and 15–19 was formed by the activated ester method, between fragments 5–10 and 11–19 by a mixed anhydride, and finally, the 5–19 fragment was acylated with the azide of the N-terminal tetrapeptide. Countercurrent distribution was extensively used for purification of the intermediates and of the end products. This nonadecapeptide proved to be even more active than the product of Schwyzer and co-workers.

In 1961, at the Fourth European Peptide Symposium in Moscow, a Hungarian peptide research team presented three papers on the preparation of protected peptides suitable for further couplings with the aim of synthesizing the N-terminal octacosapeptide of ACTH [4, 168, 233]. Selection of the 1–28 peptide as the synthetic goal was motivated by the fact that this was the smallest fragment with full activity isolated after limited peptic hydrolysis of corticotropin, the existence of a fully active 1–24 tetracosapeptide being shown only by indirect evidence (p. 123). This octacosapeptide, containing four amino acids from the species-specific sequence was no longer a common fragment of all corticotropins. With the given arrangement of amino acids in positions 25–28, it corresponded to the structure of porcine ACTH as determined by Shepherd *et al.* [317].

The three intermediates covered the sequences 1–9, 10–21 and 22–28; benzyloxycarbonyl and tosyl served as amino-protecting groups and alkyl ester groups were used for carboxyl protection (Fig. 8).

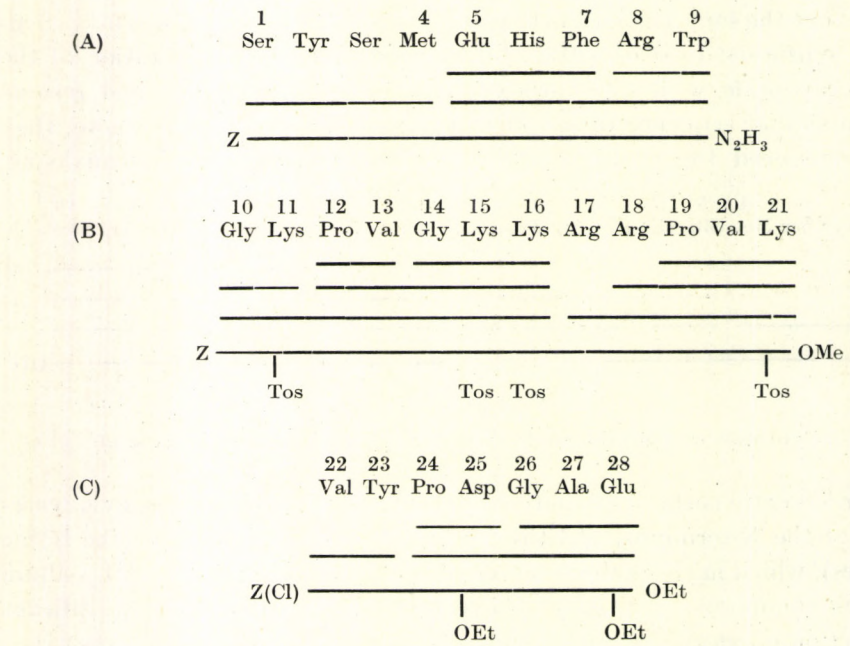


Fig. 8. Synthesis of the protected intermediates for the preparation of α_p-ACTH-(1–28)-octacosapeptide

It can be seen from the short communications containing some experimental details [5, 169, 232] that *p*-chlorobenzyloxycarbonyl protecting groups [167] often giving crystalline derivatives, and azide couplings without isolating the intermediary azides were widely used throughout the synthetic work. For the synthesis of the octacosapeptide, first components (B) and (C) were coupled followed by the acylation of (B)–(C) with peptide (A). The selected combination of protecting groups made possible the isolation of the chromatographically pure protected octacosapeptide by simple "crystallization" from hot methanol. Deblocking was accomplished by reduction with sodium in liquid ammonia, and the biologically active pure product was obtained by preparative paper electrophoresis [33].

Apart from the fact that paper electrophoresis is not the method of choice for the purification of greater amounts of polypeptides, it soon became apparent that this combination of protecting groups was not advantageous for the synthesis of a polypeptide hormone of such a complex structure. Deblocking of the homogeneous protected peptide gave, in addition to the expected free octacosapeptide, a mixture of by-products derived from unidentified cleavages along the polypeptide chain. This observation of the

Hungarian researchers coincided with those of Hofmann and Yajima [140] and of Schwyzer *et al.* [308], and seemed to be worth of further studying. Investigation of a number of model peptides containing the critical Gly-Lys-Pro amino acid sequence led to the finding that under strictly anhydrous conditions no degradation occurred [6, 102]. Correspondingly, the extensive decomposition must have been due to an uncontrolled and non-removable water content in the protected octacosapeptide. Moreover, it appeared to be very likely that the great number of protecting groups in a relatively large molecule also had some role in the extent of degradation. In spite of the fact that Ramachandran *et al.* [271] later succeeded in preparing pure hormone fragments in acceptable yields by excluding even traces of moisture from the reaction mixture, it was preferred to repeat the synthesis of the octacosapeptide using a new combination of protecting groups.

For this purpose the simultaneous application of the acid-labile *t*-butyloxycarbonyl and *t*-butyl ester protecting groups appeared to be the most promising, as they had been advantageously used by Schwyzer *et al.* in the synthesis of the N-terminal nonadecapeptide [308]. In the meantime a new line of synthetic research became of importance, namely attempts to delineate the minimal sequence possessing corticotropic activity. The new synthesis of the octacosapeptide was planned therefore in such a way that the last coupling should take place between two tetradecapeptides. Hence, the N-terminal tetradecapeptide could serve as an excellent starting material for the synthesis of N-terminal fragments of different chain length.

This compound was synthesized according to the scheme given in Fig. 9 [7, 32, 231]. The amino groups and the γ-carboxyl of glutamic acid were permanently protected by *t*-butyloxycarbonyl and *t*-butyl ester groups, and nitration was employed for temporary protection of the arginine guanidino groups. Elongation of the C-terminal heptapeptide was achieved by acylation with the azides prepared *in situ* of the protected 5–7 and 1–4 oligopeptide derivatives. For purification of the protected tetradecapeptide

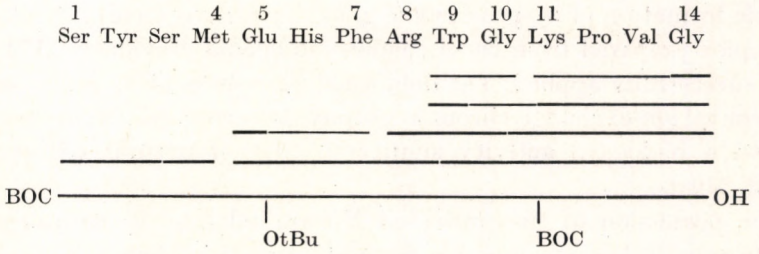


Fig. 9. Synthesis of the protected N-terminal tetradecapeptide

column chromatography on silica gel was used. Deblocking of the protected compound by dissolving it in trifluoroacetic acid afforded the pure, free tetradecapeptide [231].

The main steps in the synthesis of the tetradecapeptide containing the C-terminal amino acids are schematically shown in Fig. 10. Obviously, there was no danger of racemization in the coupling of the intermediary 15–19, 20–24 and 25–28 peptides, since both activated peptides had a proline residue at their C-terminus. Therefore, activated esters, especially N-hydroxysuccinimide derivatives [225] were predominantly used throughout the synthesis. Protection of the side chains was similar to that of the N-terminal tetradecapeptide [32].

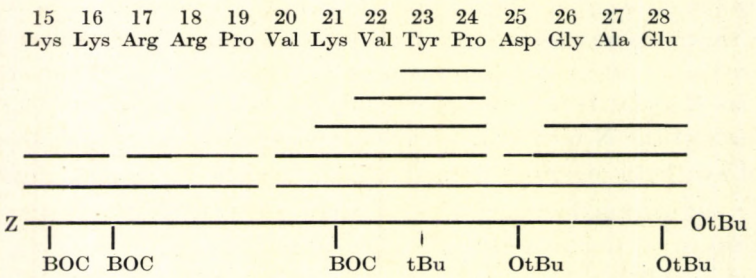


Fig. 10. Synthesis of the protected C-terminal tetradecapeptide

Coupling of large peptide fragments often presents additional difficulties due to the relatively small concentration of activated carboxyl and free amino groups. In addition, limited stability of the activated compounds leads to by-products in the slow coupling reaction. Medzihradszky and Bajusz [229] investigated the condensation step between the two tetradecapeptides in detail, and acylation with the N-terminal tetradecapeptide, in general. They found that the use of dicyclohexylcarbodiimide in the presence of pentachlorophenol gave almost quantitative yields with only negligible formation of by-products (e.g. acylurea derivative) [7]. Similarly, the complex prepared from these condensing agents previously [174] could also be successfully applied. The deblocked octacosapeptide was isolated in pure form by ion-exchange chromatography on carboxymethylcellulose and possessed a biological activity similar to that of natural corticotropins [2, 7, 32, 230].

In the possession of the protected N-terminal tetradecapeptide of the corticotropins it became possible to synthesize the whole series of oligopeptide amides containing the N-terminal 1–14, 1–15, 1–16, 1–17, 1–18,

1–19, 1–20 and 1–21 amino acids [7]. The amino components (C-terminal peptide amides) necessary for these syntheses were prepared according to the scheme shown in Fig. 11. For the synthesis of the compounds comprising the 15–17, 15–18 and 15–19 sequences Z-Lys(BOC)-Lys(BOC)-N_3, a reactive derivative of the Lys-Lys dipeptide, was used and the Z-Lys(BOC)-Lys(BOC)--Arg(NO_2)-Arg(NO_2)-Pro-OPCP activated ester prepared in this way served as starting material for the synthesis of the 15–20 and 15–21 peptide amides.

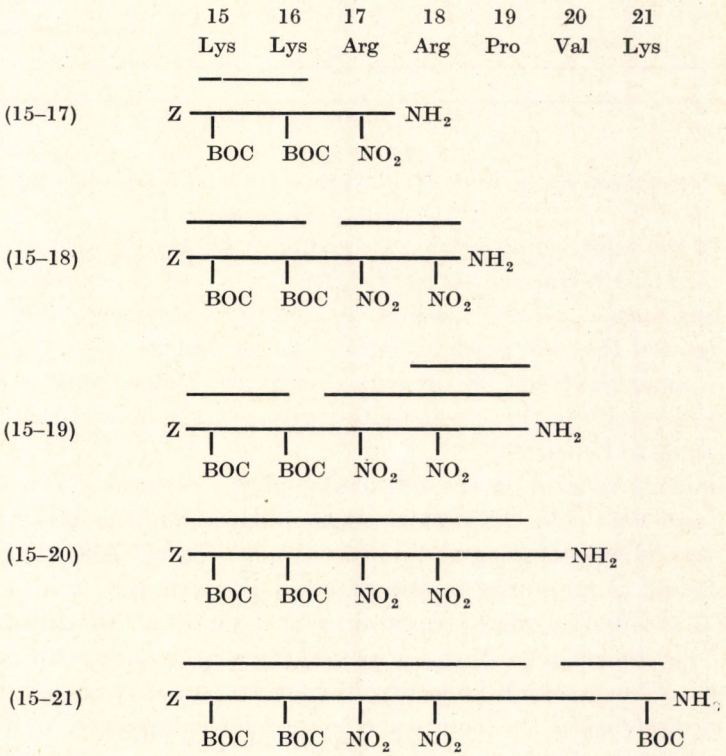


Fig. 11. Synthesis of the protected C-terminal peptide amides

Condensation of these C-terminal amides with the protected N-terminal tetradecapeptide by the dicyclohexylcarbodiimide-pentachlorophenol procedure yielded the desired N-terminal fragments. Investigation of the biological properties of these peptide amides has been of great value in determining the active centre of corticotropins.

In the meantime, in order to define the smallest structural unit of adrenocorticotropins necessary to exhibit biological activity, numerous fragments

of ACTH have been synthesized by several research groups. Schwyzer *et al.* [307] prepared the N-terminal hexadecapeptide methyl ester, a derivative which contained only one half of the basic core located at the 15–18 positions. Starting from the N-terminal decapeptide described earlier, this synthesis was performed as shown in Fig. 12. The fragment possessed only a very limited corticotropic activity.

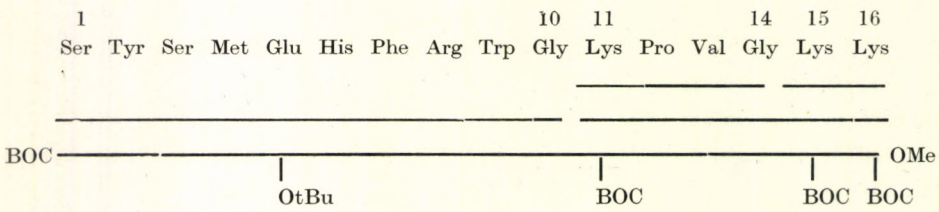


Fig. 12. Synthesis of the protected ACTH-(1–16)-hexadecapeptide methyl ester

One of the most significant corticotropin fragment syntheses was that of the N-terminal tetracosapeptide by Schwyzer and co-workers. As it was mentioned earlier, on the basis of hydrolytic experiments Bell *et al.* [14] had suggested that the peptide containing the first 24 amino acids of corticotropin must have the full biological activity. Determination of the corticotropic activity of the synthetic tetracosapeptide showed the assumption of Bell *et al.* to be correct.

Preliminary reports on the tetracosapeptide synthesis appeared as early as 1961 and 1962 [164, 297]; experimental details and the biological properties of this substance became known two years later [302]. This synthesis is significant from several points of view. For example, it is worth mentioning that only five years elapsed since Shepherd *et al.* had given expression to their pessimism regarding the possibility of the successful synthesis of a peptide of such a high molecular weight and great complexity. Furthermore, in many cases this compound served for comparison in testing different substances for corticotropic activity, and also found application for therapeutical purposes.

The protection system characterized by a combination of blocking groups derived from *t*-butanol was developed mainly in the course of the tetracosapeptide synthesis, and was later widely used in the preparation of other polypeptides as well. Advantages of the *t*-butyloxycarbonyl and *t*-butyl esters over tosyl or methyl ester protection are well known. The significant amount of labour invested into the preparation of the protected derivatives generally find recompensation in the simple and unequivocal removal of these protecting groups, affording the pure polypeptides in excellent yields.

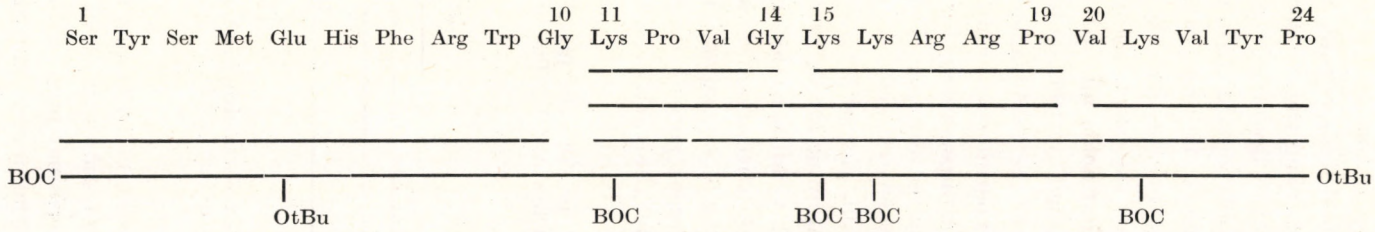


Fig. 13. Synthesis of the protected N-terminal tetracosapeptide

The key product for the preparation of the tetracosapeptide was again the suitably protected N-terminal decapeptide, and other fragments were selected such that on further coupling the danger of racemization could be avoided. This was possible by the favourable distribution of the glycine and proline residues along the polypeptide chain of the corticotropins, as it is shown in the synthesis scheme of the tetracosapeptide (Fig. 13).

Similar successful fragment syntheses have been reported by Hofmann *et al.* Their preliminary results on the preparation of a tricosapeptide amide containing the N-terminal amino acids and possessing the full biological activity of ACTH were published in 1961 [146], preceding with a few months only the announcement of the 1–24 fragment synthesis by Schwyzer *et al.* In keeping with earlier experiments, formyl group was used for protection of the lysine ε-amino groups, amide for the γ-carboxyl group of glutamic acid and acetyl for the N-terminus. Deblocking was achieved by treatment with hot dilute hydrochloric acid. As it was later demonstrated by carboxypeptidase degradation, partial acid hydrolysis cleaved not only the N-acetyl, N-formyl and glutamine amide groups, but the amide on the C-terminal lysine residue as well, yielding a tricosapeptide with free carboxyl terminal instead of the expected tricosapeptide amide [142].

In addition, examination of the biological activity of the products treated with hydrochloric acid for different periods showed that after an optimal time of 40–80 minutes significant destruction of the polypeptide had taken

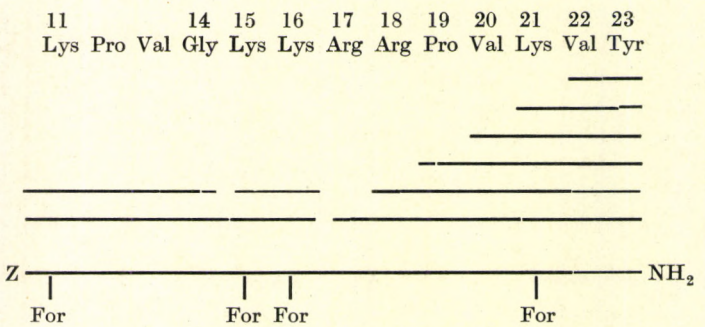


Fig. 14. Synthesis of the protected corticotropin fragment containing the 11–23 amino acids

place. Isolation of the main product from the reaction mixture required extensive purification. Figure 14 shows the synthetic steps leading to the 11–23 sequence [128]. The C-terminal part of the molecule was built up in a step-wise manner using the mixed anhydride procedure, and was

subsequently acylated by the hexapeptide azide containing the 11–16 amino acid residues. Synthesis of the decapeptide representing the N-terminal 1–10 sequence was described earlier [140]; condensation of the two larger fragments was effected by the dicyclohexylcarbodiimide technique.

Following the observation that the N-terminal tridecapeptide of ACTH is practically devoid of corticotropic activity [140], Hofmann *et al.* tried to find the smallest fragment of corticotropin which still exhibited the full biological activity of the native hormone. For this purpose the synthesis of two ACTH fragments was initiated, that of the N-terminal hexadecapeptide [147], and of the eicosapeptide amide, containing the 1–20 amino acids [129, 143]. Again, an acetyl group served for protection of the terminal amino group; the lysine ε-amino groups were formylated, and glutamine was used instead of glutamic acid in the synthesis of the intermediates. Accordingly, deprotection could be effected by treatment with hot dilute hydrochloric acid. The main steps of the synthesis of the hexadecapeptide are shown in Fig. 15; for coupling with the undecapeptide, activation of the

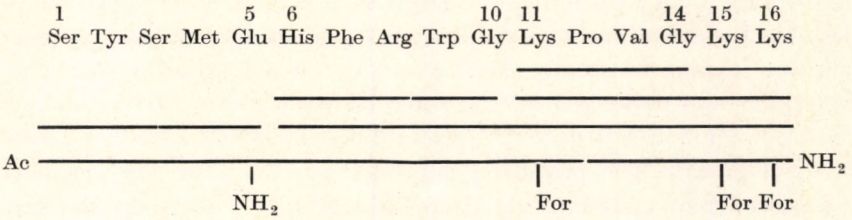


Fig. 15. Synthesis of the protected ACTH-(1–16)-hexadecapeptide amide

N-terminal pentapeptide was achieved *via* the azide method. The undecapeptide itself was prepared in two alternative routes, one of them using unprotected (or better, protonated) arginine derivatives, whereas in the other the guanidino function was blocked by a nitro group. It is noteworthy that while the acid treatment removed the amide group from the terminal lysine residue in the hexapeptide, the same group on the terminal valine residue in the eicosapeptide proved to be resistant to mild acid hydrolysis, very likely owing to the shielding effect of the bulky valine side chain. As an advantage of this protecting group combination, the intermediary peptides are hydrophilic to such an extent that they can be purified by ion-exchange chromatography on carboxymethylcellulose column using ammonium acetate buffer solutions.

As a key intermediate for the synthesis of the eicosapeptide amide the N-terminal decapeptide was used (Fig. 16), whose preparation has been described earlier [140].

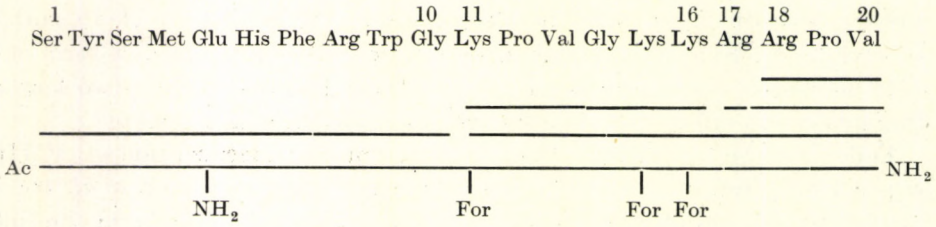


Fig. 16. Synthesis of the protected eicosapeptide amide according to Hofmann *et al.* [143]

Investigations of the relationship between chemical structure and biological activity of polypeptide hormones were also carried out by C. H. Li and co-workers, who synthesized a series of corticotropin fragments. Following the preparation of the N-terminal nonadecapeptide [214] (p. 127), preliminary [206] and detailed [217] publications appeared about the synthesis of a heptadecapeptide containing the 1–17 amino acid residues of the corticotropins. The N-terminal decapeptide, protected with blocking groups removable by reduction with sodium in liquid ammonia, was obtained in crystalline form. Improved synthesis of the Glu-His-Phe-Arg-Trp-Gly hexapeptide was published by the same research group [210]. Side chains of the basic amino acids in the C-terminal heptapeptide were also protected with tosyl groups. The protected heptadecapeptide (Fig. 17) was purified by countercurrent distribution. After deblocking, ion exchange chromatog-

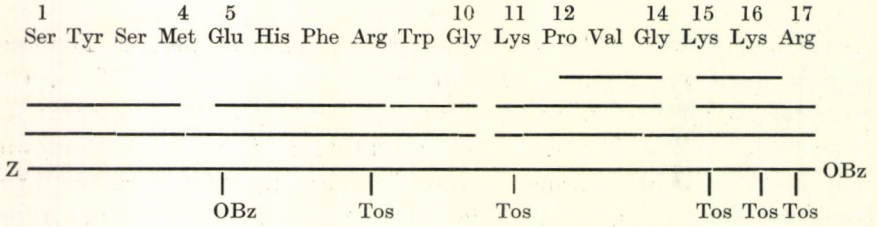


Fig. 17. Synthesis of the protected ACTH-(1–17)-heptadecapeptide

raphy afforded the pure, free ACTH-(1–17)-heptadecapeptide in a good yield, which demonstrates that under carefully controlled conditions this combination of protecting groups is also suitable for the synthesis of polypeptides of more complex amino acid composition.

The synthesis of the ACTH-(1–19)-nonadecapeptide was repeated later [205], protecting this time the lysine ε-amino groups with *t*-butyloxycarbo-

nyl substituents. The crystalline N-terminal decapeptide also used earlier for making the heptadecapeptide was chosen as the starting compound (Fig. 18). The biological properties of this product were similar to those shown by the nonadecapeptide synthesized years before *via* the alternative route (p. 127), pointing to the identity of the compounds built up by different methods.

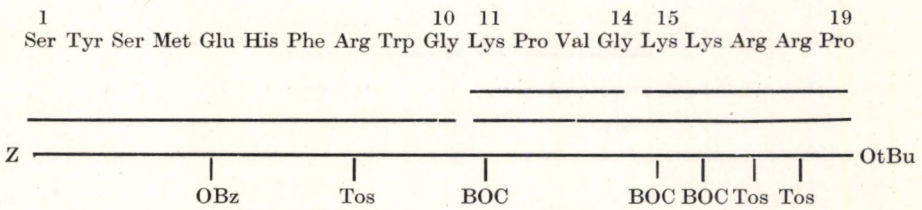


Fig. 18. The second synthesis of the protected nonadecapeptide according to Li *et al.* [205]

As it will be discussed later, the synthetic approach clearly indicated the role of the basic amino acid core in positions 15–18 in displaying the corticotropic activity. In view of this result, the question emerged whether the increasing biological activity could be brought into connection with the increasing net positive charge of the hormone fragment. In a study of this point, Ramachandran *et al.* [271] synthesized the oligopeptide amides containing the 1–17, 1–18 and 1–19 amino acids of corticotropins. Being amides, all these peptides had a greater positive charge than the corresponding fragments with free carboxyl terminal group, and all were superior to their more acidic derivatives in respect to their corticotropic activity.

In these experiments the starting material was the same N-terminal decapeptide protected with benzyloxycarbonyl, tosyl and benzyl ester groups as in the synthesis of the corticotropin fragments with free carboxyl [206, 217]. Dicyclohexylcarbodiimide was used as the condensing agent in the peptide forming step between the decapeptide and the C-terminal peptide amides, whose syntheses are represented in Fig. 19.

Coming back to the problem of peptide chain cleavage during the deblocking of tosyl derivatives, the experiments of Ramachandran *et al.* clearly demonstrated that, in accordance with earlier observations [6, 102] reduction with sodium in liquid ammonia under carefully controlled anhydrous conditions did not lead to significant decomposition. After desalting, the free peptide amides were purified by ion-exchange chromatography on carboxymethylcellulose.

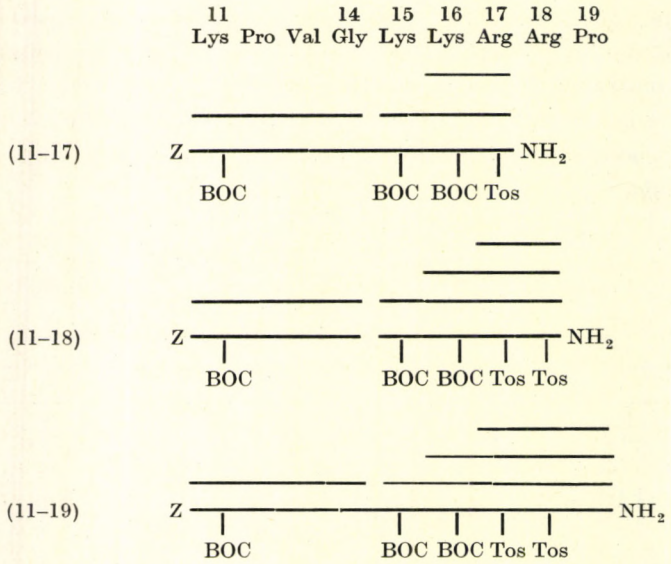


Fig. 19. Synthesis of the protected C-terminal peptide amides (Ramachandran *et al.* [271])

In addition to the preparation of the afore-mentioned peptide amides, Ramachandran and Li [275] also synthesized the α_b-ACTH-(1–26)-hexacosapeptide, containing two amino acids from the species-specific sequence characteristic of bovine corticotropin. The synthetic scheme was similar to the others elaborated by the San Francisco group; in this case all basic side chains were protected with the tosyl group (Fig. 20). Syntheses of the peptide fragments 11–19 [205] and 20–24 [274] were described earlier by the same investigators.

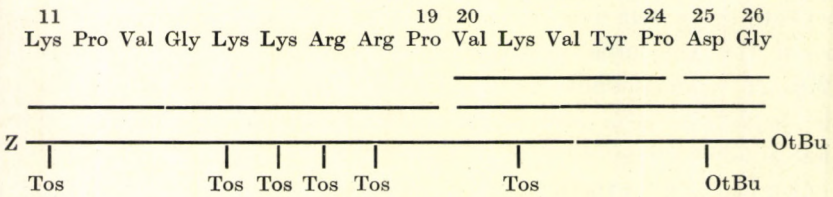


Fig. 20. Synthesis of the protected corticotropin fragment containing the 11—26 amino acids of bovine ACTH

In 1963, a new research group entered the field of corticotropin synthesis; the main object of the Hoechst investigators was the economical preparation of biologically active ACTH fragments. In the synthesis of the N-terminal

tricosapeptide amide, Geiger *et al.* [85] started from the *p*-nitrophenyl ester of the N-terminal decapeptide protected with *t*-butyloxycarbonyl and *t*-butyl ester groups [84]. The decapeptide itself was put together from the N-ter-

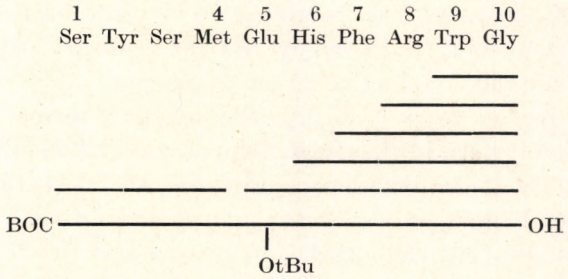


Fig. 21. Synthesis of the protected N-terminal decapeptide according to Geiger *et al.*[84]

minal tetrapeptide prepared previously [157] and from the hexapeptide containing the 5–10 amino acids, the latter having been built up in a stepwise manner (Fig. 21). In the synthesis of the C-terminal part of the tricosapeptide amide [325], the lysine ε-amino groups were protected with *t*-butyloxycarbonyl groups and the guanidino function either by nitration or merely by protonation (Fig. 22).

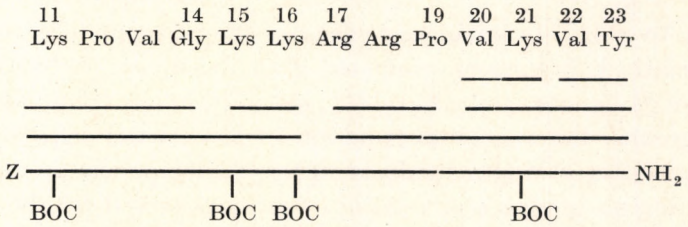


Fig. 22. Synthesis of the protected corticotropin fragment containing the 11–23 amino acids

The same research group also intended to prepare a series of N-terminal polypeptide fragments, for which the synthesis of some C-terminal components (peptide amides comprising the 11–20, 11–21, 11–23 [325] and 11–24 amino acids [324], and peptide *t*-butyl esters with the 11–20 and 11–22 sequences [325]) was described. Preparation of the planned fragments comprising the amino acids 1–20, 1–21, 1–22 and 1–24 was only mentioned in a paper [85]; experimental details were not published.

Synthesis of the tricosapeptide amide was repeated later in an alternative route [83]. This time the N-terminal decapeptide was made by acylation of the 2–10 nonapeptide with the N-terminal amino acid, hence this procedure offered an easy way to obtain corticotropin fragments with various amino acids on the N-terminus. The combination of dicyclohexylcarbodiimide with pentachlorophenol, proposed by Bajusz *et al.* [7, 10], was advantageously used for the coupling of larger fragments.

In the most recent years two Japanese research teams also published successful synthetic work in this field. Otsuka *et al.* [259] reported the synthesis of the ACTH-(1–18)-octadecapeptide and ACTH-(1–18)-octadecapeptide amide, after having published the preparation of some intermediates in preceding articles [152, 255, 258]. The synthesis of the N-terminal decapeptide was also published [260]. Selection of the fragments for the octadecapeptide synthesis was essentially the same as in previous works (Fig. 23).

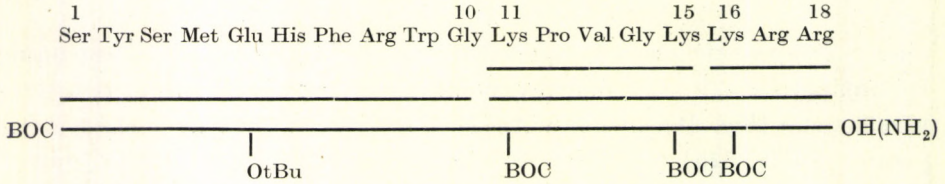


Fig. 23. Synthesis of the protected N-terminal octadecapeptide and octadecapeptide amide

Fujino *et al.* [68, 69] again synthesized the tricosapeptide amide corresponding to the N-terminal sequence of corticotropins. The route chosen showed marked differences compared with the approach by Geiger *et al.* [83, 85] or Hofmann *et al.* [142, 146], not only in the combination of protecting groups, but in the distribution of the partial sequences as well. *t*-Butyloxycarbonyl was generally used for the temporary protection of the terminal amino groups and benzyloxycarbonyl served for the lysine side chain acylation. Nitro groups protected the guanidino functions throughout the whole synthesis. Removal of all the blocking groups could be effected by the newly developed method of Sakakibara *et al.* [288], by means of acidolysis in liquid hydrogen fluoride. The outlines of the synthesis are shown in Fig. 24. Deblocking afforded the hydrofluoride which was transformed into the acetate and purified by chromatography on carboxymethylcellulose. Syntheses of the intermediates were described in a separate paper [74].

Following the successful synthesis of human ACTH [10] (p. 146), it seemed reasonable to prepare some larger fragments of this hormone, which contain shorter or longer amino acid sequences also from the species-specific part.

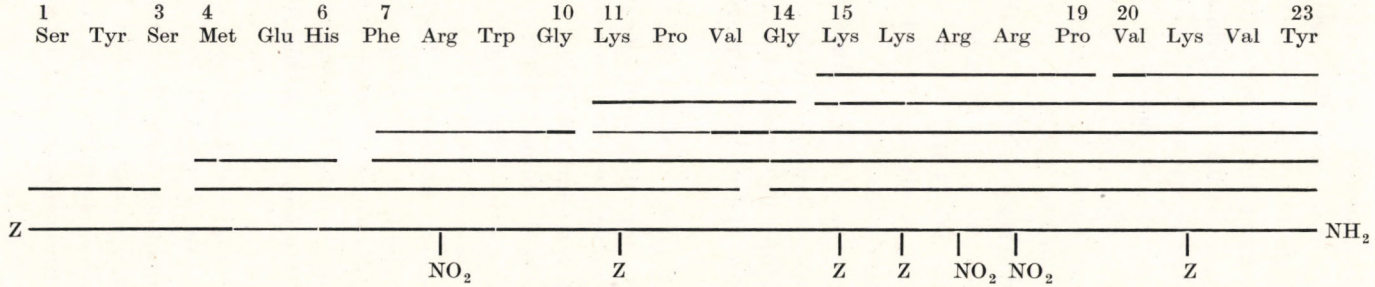


Fig. 24. Synthesis of the protected N-terminal tricosapeptide amide

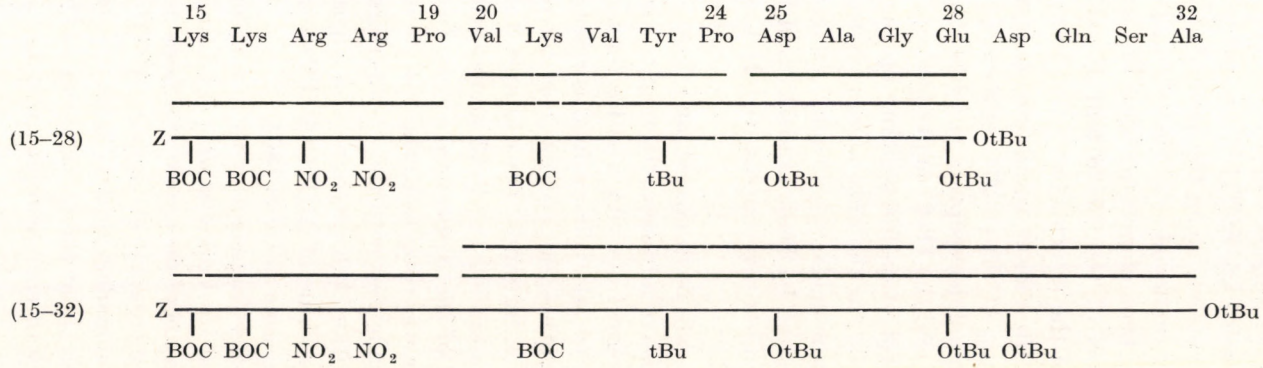


Fig. 25. Synthesis of the protected corticotropin fragments containing the 15–28 and 15–32 sequences of human ACTH

Such fragments could be used for studying the immunological properties of the human hormone in relation to its chemical structure and the possible role of the C-terminal sequence in the biological activity. Kisfaludy and Lőw [170] prepared the N-terminal peptides comprising the 1–28 and 1–32 amino acids (α_h-ACTH-(1–28)-octacosapeptide and α_h-ACTH-(1–32)-dotriakontapeptide), using the protected N-terminal tetradecapeptide [7] as the starting material. As the synthetic route of this tetradecapeptide has already been shown (p. 129), Fig. 25 illustrates only the main steps in the synthesis of the 15–28 and 15–32 C-terminal sequences. Preparation of some of the intermediates had been described earlier [10, 225].

In 1970 a short communication reported the synthesis of the 1–27 fragment (α_h-ACTH-(1–27)-heptacosapeptide). Otsuka *et al.* [262] used the N-terminal decapeptide protected with benzyloxycarbonyl and benzyl ester groups and the 11–27 heptadecapeptide with *t*-butyloxycarbonyl and *t*-butyl ester protection for building up the heptacosapeptide (Fig. 26).

Finally, as the only missing N-terminal fragments containing more than ten amino acids, the terminal undeca- and dodecapeptide amides have been synthesized by Medzihradszky and Pongrácz [236]; for this, the N-terminal decapeptide was coupled with H-Lys(BOC)-NH_2 and H-Lys(BOC)-Pro-NH_2 amides.

As it will be discussed later, importance of the integrity of the N-terminal structure of adrenocorticotropins and corticotropin fragments in the display of biological activity was for long rather questionable. In order to obtain experimental evidence in this respect, not only N-terminal analogues have been synthesized, but also corticotropin fragments with amino acids missing on the N-terminus. For example, Geiger *et al.* [87] prepared the docosapeptide containing the 2–23 amino acid residues of corticotropin, a fragment devoid of the N-terminal serine. In the course of this synthesis the 2–4 tripeptide was coupled to the 5–10 hexapeptide, and the resulting nonapeptide was condensed with the 11–23 tridecapeptide amide described earlier [325]. In addition, the synthesis of a tricosapeptide amide, lacking the N-terminal serine residue of ACTH (ACTH-(2–24)-tricosapeptide amide) was described by the same authors [86].

Further fragments with incomplete N-terminal sequence have been synthesized by Fujino *et al.* [71], using intermediates from the ACTH-(1–23)-tetracosapeptide amide synthesis [69]. Such compounds were the ACTH-(4–23)-eicosapeptide amide, ACTH-(5–23)-nonadecapeptide amide, ACTH-(6–24)-nonadecapeptide and ACTH-(7–23)-heptadecapeptide amide. Conclusions about the contribution of the terminal amino acids to the biological activity of corticotropins will also be discussed later.

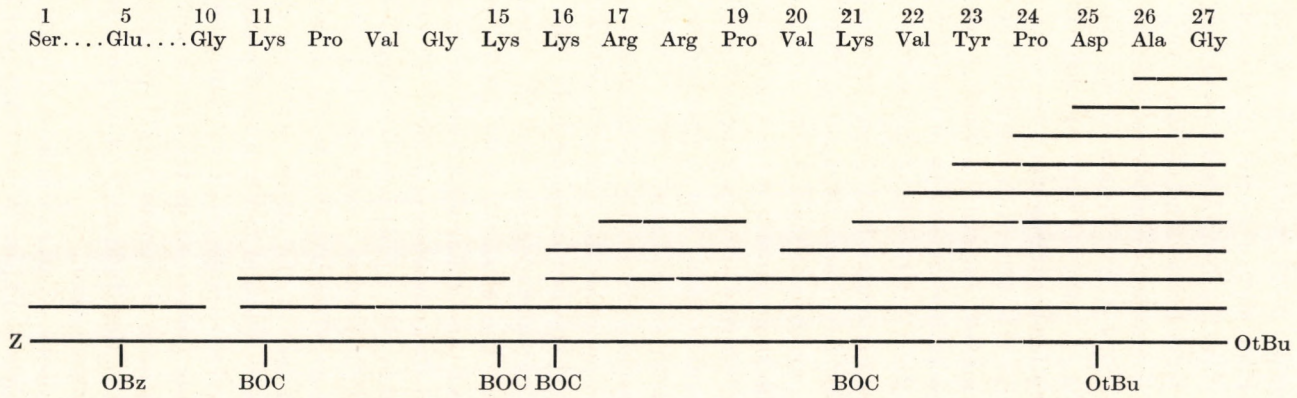


Fig. 26. Synthesis of the protected N-terminal heptacosapeptide of human ACTH

Two protected peptide fragments derived from other parts of corticotropin should be mentioned briefly, a heptapeptide ester H-Glu(OEt)-Ala--Phe-Pro-Leu-Glu(OEt)-Phe-OMe of Oertel [251], and an undecapeptide H-Pro-Val-Gly-Lys(Tos)-Lys(Tos)-Arg-Arg-Pro-Val-Lys(Tos)-Val-OMe (sequence 12–22) prepared by Schröder and Gibian [294].

Fragments with even fewer amino acid residues have been synthesized by various authors (see [295] p. 239 for a review). None of these peptides has ever been used in the synthesis of larger biologically active molecules.

Recent successes in peptide synthesis using the solid-phase method [241] led Li *et al.* to investigate this procedure for the preparation of peptides related to pituitary hormones. Selecting a shorter part of corticotropins, which was believed to be rather critical from the synthetic point of view, they synthesized the Met-Glu-His-Phe-Arg-Trp-Gly heptapeptide [18], whose synthesis *via* classical routes had been described previously [219]. After cleavage from the resin and deblocking ($Na—NH_3$), the purified product was obtained in an overall yield of 18%.

Blake *et al.* [22] in the same laboratory applied the solid-phase method for the synthesis of the N-terminal nonadecapeptide and of two analogues thereof, in which the peptide chain was lengthened on the N-terminus by a proline and an alanine residue, respectively. Compounds of this kind, slightly differing from each other on the N-terminus, can advantageously be built up on the solid support, as the synthetic pathway branches off from a common intermediate in the last coupling only. The chemical and biological properties of the nonadecapeptide were the same as those shown by the product prepared by the classical route [205]. In addition, Li and Hemmasi [211] described the similar synthesis of the glutamine analogue — [$Glu(NH_2)^5$]-ACTH-(1–19)-nonadecapeptide — by coupling the nonapeptide H-Lys(Tos)-Pro-Val-Gly-Lys(Tos)-Lys(Tos)-Arg(Tos)-Arg(Tos)-Pro-OH with the decapeptide hydrazide BOC-Ser(Bzl)-Tyr(Bzl)-Ser(Bzl)-Met--Glu(NH_2)-His(Bzl)-Phe-Arg(Tos)-Trp-Gly-N_2H_3. As it is seen both intermediates are fully protected in the amino acid side chain, which is a requirement for unequivocal reaction route in solid-phase peptide synthesis. Preparation of the protected decapeptide was published separately [212]. These successful syntheses performed in the San Francisco laboratory (see also the solid-phase synthesis of human ACTH, p. 149) clearly demonstrated the applicability of the Merrifield method for preparing polypeptide hormones of rather complex structure.

For methodological reasons, as an example of peptide fragment condensation on the polymer support, Yajima *et al.* [352] prepared the 5–14 decapeptide of ACTH. The 11–14 tetrapeptide, protected on the amino groups

(BOC-Lys(Z)-Pro-Val-Gly-OH [74]) was coupled to the chloromethylated polymer; removal of the *t*-butyloxycarbonyl was followed by acylation with the hexapeptide Z-Glu(OBz)-His-Phe-Arg(NO_2)-Trp-Gly-OH. The free decapeptide was obtained after the necessary cleavages and purification in a yield of 40%, which was much lower than expected. Yet, solid-phase fragment condensation offers some advantages such as easy removal of side reaction products (acylurea). N-terminal corticotropin fragments synthesized thus far are summarized on the following pages.

Synthetic Corticotropin Fragments

Peptide	Year of publication	References
ACTH-(1–10)-decapeptide	1961	[140, 301]
	1966	[260]
ACTH-(1–11)-undecapeptide amide	1975	[236]
ACTH-(1–12)-dodecapeptide amide	1975	[236]
ACTH-(1–13)-tridecapeptide amide	1961	[105, 140]
ACTH-(1–14)-tetradecapeptide	1975	[231]
ACTH-(1–14)-tetradecapeptide amide	1966	[7]
ACTH-(1–15)-pentadecapeptide amide	1966	[7]
ACTH-(1–16)-hexadecapeptide	1962	[147]
ACTH-(1–16)-hexadecapeptide methyl ester	1962	[307]
ACTH-(1–16)-hexadecapeptide amide	1960	[121]
	1966	[7]
ACTH-(1–17)-heptadecapeptide	1962	[206]
	1964	[217]
ACTH-(1–17)-heptadecapeptide amide	1965	[271]
	1966	[7]
ACTH-(1–18)-octadecapeptide	1965	[259]
ACTH-(1–18)-octadecapeptide amide	1965	[259, 271]
	1966	[7]
ACTH-(1–19)-nonadecapeptide	1960	[213]
	1961	[214, 300]
	1963	[41]
	1964	[205]
	1972	[22]
ACTH-(1–19)-nonadecapeptide amide	1965	[271]
	1966	[7]
[Glu(NH_2)^5]-ACTH-(1–19)-nonadecapeptide	1960	[308]
	1961	[214]
	1972	[211]
ACTH-(1–20)-eicosapeptide	1964	[85]
ACTH-(1–20)-eicosapeptide amide	1962	[129, 143]
	1964	[85]
	1966	[7]

Synthetic Corticotropin Fragments *(cont.)*

Peptide	Year of publication	References
ACTH-(1–20)-eicosapeptide methyl ester	1956	[30]
ACTH-(1–21)-heneicosapeptide amide	1964	[85]
	1966	[7]
ACTH-(1–22)-docosapeptide	1964	[85]
ACTH-(1–23)-tricosapeptide	1962	[142]
ACTH-(1–23)-tricosapeptide amide*	1961	[146]
ACTH-(1–23)-tricosapeptide amide	1964	[85]
	1969	[68, 83]
	1970	[69]
ACTH-(2–23)-docosapeptide	1964	[87]
ACTH-(4–23)-eicosapeptide amide	1971	[71]
ACTH-(5–23)-nonadecapeptide amide	1971	[71]
ACTH-(7–23)-heptadecapeptide amide	1971	[71]
ACTH-(1–24)-tetracosapeptide	1961	[164]
	1962	[297]
	1963	[302]
ACTH-(1–24)-tetracosapeptide amide	1964	[85]
ACTH-(2–24)-tricosapeptide amide	1968	[86]
ACTH-(6–24)-nonadecapeptide	1971	[71]
α_b-ACTH-(1–26)-hexacosapeptide**	1965	[275]
α_h-ACTH-(1–27)-heptacosapeptide†	1970	[262]
α_p-ACTH-(1–28)-octacosapeptide$^+$	1962	[33]
	1966	[7, 32, 230]
	1975	[2]
α_h-ACTH-(1–28)-octacosapeptide‡	1968	[170]
α_h-ACTH-(1–32)-dotriacontapeptide§	1968	[170]

* Later [142] demonstrated to be the free tricosapeptide.

** According to the recently revised structures this compound represents the α_s-ACTH-(1–26)-hexacosapeptide.

† Correctly [Asp^{25},Ala^{26},Gly^{27}]-α_h-ACTH-(1–27)-heptacosapeptide.

$^+$ Corresponding to the structure determined by Shepherd *et al.* [317]. The correct abbreviation: [Asp^{25}]-α_p-ACTH-(1–28)-octacosapeptide.

‡ Correctly [Asp^{25},Ala^{26},Gly^{27}]-α_h-ACTH-(1–28)-octacosapeptide.

§ Correctly [Asp^{25},Ala^{26},Gly^{27},Glu(NH$_2$)^{30}]-α_h-ACTH-(1–32)-dotriacontapeptide.

3. TOTAL SYNTHESIS OF PORCINE AND HUMAN CORTICOTROPIN

Experiences gained during the syntheses of smaller corticotropin fragments made possible for two research groups to succeed in synthesizing the complete ACTH molecule composed of 39 amino acids. In 1963 Schwyzer

and Sieber [311] reported the total synthesis of the porcine corticotropin and in 1967 Bajusz *et al.* [10] that of the human hormone.

Syntheses of the complete native hormones were justified for several reasons. First, biological reactions, such as hormone–receptor interactions, certainly occur according to molecular mechanism on the molecular level. A hormone fragment having full activity on weight basis, but only half the molecular weight of the parent compound, possesses actually only half the activity of the native hormone on molar basis. In other words, it is only the native hormones with complete structure which are really fully active in biological reactions. Second, the biological role of the C-terminal part of corticotropins was largely unknown in 1963 as well as in 1967, as it is more or less so even today. However, the fact that this part of the molecule contains the different and species-specific sequence obviously shows the importance of the C-terminal part in eliciting immunological properties. Furthermore, application of the human adrenocorticotropic hormone for therapeutical purposes seems to be advantageous whenever hypersensitivity against corticotropins of animal origin can be detected [175].

Experimental details of the full synthesis of porcine corticotropin were published by Schwyzer and Sieber in 1966 [312]. The pentadecapeptide 25–39 was synthesized by stepwise elongation of the peptide chain from the C-terminal glutamic acid. Permanent protection of side chain carboxyl was provided by *t*-butyl ester groups; temporary protection of the α-amino groups was ensured by benzyloxycarbonyl. The individual amino acids were coupled to the growing peptide chain through their *p*-nitrophenyl ester derivatives. Decarbobenzoxylation by catalytic hydrogenolysis gave quantitative yields in most steps, and the overall yield of the 28-step synthesis was a remarkable 6.85%.

For the synthesis of the whole molecule this pentadecapeptide was first acylated by the 17–24 octapeptide, the resulting tricosapeptide then again with the 11–16 hexapeptide, and the synthetic route was completed by coupling with the protected N-terminal decapeptide, according to the scheme in Fig. 27. The above distribution of peptide fragments assured a racemization-free synthesis, as the C-terminal amino acids in peptides 1–10 and 17–24 are glycine and proline, respectively. The peptide bond between the 16 and 17 amino acid residues was formed by the azide procedure. The optical purity of the resulting free nonatriacontapeptide was checked by careful physical investigations and by comparison with the native porcine adrenocorticotropic hormone.

The key intermediate in the human corticotropin synthesis was the same protected N-terminal tetradecapeptide which had already been of great

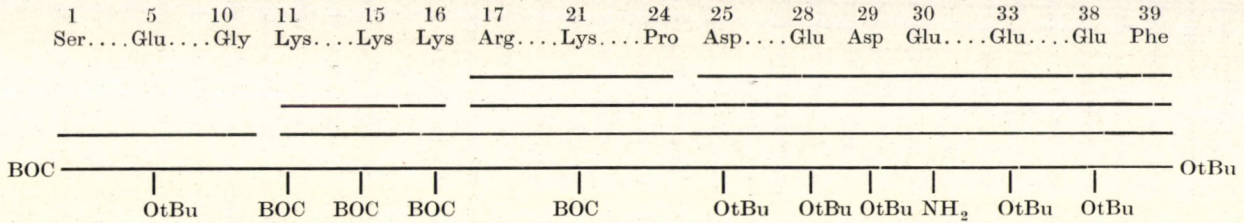


Fig. 27. The main steps of the synthesis of porcine corticotropin

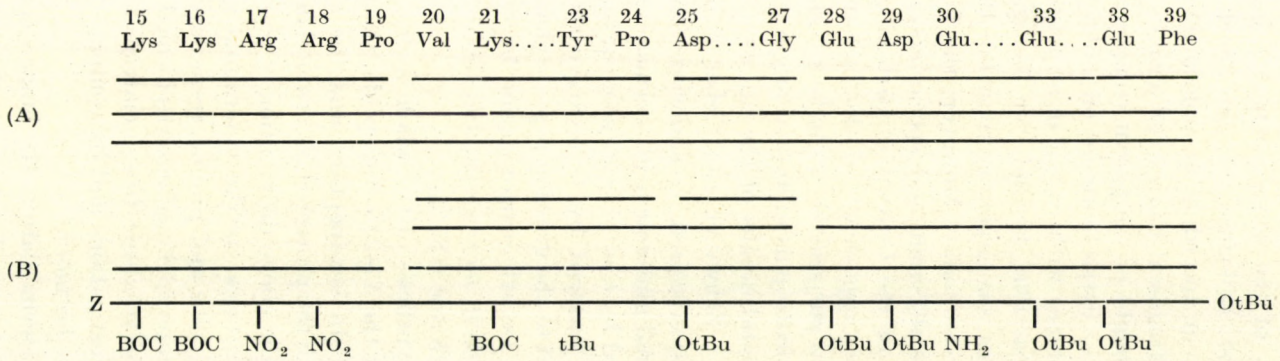


Fig. 28. Alternative routes for the synthesis of the protected C-terminal pentacosapeptide of human corticotropin

value in the preparation of biologically active N-terminal fragments [7, 32, 231]. Bajusz *et al.* [9, 10] synthesized the 15–39 pentacosapeptide in two alternative ways. Route (A) is characterized by acylationof the C-terminal 28–39 fragment with the 25–27 tripeptide, followed by coupling with the decapeptide comprising the 15–24 amino acids, which itself has been synthesized from two pentapeptides containing the 15–19 and 20–24 amino acid residues. In Route (B), first the octapeptide 20–27 was prepared from the intermediates mentioned above and was subsequently lengthened on both sides with the remaining sequences to give the desired pentacosapeptide. These steps are outlined in Fig. 28. The C-terminal dodecapeptide containing the 28—39 amino acid residues was synthesized by fragment condensation

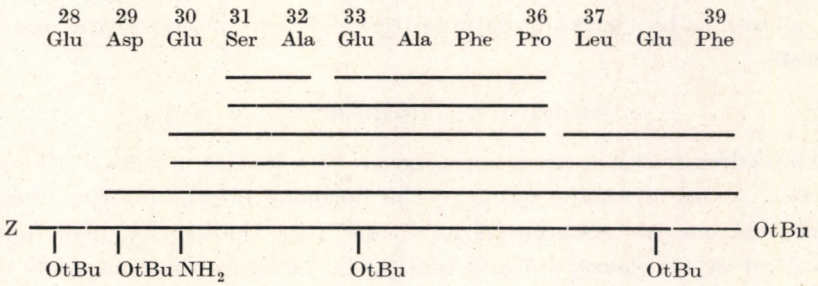


Fig. 29. Synthesis of the protected C-terminal dodecapeptide of human ACTH

as shown in Fig. 29 [9], some of the intermediates having been described earlier [3].

Combination of the protecting groups or the coupling methods did not differ significantly from those applied in the porcine corticotropin synthesis. These hormones containing 39 amino acids were at that time the largest native polypeptide molecules obtained by chemical synthesis.

As it was mentioned in Section II. 1. dealing with the structure elucidation of corticotropins, at about the same time Hungarian and Swiss authors published some observations concerning the primary structures of porcine and human ACTH. According to these results it seemed to be very likely that both hormones contained an Asn-Gly amino acid sequence in the 25–26 positions. Synthesis of human ACTH with the new structure proposed by Riniker *et al.* [278] was independently achieved soon by both the Swiss and Hungarian research groups [173, 320]. The methods applied in the course of this work did not differ essentially from those used in previous corticotropin syntheses.

Most recently, in the framework of a program for the solid-phase synthesis of polypeptide hormones, Yamashiro and Li [365] published the Merrifield

synthesis of the human ACTH of revised structure. Experimental details were similar to those used in other solid-phase syntheses from this laboratory, except that the C-terminal phenylalanine was coupled to the chloromethylated polymer through its tetramethylammonium salt, and the optical purity of phenylalanine attached in this manner had been checked before continuing the lengthening of the peptide chain. The synthesis was carried out in a peptide synthesizer, and not only a very detailed operation schedule, but also the careful characterization and comparison of the synthetic material with the native hormone have been described. The biological activity of the synthetic product will be discussed later (p. 156); the identity of the synthetic and natural hormones was proved by circular dichroism spectra and optical rotatory dispersion, paper and polyacrylamide gel electrophoresis, as well as by electrophoretic patterns of both chymotryptic and tryptic digests.

4. BIOLOGICAL PROPERTIES OF ACTH

The adrenocorticotropic hormone is best known for its action on the adrenal gland, even the name of the hormone originates from this major biological activity. As pointed out by Li [203], the variety of the biological effects of ACTH clearly indicate that the hormone does not act solely through the mediation of the adrenal cortex. Its adrenal and extra-adrenal functions were summarized and briefly reviewed by Li [203], Lebovitz and Engel [185], Engel [62], and by Ramachandran and Li [276]. It is not the task of the present paper to give a detailed account on these biological properties. Some of them, however, deserve special interest for they have widely been used as a measure of the biological potency of corticotropins and derivatives. The most remarkable properties of ACTH which proved to be the best for delineating biologically important chemical features of the hormone are the corticotropic, melanocyte-stimulating and lipid-mobilizing activities.

In the course of the isolation, structure determination and synthesis of ACTH, several methods have been developed for determining its corticotropic activity. Sayers *et al.* [289] were the first in elaborating a rapid and reliable assay on the basis that adrenal ascorbic acid is depleted upon administration of the hormone, and this assay proved to be very useful in measuring the ACTH content of pituitary extracts. On the other hand, this procedure is of limited value when comparing the biological activities of different fragments with that of the intact hormone. The reason is that our knowledge on the relation between adrenal ascorbic acid depletion and steroidogenesis is insufficient. In addition, ascorbic acid depletion is considerably dependent on the means of administration (intravenous or sub-

cutaneous), and since even this dependence can become reversed when testing different fragments or intact corticotropin, there have been significant controversies among the different research groups. For example, investigating the biological properties of ACTH-(1–19)-nonadecapeptide, Li *et al.* [203, 214] found 35 U/mg activity on intravenous and 74 U/mg activity on subcutaneous application (s.c./i.v. ratio: 2). In contrast, Hofmann *et al.* observed that the eicosapeptide amide corresponding to positions 1–20 of the corticotropin molecule and exhibiting a high level of adrenal ascorbic acid depleting potency (111 U/mg on intravenous application) possesses a s.c./i.v. ratio of 0.3 [143]. They also found that the same ratio existed when the corticotropic activity of the ACTH-(1–23)-tricosapeptide was measured, which had a potency of 116 U/mg on intravenous and a mere 38–43 U/mg activity on subcutaneous administration [142]. Since natural ACTH was known to possess a threefold potency when given subcutaneously as compared with the intravenous application (s.c./i.v. ratio: 3) [65, 120] Hofmann suggested that low s.c./i.v. ratios were a characteristic property of adrenocorticotropically active, chemically defined subunits of the ACTH molecule, and for the change in the ratio the C-terminal 23–39 sequence might be responsible. For the controversy between the findings of Li and Hofmann there is no explanation so far. It can be concluded, however, that for proper comparison of different corticotropically active compounds a far-reaching identity in the assay procedure is imperative.

As a more direct measurement of the corticotropic activity, the *in vitro* procedure of Saffran and Schally [287] is perhaps more suitable for investigation of the structure–effect relationships. In this method the corticosteroid production of isolated adrenal tissue under stimulation of the polypeptide hormone is quantitatively measured. Even more accurate is the estimation of corticosteroids after the *in vivo* application of the hormone [101], since in this case the hormone to be investigated undergoes similar conditions and intermediary processes as does the native hormone in natural circumstances. The most reliable method is a modification of the latter procedure, a very sensitive biological assay, which has been developed by Lipscomb and Nelson [221]. According to this method free corticosterone in the adrenal venous plasma is measured shortly after the administration of the hormone.

In agreement with literature data, Szporny *et al.* [326] found that comparison of various synthetic corticotropin fragments with natural ACTH involves some difficulties. For example, there are differences in the time of the peak of blood corticosterone level and in the duration of action. This finding can most probably be explained by differences in the absorption and inactivation rates of synthetic peptides and the native hormone.

Szporny *et al.* developed a new method by which a continuous corticosterone measurement in rats is possible, covering the whole period of blood corticosterone elevation. On the basis of this method valuable conclusions could be drawn concerning the role of the C-terminal sequence of corticotropins in their biological activity [8].

Among the extra-adrenal effects of corticotropins at least two can be found that are suitable for following the changes in activity which occur on changing the molecular structure. These effects are elicited not only by ACTH, but by a number of other polypeptide hormones containing common partial amino acid sequences (melanocyte-stimulating activity) or by a series of hormones with quite different structure (lipid-mobilizing activity).

Melanocyte-stimulating activity is measured *in vivo* in intact frogs (mostly in *Rana pipiens*) according to the procedure of Hogben and Slome [148]. Upon administration of the hormone the dermal melanocytes — the melanin-forming cells in the skin — become dark, in consequence of the dispersion of melanin granules throughout the cytoplasm. This dispersion can be followed by microscopic observation and its different stages are characterized by the arbitrary Hogben index. As separation of the effects of injected melanotropin from the endogeneous hormone in the intact frog is difficult, unequivocal results can be obtained in hypophysectomized animals [182]. The activity is usually expressed in terms of the minimum dose required to produce a significant change in the Hogben index within a definite period of time.

In 1954 an *in vitro* quantitative bioassay for the melanocyte-stimulating hormone was described ([319]; see also [199]). This method depends upon the changes in light reflection from isolated frog skin before and after immersion in solutions containing melanotropic substances. Decrease in reflectance, i.e. darkening can be measured photometrically. This method is much more rapid and easier to carry out than the *in vivo* assay.

Melanotropic activity is generally expressed as units per gram substance. One unit of MSH is defined as the amount which produces a darkening equivalent to that produced by 0.04 μg of a standard lyophilisate, which is obtained from 2.0 g of acetone-dried bovine posterior pituitary powder (Armour) by shaking for 1 hour in 200 ml of water, centrifugation and lyophilisation of the supernatant. 0.3 units per ml produce measurable darkening of the isolated frog skin.

Of the extra-adrenal effects of adrenocorticotropic hormone, the *in vitro* release from rat, rabbit or mouse adipose tissue of free fatty acids, and the *in vivo* adipokinetic effects in mice were also studied in respect to the chemical features of the hormone. The *in vitro* procedure is much more simple

and therefore more often used; slices of the susceptible adipose tissue incubated with the adipokinetic substance release free fatty acids and glycerol into the medium, where both can be quantitatively measured [341, 342]. Relative potencies of the lipid-mobilizing substances are expressed as the minimal effective dose which causes a statistically significant rise in free fatty acid content in excess of that released by saline-treated controls.

The survey of data concerning biological activities of natural corticotropins is fairly difficult. This is due primarily to the fact that the various research groups were able to isolate from the hypophysis of the same species more than one corticotropic substances, most of them differing only in their amide contents. In addition, different biological assays are also responsible for divergencies in estimation of the biological potencies. Some directions in this respect can be found in reviews compiled by Hofmann [122] and Schröder and Lübke (p. 196 in [295]).

The specific activites of the main components of corticotropic substances measured by the *in vitro* steroidogenesis assay method of Saffran and Schally [287] were summarized by Li [203] (Table I).

Table I

Biological Activity of Natural Corticotropins

Peptide	*In vitro* steroidogenesis, U/mg	*In vivo* ascorbic acid depletion, U/mg
α_h-ACTH	52	26 [190]
α_p-ACTH	150, 95 [100]	100–150 [343], 80–100 [316]
α_s-ACTH	177	150 [209], 100 [285]
α_h-ACTH	140	

Activities determined by the *in vivo* ascorbic acid depletion assay gave similar results. As it is seen from Table I, all corticotropins gave comparable potencies in both assays with the only exception of the human hormone. Li supposed that this could be a consequence of different structures, while Hofmann stated [122] that the relatively minor variations between the structures of human and animal hormones failed to provide a satisfactory explanation for the significant differences in corticotropic activity. In his opinion the reason might lie in some indetectable contamination (e.g. methionine S-oxide derivative) from the isolation process. Hofmann's prediction was later justified by the preparation of a pure sample of human

ACTH in 1962 by Li *et al.* (cited in [215]) which possessed an activity of about 130 U/mg, measured by both *in vitro* and *in vivo* assays.

In order to obtain a reliable and meaningful picture on the biological activity of natural and synthetic corticotropins of different origin, Ney *et al.* [247] determined their *in vivo* steroidogenic potencies using the assay procedure of Lipscomb and Nelson [221] (Table II).

Table II

In vivo Steroidogenic Activity of Natural Corticotropins

Peptide	*In vivo* steroidogenesis, U/mg
α_b-ACTH	140
α_s-ACTH	106
α_h-ACTH	133
α_p-ACTH	85
α_p-ACTH (synth.)	90

Supposing that the corticotropins measured were all chemically homogeneous, pure products, the activity values ranging from 85 to 140 U/mg, corresponding to activities of about 400 and 600 units per micromole, although comparable, yet reflect some effects of the C-terminal structure on the corticotropic activity. Further careful investigations are needed to clarify this point. Molar activities of the synthetic human ACTH and its fragments revealed in different tests will be presented later (p. 178) and the possible role of the C-terminal part of the molecule will be discussed.

The melanocyte-stimulating activities of natural corticotropins are identical within the limits of error, corresponding to a value of about 1×10^8 MSH units per gram hormone. These activities were compiled by Hofmann [122] and measured again by Ney *et al.* [247] in their comprehensive program striving for reliable comparison. The results are shown in Table III. These values are by two orders of magnitude lower than the potency of α-melanotropin, the most active melanotropic agent. For the sheep ACTH Lohmar and Li [223] reported an activity of 4×10^7 U/g which is practically the same as given in Table III.

The extra-adrenal lipolytic potencies of corticotropins are also very similar, when tested in a given species. Nevertheless, adipose tissues of different species exhibit marked differences in sensitivity against lipolytic substances and even the seasonal variation can be significant: it is known from the

Table III

Melanocyte-stimulating Activity of Natural Corticotropins

Peptide	*In vitro* activity, MSH U/g	
	Hofmann	Ney
α_p-ACTH	1.7×10^8	1.3×10^8
α_s-ACTH	0.6×10^8	1.0×10^8
α_b-ACTH	0.5×10^8	0.5×10^8 [59]
α_h-ACTH	0.4×10^8	1.2×10^8

experiments of Lebovitz and Engel [185] that the minimum dose that gives a measurable effect may vary by as much as 20-fold. The following data can be compiled from the literature:

Table IV

Lipolytic Activity of Natural Corticotropins

Peptide	Minimal effective dose (μg/ml)		
	Rabbit	Rat	Mouse
α_s-ACTH	0.008 [223]	0.001 [223]	0.1–1 [223]
	0.0064 [330]	0.057 [330]	
α_p-ACTH		0.001 [149]	
α_h-ACTH (synth.)	0.002 [49]	0.002 [49]	

Biological activities of synthetic corticotropins can be summarized as follows. The activity of the synthetic porcine hormone [311, 312] was investigated by Barthe *et al.* [12] in various experimental assay procedures; the Third International Standard was used for comparison. From the adrenal ascorbic acid depletion assay this synthetic hormone was estimated to have a potency of 115 U/mg. Secretion of corticosteroids from the adrenal gland was measured *in vivo* and *in vitro*; the effects of the synthetic peptide were similar to those of the natural hormone as can be judged from graphical illustrations. The melanophore-stimulating activity of the synthetic product was also identical with that of the standard.

Synthetic human ACTH [10] was measured using both the adrenal ascorbic acid depletion method of Sayers [289] modified by Hamburger [112] and the *in vitro* steroidogenesis assay [287]; 109 and 107 U/mg were found, respectively [327, 328]. This is in good agreement with the activity of the

natural hormone. Assay of potency based on elevation of the corticosterone level in the peripheral blood of rat showed a similar activity (106 U/mg i.v. and 108 U/mg s.c.) [327].

The *in vivo* steroidogenic activity [336] of synthetic human ACTH obtained by the solid-phase method [365] did not differ significantly from that of the natural hormone (140 and 156 U/mg, respectively). They were also identical in their lipolytic effects in isolated rat or rabbit fat cells, the concentrations at one-half maximal response being 10 and 40 ng/ml.

The biological activity of the synthetic human corticotropin with revised amino acid sequence was determined by Schenkel–Hulliger *et al.* [291] and found to be 188 U/mg in the *in vitro* steroidogenesis assay. This value, as well as the melanocyte-stimulating and lipolytic activities did not differ from those shown by the synthetic porcine ACTH, indicating that the amino acid sequence 25–31, where the species difference resides, does not influence these biological properties.

III. MELANOCYTE-STIMULATING HORMONES

1. STRUCTURE

Several polypeptide hormones can be found in the pituitary which all possess a characteristic biological activity, the ability to darken the skin of some amphibians resulting from stimulation of melanocytes. Besides the adrenocorticotropic hormone such a principle is the melanocyte-stimulating hormone or melanotropin,* named just after this peculiar property.

Two kinds of melanotropins could be isolated from the hypophyses of various species, α-MSH, which has a common structure irrespective of its origin, and β-melanotropins, having an amino acid sequence characteristic of the species serving as the source of isolation, and possessing at the same time some structural features common with α-MSH and with the corticotropins as well.

The structure of α-melanotropin determined by Harris and Lee [116, 118] (porcine), Li [202] (bovine), Dixon and Li [58] (equine) and Lee *et al.* [191]

* This hormone family has had several different names during the last decades. As their main source is the intermediary lobe of pituitary (pars intermedia), Zondek and Krohn [367] called them intermedins. These hormones, however, can also be found in the anterior and posterior lobes; in fact, in several species, where the intermediary lobe cannot be distinguished (e.g. whale, chicken) they can be isolated only from other parts of the hypophysis.

Accordingly, it seemed more consequent to name them after their characteristic activity, thus the names melanocyte-stimulating hormone (MSH) (Lerner *et al.* [198]) and melanotropin (Li [201]) have widely been accepted and will be used throughout this review.

Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

Fig. 30. Structure of α-melanotropin

Human	H-Ala-Glu-Lys-Lys-Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH
Bovine, Ovine	H-Asp-Ser-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH
Porcine, Ovine	H-Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH
Equine	H-Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Arg-Lys-Asp-OH
Monkey	H-Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH

Fig. 31. Structure of the β-melanotropins

H-(Tyr)-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Met-OH(-NH_2)

Fig. 32. Structure of a melanocyte-stimulating hormone from the dogfish *Squalus acanthias*

(monkey) is shown in Fig. 30. It is most peculiar that although the presence of α-melanotropin in human pituitary has been demonstrated with great probability, it has never been isolated in pure state and thus, its actual structure is unknown [191].

Recent investigations by Lee *et al.* [188] led to the isolation from human pituitary of a peptide containing the entire sequence of α-MSH and six additional amino acids. As the primary structure of this peptide is unknown, it cannot be decided whether this compound belongs to the α- or β-type of the melanocyte-stimulating hormones.

The structure of β-MSH of porcine origin was determined by Harris and Roos [119] and by Geschwind *et al.* [90], that of the bovine β-melanotropin by Geschwind *et al.* [91], the equine by Dixon and Li [59], the monkey by Lee *et al.* [191], and the structure of the human hormone by Harris [115]. Interestingly enough the sheep hypophysis contains, in addition to ACTH, three melanotropic substances: α-MSH and two β-melanotropins, the latter having structures identical with those of the bovine and porcine hormones [88]. Accordingly, ovine β-MSH with a sequence characteristic to the species does not exist. The structures of the known β-melanotropins are summarized in Fig. 31.

There is only one known melanotropin whose structure does not fit into the scheme given above and perhaps resembles rather α-MSH; this is the melanotropic principle isolated from the pituitary of dogfish *(Squalus acanthias)* [224] (Fig. 32). According to Lowry and Chadwick this hormone is a mixture of polypeptides, one of them containing an amide group at the C-terminus, an other containing an additional tyrosine residue on the N-terminal end.

It should be mentioned here that according to recent investigations by Scott and Lowry [313] neither α-MSH nor β-MSH exists in the human organism. Human β-MSH isolated previously seems to be an artefact produced by partial enzymic cleavage of the β-lipotropin molecule during the isolation process.

2. SYNTHESIS OF α-MELANOTROPIN

The first synthesis of α-melanotropin was described by Guttmann and Boissonnas [104]. The protected tridecapeptide has been condensed from the N-terminal pentapeptide [103] and the C-terminal octapeptide [24] by means of the dicyclohexylcarbodiimide technique. Partial fragments and the protected tridecapeptide were synthesized by the following route (Fig. 33). In the course of the synthesis of intermediates trityl protection and dicyclohexylcarbodiimide as the condensing agent were extensively used.

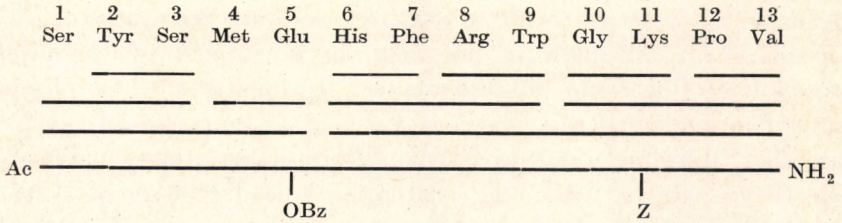


Fig. 33. Synthesis of the protected α-melanotropin according to Guttmann and Boissonnas [104]

Since the presence of the methionine residue made the removal of the protecting groups by catalytic hydrogenolysis or by reduction with sodium in liquid ammonia difficult, the authors elaborated a new deblocking method. Among the various conditions tested, hydrogen bromide in trifluoroacetic acid at −5°C proved to be the best, and side reactions were prevented by the addition to the reaction mixture of methyl ethyl sulfide and diethyl phosphite. A pure product was obtained after countercurrent distribution and preparative paper electrophoresis.

Based on the experience that carbodiimide coupling is very often accompanied by partial racemization, Hofmann *et al.* [145] disputed the optical purity of the product. Although the biological activity of the synthetic α-melanotropin was identical with that of the native hormone, this result still did not exclude such a possibility (*cf.* Section V. 3. p. 212).

Several years later Schwyzer *et al.* also described two new syntheses for α-melanotropin [297, 298], using protecting groups easily removable by acidolysis and hydrazinolysis. Some peptide intermediates already used in corticotropin syntheses could also be adapted to the preparation of α-melanotropin; the last steps of the synthesis are shown in Fig. 34.

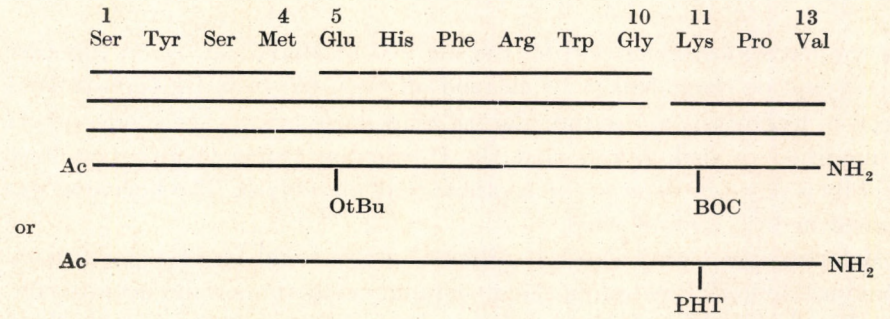


Fig. 34. Synthesis of the protected α-melanotropin according to Schwyzer *et al.* [298]

The successful application of the phthalyl group for the protection of the lysine side chain demonstrated not only the advantages of this unjustly neglected protecting group, but also offered a good opportunity for obtaining melanotropin analogues differing only in the acyl functions on the N-terminus. The N-terminal *t*-butyloxycarbonyl protecting group could namely be selectively removed from the ε-phthalyl-lysine derivative allowing the acylation of the N-terminus with various acyl groups for studying their effects on the melanocyte-stimulating activity. Some results of these investigations will be discussed in the Chapter dealing with the structure–activity relationships of melanotropins.

In 1968 a new synthesis for α-MSH was described by Yajima *et al.* [351]. Although they used the same N-terminal Ac-Ser-Tyr-Ser-Met-N_2H_3 tetrapeptide hydrazide as Schwyzer *et al.*, they prepared it in an alternative route, and coupled the azide directly with the C-terminal nonapeptide containing the 5–13 amino acid residues. Formyl group served for protection of the lysine ε-amino function, and it could be preferably removed by treatment with hydrazine acetate. The synthetic route is outlined in Fig. 35. Finally, the successful synthesis of α-melanotropin on solid phase deserves mentioning: Blake *et al.* [16] synthesized first the [Glu(NH_2)^5]-α-melanotropin and

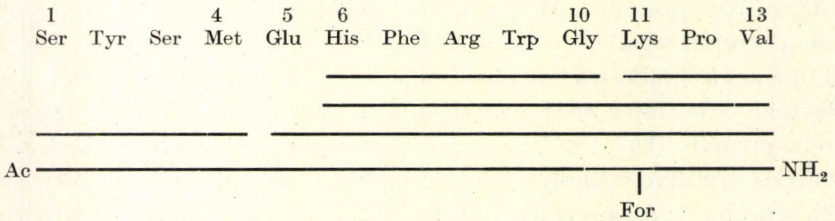


Fig. 35. Synthesis of the protected α-melanotropin according to Yajima *et al.* [351]

later the native hormone [19] by the method of Merrifield. As in this case the polypeptide chain had to be cleaved off from the resin by ammonolysis instead of hydrolysis to produce the tridecapeptide amide, the solid support was modified in such a way that the C-terminal valine residue had been bound by a phenolic ester group to allow easy aminolysis. The hormone was obtained in 10% overall yield.

α-Melanotropin, synthesized in different ways could be best purified on carboxymethylcellulose ion-exchange column using ammonium acetate concentration and pH gradient for elution. The pure hormone emerges from the column with a 0.2 molar buffer of pH 6.7.

3. SYNTHESIS OF β-MELANOTROPINS

The first attempts to synthesize bovine β-MSH were made by Schwyzer *et al.* [158, 303]. Having established that the natural hormone survives the treatment with sodium in liquid ammonia with only minor decrease in biological potency, Schwyzer *et al.* selected benzyloxycarbonyl and tosyl protection for the basic side chains, and elaborated a synthetic route shown in Fig. 36.

Contrary to the expectations, the protecting groups could not be split off without significant destruction of the peptide chain. Schwyzer *et al.* [299], therefore, repeated the synthesis using blocking groups derived from *t*-butanol. The fragment distribution was the same as represented in Fig. 36; the structure of the protected octadecapeptide is shown in Fig. 37.

Preliminary data on the synthesis of monkey β-melanocyte-stimulating hormone were reported by Yajima *et al.* [362], and the synthesis of the human β-MSH was also announced by the same research group [350]. In the meantime, in a lecture on the International Symposium on the Pharmacology of Hormone Polypeptides in Milan Rittel also presented a synthesis of the human hormone [280].

As a common intermediate for the synthesis of both hormones Yajima *et al.* [364] prepared the C-terminal decapeptide according to Fig. 38. The lysine ε-amino groups were blocked by formyl, whereas the arginine guanidino functions by nitro group.

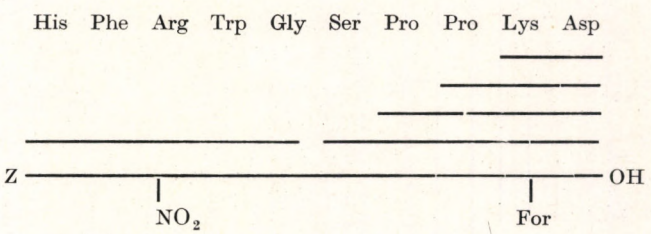


Fig. 38. Synthesis of the protected C-terminal decapeptide of β-melanotropins

Formylation rendered the protected compound water-soluble, thus purification could be effected by ion-exchange chromatography in aqueous solution. Preparation of the N-terminal half of this decapeptide had been described earlier by Hofmann and Lande [127].

Removal of the benzyloxycarbonyl and nitro groups was followed by elongation of the chain on the amino terminal group *via* a stepwise procedure [361] resulting in a pentadecapeptide common in both human and

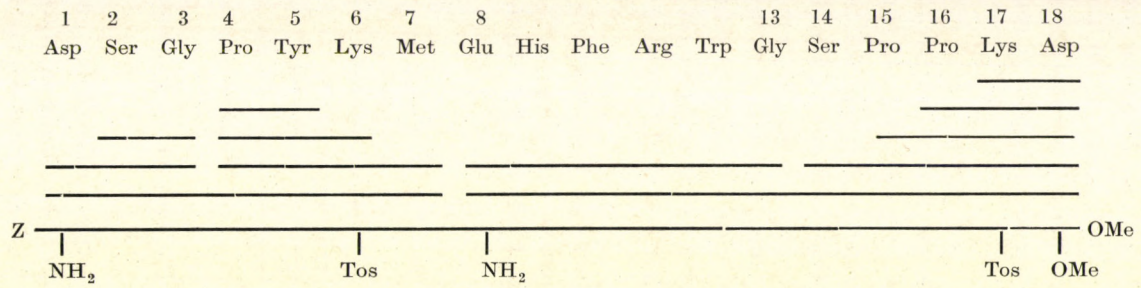


Fig. 36. Synthesis of the protected bovine β-melanotropin

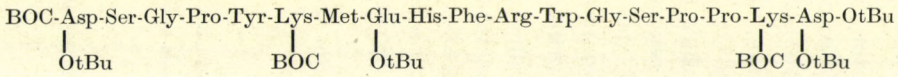


Fig. 37. Structure of the protected bovine β-melanotropin prepared by an alternative route

monkey β-melanotropins (Fig. 39). For deprotection, liquid hydrogen fluoride proved to be an excellent reagent, as catalytic hydrogenolysis was prevented by the presence of the methionine residue. Peptide homologues

H-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys(For)-Asp-OH

Fig. 39. Structure of the C-terminal pentadecapeptide used for the synthesis of human and monkey β-melanotropins

obtained after each elongation step were used for studying the relationship between the chain length and melanotropic activity (p. 218). Preparation of the tetrapeptide Pro-Tyr-Arg-Met was described in a separate paper [353].

Starting from the common intermediary pentadecapeptide and lengthening it on the N-terminal with the Asp-Glu-Gly tripeptide, Yajima *et al.* [363] succeeded in synthesizing the monkey β-melanotropin. One year later the same research group reported the synthesis of the human hormone [349]; this time the pentadecapeptide was acylated with a heptapeptide derivative, comprising the amino acid sequence Ala-Glu-Lys-Lys-Asp-Glu-Gly. Both the tripeptide and the heptapeptide derivatives have been prepared using *t*-butyloxycarbonyl and *t*-butyl ester protecting groups. The synthetic scheme for the fully protected heptapeptide is given in Fig. 40. Coupling

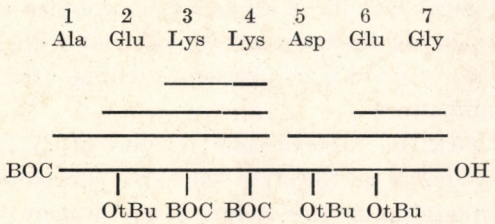


Fig. 40. Synthesis of the protected N-terminal heptapeptide of human β-melanotropin

of the tripeptide to the pentadecapeptide was accomplished *via* the N-hydroxysuccinimide ester, that of the heptapeptide with the aid of a complex prepared from dicyclohexylcarbodiimide and pentachlorophenol. Final deblocking was effected by acidolysis with trifluoroacetic acid and deformylation with hydrazine acetate. Both melanotropins could be purified by ion-exchange chromatography on carboxymethyl cellulose column.

No detailed description of the Rittel synthesis has appeared yet. The short presentation [280] points to a synthetic route based mainly on intermediates prepared earlier by Schwyzer *et al.* [299], such as the undecapeptide con-

taining the 12–22 amino acid sequence. This C-terminal part was first acylated with the 7–11 pentapeptide, then with the N-terminal hexapeptide, both protected with groups derived from *t*-butanol. Finally, the protected docosapeptide was purified by countercurrent distribution and the free hormone with ion-exchange chromatography.

Some discrepancies in the optical rotations of human β-MSH were recorded; the synthetic hormone prepared by Yajima *et al.* had a value of −76.4°, while Rittel's product showed a rotatory power of −99.8°, the rotation of the native hormone being −104° [280].

The syntheses of human and monkey β-melanocyte-stimulating hormones have been accomplished also by the solid-phase method [340].

A semisynthetic procedure, transformation of porcine β-melanotropin into the lysine[10] analogue of the human hormone has been described by Burton and Lande [38].

4. THE BIOLOGICAL ACTIVITY OF MELANOTROPINS

As it was mentioned earlier (p. 152), various methods have been published for measuring the melanocyte-stimulating activity. In most cases the *in vitro* procedure of Shizume *et al.* [319] is applied, sometimes with slight modifications. As the biological activity of all natural hormones and their derivatives has been determined by this method, in the following only the biological potencies obtained by the *in vitro* procedure will be used for the comparison of melanotropic substances.

When dealing with the corticotropic activity of ACTH and ACTH fragments, it has been shown (p. 150) that the same compound may elicit different responses depending on the means of application. From this point of view the *in vitro* experiments seem to be somewhat more reliable, as these are performed in less complicated systems, possibly avoiding some of the factors which may influence the biological activity and cannot be controlled or foreseen. In spite of this, when measuring melanocyte-stimulating activity, even qualitative agreement is difficult to attain, especially in the study of weakly active peptides, where the substance can sometimes be found by an order of magnitude less active than the same compound tested in another laboratory. Such variations may be caused by slightly different test methods, differences in the test animals, as well as seasonal changes in skin response. Since analogues of the hormones may be affected differently by the experimental conditions, it is difficult to assess their exact potency with respect to each other or to a standard. These points should be kept in mind when

conclusions concerning structure–function relationships between hormone analogues are to be drawn.

Homogeneous α-MSH from porcine pituitary gland has an activity of about 2×10^{10} U/g [189, 196, 322]. For the synthetic α-MSH Guttmann and Boissonnas [104] found an activity of 3.3×10^{10} U/g, Schwyzer *et al.* [298] $1.2\text{–}4\times10^{10}$ U/g, Blake and Li [19] 2×10^{10} U/g and Ney *et al.* [247] 1.46×10^{10} U/g. The latter authors reported an activity of 1.0×10^{10} U/g for the native compound. In the light of the above statements these potencies can be regarded as identical.

It is remarkable, however, that a sample of porcine α-MSH prepared by Schally *et al.* [290] had an *in vitro* frog skin darkening activity of at least 2×10^{11} U/g [197], and the synthetic product made by Schwyzer *et al.* also showed a very high (up to 10^{12} U/g) activity when tested in the laboratory of Lerner [298]. Later Lande and co-workers obtained values in the range of 10^{12} U/g for the natural product, and homogeneous synthetic melanotropin preparations from Hofmann also assayed about 10^{12} U/g [180]. For the synthetic hormone Yajima *et al.* [351] reported an activity of $2.3\text{–}5.4\times10^{12}$ U/g. As all these native and synthetic products proved to be otherwise identical and could not be distinguished by chemical means, there is no explanation so far for the discrepancy between these values; it is very likely that the reason has to be sought in the biological testing. Thus, it is again obvious that meaningful conclusions concerning structure–function relationships between the hormone and its derivatives can only be drawn, when the potencies measured are compared with those obtained by simultaneous testing of the chemically pure natural or synthetic hormone. Correspondingly, in this review the activities of MSH analogues and derivatives will be related to that of pure α-melanotropin supposed to have a potency of 2×10^{10} U/g.

The melanocyte-stimulating hormones of the β-type are somewhat less active biologically than α-MSH. The *in vitro* activities recorded in the literature are summarized in Table V.

The corticotropic effect of α-melanotropin has been investigated by Steelman and Guillemin [322], who found an activity of 0.1 U/mg *in vivo* and 3.5 U/mg *in vitro*. The protected melanotropin ([$Glu(NH_2)^5$, $Lys(For)^{11}$]-α-MSH) synthesized by Hofmann and Yajima [140] proved to be inactive, and this inactivity might be explained by the differences between the structures of the free and protected hormones. As Hofmann and Yajima pointed out, the possibility could not be excluded that the natural product assayed by Steelman and Guillemin might have contained impurities having the ability to bring about corticotropic responses. Later investigations by Ney *et al.* [247] indicated that α-melanotropin does possess intrinsic corticotropic

Table V

Melanocyte-stimulating Activity of β-Melanotropins

β-MSH	MSH-activity, U/g	References
Man	3.3×10^9	[263]
Man (synthetic)	6.2×10^9	[349, 350]
	1.6×10^9	[340]
	$3–6 \times 10^9$	[280]
Monkey	$3–5 \times 10^9$	[191, 319]
Monkey (synthetic)	$0.6–2.5 \times 10^{10}$	[362, 363]
	2.5×10^9	[340]
Horse	1.2×10^9	[59]
Pig	3.8×10^9	[263]
	5.0×10^9	[202]
Sheep (Asp-Glu-Gly...)	3.8×10^9	[263]
Sheep (Asp-Ser-Gly...)	9.7×10^9	[247]
Beef	9.7×10^9	[247]
	2.0×10^9	[202]
Beef (synthetic)	8.3×10^9	[247]
	$1.2–4 \times 10^9$ (and 2×10^{11})	[299]

activity (0.04 U/mg and 0.17 U/mg for porcine and synthetic α-MSH, respectively) in the *in vivo* steroidogenesis assay, but the relatively high potency shown by Steelman and Guillemin could not be verified.

An adrenocorticotropin-like action of β-MSH, corticosterone release into the adrenal vein, was noted by Li [203]. Closer examination performed by Ney *et al.* [247] showed a 0.02–0.03 U/mg adrenal-stimulating activity for various β-melanocyte-stimulating hormones.

IV. RELATIONSHIP BETWEEN THE CHEMICAL STRUCTURE AND BIOLOGICAL ACTIVITY OF CORTICOTROPINS

1. BIOLOGICAL ACTIVITY OF CORTICOTROPIN FRAGMENTS

Synthetic work on corticotropin fragments was primarily aimed at the preparation of polypeptides with intact N-terminal sequences, as even small alterations in this part of the molecule led to a marked decrease of the biological activity. In order to delineate the shortest sequence of amino acids still possessing corticotropic activity, N-terminal polypeptides lacking

shorter or longer fragments from the C-terminal part have been synthesized. As a further possibility the question emerged, whether the individual effects of ACTH such as the corticotropic, melanocyte-stimulating and lipid-mobilizing activities could belong to separate structural units, or these are properties of the hormone as a whole. As it will be shown in this Chapter, synthesis of systematically selected corticotropin fragments leading to an almost complete series of sequential homologues and their biological investigation gave valuable information about the location of the active centre along the peptide chain. In fact, closer examination of the biological properties of these fragments substantiated some hypotheses concerning the mechanism of their action at molecular level, such as binding to the hypothetical receptor or interaction with plasma proteins. It can be said, therefore, that this enormous amount of time-consuming synthetic work demanding efforts of numerous research groups has not been wasted, since these results could not have been achieved in any other way than by studying the biological activity of corticotropin fragments obtained by chemical synthesis. The structure–activity relationships were significantly clarified by the biological examinations of synthetic corticotropin analogues and fragments containing modified sequence or altered amino acids.

Syntheses of the N-terminal corticotropin fragments have been reviewed in Chapter II, their biological properties and some conclusions which could be drawn will be discussed below. It should be emphasized that the great variety of biological assays and the uncertainties derived from their nature allow reliable comparisons of different fragments in that case only, when the measurements had been conducted under strictly identical circumstances. As this demand could not be fulfilled in the early, pioneering work, controversial data are not too rare from this time. For the same reason, exceptional value is attached to systematic investigations, which were directed to the simultaneous testing and comparison of a larger group of related polypeptide fragments.

The first synthetic peptide exhibiting corticotropic activity was the eicosapeptide methyl ester prepared by Boissonnas *et al.* [30]. Assayed by the method of Saffran and Schally [287], this product possessed a positive, though limited, ACTH activity (2–3 U/mg). Since the tetracosapeptide containing the N-terminal 24 amino acids was with great probability assumed to have full corticotropic potency (80–100 U/mg), there were three possibilities to explain the diminished activity of the eicosapeptide. First, the fully active fragment is larger than the first twenty amino acids; second, activity is influenced by the presence of the C-terminal methyl ester group, and third, the possibility of a certain amount of racemization at some steps

of the synthesis could not be excluded. As it turned out later, this last explanation proved to be the right one.

Corticotropic activity was also found when testing a polypeptide of much smaller molecular weight. After Steelman and Guillemin [322] had determined the *in vitro* and *in vivo* corticotropic activity of the purified α-melanocyte-stimulating hormone isolated from natural sources and found it to be active (0.1 U/mg *in vivo* and 3.5 U/mg *in vitro*), Hofmann and Yajima [140] measured the activity of an α-melanotropin derivative containing glutamine in position 5 and ε-formyl-lysine in position 11. While this analogue was inactive, the N-terminal tridecapeptide amide of ACTH proved to be active, exhibiting some 0.1 U/mg corticotropic activity both *in vivo* and *in vitro*. On testing the ACTH-(1–13)-tridecapeptide prepared by Guttmann and Boissonnas [105], no unequivocal ACTH activity was detected either *in vivo* or *in vitro*.

The first synthetic peptide possessing significant corticotropic effect was the glutamine derivative of the N-terminal nonadecapeptide prepared by Schwyzer *et al.* [308], with an activity of 20–30 U/mg, estimated by the method of Sayers *et al.* [289]. According to the authors it would have been really peculiar for the lysine residue in position 21 to play such a decisive role in eliciting corticotropic activity in a molecule already containing four basic amino acid near the C-terminus, as it could have been concluded from the low level of activity of the eicosapeptide prepared by Boissonnas and Guttmann, ending just before this lysine residue. The obvious shortcomings of the Boissonnas peptide were confirmed later by further synthetic work. The N-terminal nonadecapeptide and its glutamine analogue prepared by Li and associates showed similar activities [203, 213, 214]. Activity data of the nonadecapeptides and of their derivatives are summarized in Table VI.

Table VI

Biological Activity of the N-Terminal Nonadecapeptide

Peptide	MSH, U/g	Ascorbic acid depletion, U/mg	*In vitro* steroidogenesis U/mg
[Glu(NH_2)^5]-ACTH-(1–19)-OH [308]		20–30	20–30 (cited in [214])
[Glu(NH_2)^5]-ACTH-(1–19)-OH [203, 214]		20.8 (s.c.) 11.8 (i.v.)	24.7
ACTH-(1–19)-OH [203, 214]	1.4×10^7	34.6 (i.v.) 74.2 (s.c.)	39.8
ACTH-(1–19)-OH [205]	7×10^7		33

Accordingly, it was apparent that replacement of the glutamic acid by glutamine in position 5 caused a decrease of the adrenal-stimulating activity. Controversies originating from the different means of application have already been briefly mentioned. *In vitro* lipolytic assay indicated that the nonadecapeptide was highly active as a lipolytic agent. In addition, it has been assayed in man and again shown to possess significant ACTH potency. Thus, the synthetic nonadecapeptide exerts the same biological functions as the natural ACTH molecule. This was the first synthetic peptide which had both adrenocorticotropic and melanotropic activities in high potency; hence, it represented the most convincing evidence in favour of intrinsic melanocyte-stimulating activity in adrenocorticotropins.

Three years later the synthesis of the nonadecapeptide was repeated; the new peptide showed similar corticotropic and somewhat higher melanotropic activity [205] (Table VI). This latter observation can be explained by some possible changes in the assay procedure, as control ACTH was also found to have greater melanotropic potency. The molar activities of the nonadecapeptide compared with those of some sequential homologues determined *in vivo* by Ney *et al.* [247] are shown in Table X (p. 174).

It is to be mentioned here that in another *in vivo* steroidogenesis assay [336] the nonadecapeptide was compared with sheep ACTH (assumed to have a potency of 100 U/mg) and found to possess 111 U/mg activity [22]. Similarly, ACTH-(1–19)-nonadecapeptide synthesized by the solid-phase method had an activity of 92 [22] and the glutamine analogue [211] 31.2 U/mg. All these data as well as the melanocyte-stimulating activities are summarized in Table VII.

Table VII

Biological Activity of the N-Terminal Nonadecapeptides

Peptide	*In vivo* steroidogenesis, U/mg	MSH activity U/g
ACTH-(1–19)-OH (conventional synth.)	111	1×10^7
ACTH-(1–19)-OH (solid phase)	92	
[Glu(NH_2)^5]-ACTH-(1–19)-OH	31.2	1×10^7
α_S-ACTH	100	1×10^7

The N-terminal tricosapeptide of Hofmann *et al.* [142, 146] and the tetracosapeptide prepared by Schwyzer and co-workers [164, 302] proved to be identical with the natural hormone not only in the qualitative biological

effects, but also in their quantitative proportions. Thus these peptides furnished unequivocal proof for the assumption made by Shepherd *et al.* on the basis of partial enzymic and acidic hydrolysis of corticotropin [317] that the sequence containing the first 24 amino acids should be enough for eliciting the full biological activity of the adrenocorticotropic hormone.

The tricosapeptide exhibited marked melanocyte-stimulating activity (2.0×10^8 U/g *in vitro*), and in this respect it was essentially as potent as the natural corticotropins. It possessed 116 U/mg activity in the rat adrenal ascorbic acid assay compared with the reported activity of 80–100 U/mg shown by pig corticotropin. When assayed for its ability to bring about elevation in plasma corticosterone levels in the rat, an activity of 91 U/mg was found for the synthetic hormone. Pig corticotropin is reported to possess a potency of 94.5 U/mg in this assay [100]. It is remarkable that the tricosapeptide exhibited only 40 U/mg in the same test when assayed subcutaneously (*cf.* p. 151). When administered intramuscularly to human subjects, it elicited similar responses as natural ACTH. Biological properties of both N-terminal peptides are presented in Table VIII.

Table VIII

Biological Activity of the Tricosa- and Tetracosapeptides

Peptide	*In vitro* steroidogenesis U/mg	*In vivo* steroidogenesis U/mg	Ascorbic acid depletion U/mg	MSH U/g
α_p-ACTH		94.5 [100]	80–100 [316]	1.7×10^8 [202]
ACTH-(1–23)-OH [142]		91	116 i.v.	2.0×10^8
			40 s.c.	
ACTH-(1–24)-OH [302]	110		106 s.c.	$1–2 \times 10^8$

Hofmann *et al.* [142] also investigated the biological activity of the protected tricosapeptide amide, a derivative bearing acetyl group on the N-terminus, formyl groups on the lysine side chains and containing glutamine instead of glutamic acid in position 5. This peptide was practically devoid of adrenal cortex stimulating potency (0.05–0.1 U/mg in the ascorbic acid depletion assay), but exhibited a potent melanocyte-expanding activity *in vitro* in frogs (2×10^8 MSH U/g), and *in vivo* in man; on administration to a light coloured negro it brought about darkening of the skin as studied by visual observation.

As it is indicated in Table VIII, the biological activities of the tetracosapeptide [302] are comparable with those of the tricosapeptide and natural

corticotropin. It is somewhat surprising that this peptide exhibits an activity of 106 U/mg in the Sayers subcutaneous test in contrast to the 40 U/mg shown by the tricosapeptide. It is not very likely that the C-terminal proline residue in the tetracosapeptide could be responsible for the enhanced activity. Comparison of these peptides using the *in vivo* steroidogenesis assay revealed similar responses [247]. Schwyzer and Kappeler [302] made the interesting observation that heat sterilization of the tetracosapeptide led to a significant rise in corticotropic potency (up to 225 U/mg). It is supposed that partial racemization or reaction with additives rather than conformational changes are responsible for this effect.

In order to locate the minimal sequence of amino acids necessary for the corticotropic activity, both research groups synthesized N-terminal peptides of smaller molecular weight. For this reason, the hexadecapeptide and the hexadecapeptide methyl ester were selected, both containing only the half of the basic core of ACTH. For the free hexadecapeptide Hofmann *et al.* [147] found that assays by both the *in vivo* adrenal ascorbic acid depletion technique and elevation of the plasma corticosterone level indicated a low but reproducible activity of about 0.1 U/mg. This corresponds to the activity of the N-terminal tridecapeptide amide, hence, this seems to be a general property of N-terminal oligopeptides of ACTH containing at least 13 amino acid residues.

The hexadecapeptide possessed an *in vitro* melanotropic activity at the level of 3.7×10^8 U/g, which should be compared with that of the protected product (p. 135) which is 2.0×10^9 U/g. Interpretation of the melanocyte-stimulating activity in connection with structural features will be given later.

The methyl ester derivative [307] had also less than 1 U/mg activity when assayed by the ascorbic acid depletion method. In contrast, an activity of 5–10 U/mg was found by the *in vitro* steroidogenesis assay. It is conceivable that the rise in potency was effected by the blocked carboxyl terminus. The melanocyte-stimulating activity of the hexadecapeptide methyl ester was found to be approximately 4×10^8 U/g [298].

It could therefore be concluded from the biological data reviewed above that the smallest fully active fragment of ACTH must be longer than 16 but may be shorter than 23 amino acids. From this point of view, the previously discussed biological investigations of the N-terminal nonadecapeptides prepared by Li and Schwyzer [214, 308] also greatly contributed to further characterizing the structural features of the active centre.

Another significant step in this direction involved the synthesis of the eicosapeptide amide by Hofmann *et al.* [129, 143], possessing an adrenal

ascorbic acid depletion potency of 111 U/mg and an activity of 83 U/mg in plasma corticosterone elevation. Thus Hofmann was able to demonstrate that elongation of the N-terminal 16 amino acid residues of ACTH to a chain corresponding to 20 amino acids brings about a more than thousand-fold increase in adrenocorticotropic potency. Accordingly, the -Arg-Arg-Pro-Val- sequence, which occupies positions 17–20 in the ACTH molecule (or a portion thereof) must be essential for high level activity. The compound has also been administered to humans by intravenous infusion and gave a significant rise in plasma 17-hydroxysteroids [143].

The melanocyte-stimulating activity of the eicosapeptide amide is reported to be 1.1×10^8 U/g, that of the protected amide (p. 136) is again somewhat higher: 4.2×10^8 MSH U/g. It should be mentioned that Lebovitz and Engel [186] compared the biological potency of the eicosapeptide amide with that of purified pig ACTH and expressed it as percentage of activity of the natural hormone. Porcine corticotropin used for this comparison was assayed by the same researchers [31] and had an activity of 65–70 U/mg *in vivo* and 49 U/mg *in vitro*. On this basis the percentages found (steroidogenesis 181% *in vivo*, and 78% *in vitro*) correspond to 120–125 U/mg *in vivo* and 38 U/mg *in vitro* activity. Similarly, the adipokinetic effect was also measured and found to be 160% *in vivo* and 39% *in vitro*. Thus, by *in vivo* assays the eicosapeptide had the same molar activity as corticotropins in respect of both the adrenal and extraadrenal effects. Problems involved in comparing the activities on a molar and a weight basis will be dealt with later.

A comparison of the eicosapeptide amide with the nonadecapeptides of Li and Schwyzer, led Hofmann and Yajima to some conclusions [141]. Thus, differences in the biological activity might point to an unusual significance of the valine residue in position 20, or it might be a reflection of the difference between a free C-terminal carboxyl in the nonadecapeptides and an amide function in the eicosapeptide amide. As it will be shown later, this latter possibility proved to be correct.

After the finding that the hexapeptide does not possess significant corticotropic activity and the first nineteen residues probably represent the minimal structural requirements for steroidogenesis, Li *et al.* [205] attempted to further define the structural features of ACTH essential for eliciting the major biological activity of this hormone. Another objective of this synthetic approach was to investigate the possibility of separation of the various biological effects. In order to find a derivative lacking some of these properties, Li *et al.* [217] synthesized the N-terminal heptadecapeptide which exhibited a low degree of potency in stimulating the adrenals (6.4 U/mg

in vitro and 1.7 U/mg *in vivo*), indicating that the integrity of the basic amino acid core is important for the manifestation of high level adrenal-stimulating activity. The heptadecapeptide exhibited a melanocyte-stimulating potency of 2×10^8 U/g, similar to those shown by other N-terminal fragments discussed previously. The minimal effective dose of the heptadecapeptide in *in vitro* lipolysis was 0.0033 μg/ml in the rat, and the compound has been shown to be more active than the natural hormone when tested on rabbit adipose tissue (0.0004 μg/ml) [330].

It appeared also interesting to see whether a peptide, combined from the N-terminal decapeptide sequence essential for stimulating the adrenal cortex and from the basic core of corticotropins consisting of the 15–18 amino acid residues, would elicit any biological activity. Biological investigation of such a pentadecapeptide would give information concerning the importance of the 11–14 Lys-Pro-Val-Gly sequence, which connects the above mentioned two parts in natural corticotropins. The pentadecapeptide (ACTH-(1–10 + 15–19)-pentadecapeptide) was synthesized by Li *et al.* [216] and the observed biological activities were remarkable. By bioassay procedures both *in vivo* [221] and *in vitro* [287] the peptide was found to exhibit an adrenal-stimulating potency of less than 1 U/mg and the MSH activity was also very limited: 1.9×10^6 U/g. The *in vivo* steroidogenic potency determined by Ney *et al.* [247] was insignificant, amounting to 0.0055 U/mg. However, the peptide was found to be as potent a lipolytic agent as natural ACTH on rabbit adipose tissue. Thus the synthetic pentadecapeptide was the first substance, where a separation of the MSH and lipolytic activities could be achieved. These activities compared to those of sheep ACTH are presented in Table IX. The importance of the tetrapeptide sequence at positions 11–14 will be discussed later (p. 228).

Table IX

Comparison of the Biological Activity of Ovine ACTH and ACTH-(1–10 + 15–19)-OH

Effect	α_3-ACTH	ACTH-(1–10+15–19)-OH
In vitro steroidogenesis, U/mg	135	0.42
In vivo steroidogenesis, U/mg	113	0.11
In vivo steroidogenesis, U/mg [247]		0.0055
In vitro MSH activity, U/g	6.4×10^7	1.9×10^6
In vivo MSH activity, μg	0.2	20
Lipolysis, min. effect. dose (rabbit), μg/ml	0.0064	0.0037

The biological activities of corticotropin fragments discussed so far clearly demonstrate that the positive charge contributed by the basic amino acids may play an important part in the action of this hormone. Further evidence in support of this hypothesis was presented by Ramachandran *et al.* [271] by the synthesis and biological assay of the N-terminal heptadecapeptide, octadecapeptide and nonadecapeptide amides. In their paper, the activity values were calculated on the molar basis as it seemed to be more appropriate and more meaningful to state the biological activity in units per millimol rather than per milligram. For better comparison, in Table X the biological properties of some corticotropin fragments discussed previously and corticotropic activities expressed as units/mg are also included. The *in vivo* melanotropic activity means again the dose required for the usual change in the melanophore index. The *in vivo* steroidogenesis values shown in Table X were determined by Liddle and associates and published also separately in London, on the occasion of the International Congress of Endocrinology in 1964 [247]. It is seen from the data that the corticotropic activity of the peptides of similar structural features continuously increases with the molecular weight if the activities are calculated on the molar basis. As a consequence, only the complete natural hormone has full biological activity. This implies at the same time that the C-terminal part, although not essential, yet plays a significant part in evoking biological responses. It is also evident that peptide amides are, as a rule, more active than the corresponding free acids, due to their greater net positive charge. This effect seems to be quite considerable, since even the smaller octadecapeptide amide possesses a higher potency than the nonadecapeptide containing one more amino acid but bearing free terminal

Table X

Molar Activity of N-Terminal Corticotropin Fragments

Peptide	Steroidogenesis				Melanotropic activity	
	in vivo		*in vitro*		*in vivo*	*in vitro*
	U/μmole	U/mg	U/μmole	U/mg	μmole	U/μmole
α_S-ACTH	481	106	617	136	4.4×10^{-5}	2.9×10^{5}
ACTH-(1–19)-NH_2	345	146	122	54	8.7×10^{-5}	4.2×10^{5}
ACTH-(1–19)-OH	110	47	82	35	8.7×10^{-5}	3.3×10^{5}
ACTH-(1–18)-NH_2	299	133	74	33	8.9×10^{-5}	4.5×10^{5}
ACTH-(1–17)-NH_2	69	33	88	42	9.6×10^{-5}	4.2×10^{5}
ACTH-(1–17)-OH	11.3	5.3	10.9	5.2	9.6×10^{-5}	2.1×10^{5}

carboxyl group. Furthermore, in the *in vivo* assay there is no difference between the activities of the octadecapeptide amide and natural ACTH on weight basis, indicating that the Pro-Val sequence in positions 19–20 in the eicosapeptide amide has no role in eliciting its high corticotropic activity. No unequivocal conclusions can be drawn from a comparison of the *in vivo* and *in vitro* measurements: the heptadecapeptides are quite similar in both applications, and so are the nonadecapeptide acid and the intact hormone, whereas the nonadecapeptide and octadecapeptide amides are both much more active *in vivo* than *in vitro*.

From Table X it is also seen that melanocyte-stimulating activities of the synthetic peptides are comparable with that of natural corticotropin and are independent of the neutral or acidic character of the C-terminus. As the molecular weights of the fragments listed in Table X range from 2092 to 2346, the *in vitro* values correspond to an activity of $1–2\times10^8$ MSH U/g.

The synthetic hexacosapeptide with two amino acids from the species-characteristic sequence of bovine corticotropin [275] had biological properties which were in accord with the data presented in Table X, i.e. steroidogenesis *in vivo* 390 U/μmole, *in vitro* 279 U/μmole, MSH activity *in vitro* 4.8×10^5 U/μmole and *in vivo* 6.6×10^{-5} μmole.

In Table XI the results of the systematic measurements of Ney *et al.* [247] are summarized, which have not been reviewed in this paper previously. The peptides tested were from the laboratories of Li, Hofmann and Schwyzer, and assayed for ACTH activity by the method of Lipscomb and Nelson [221].

Table XI

Biological Activity of Different Peptides as Determined by Ney *et al.* [247]

Peptide	Steroidogenesis U/mg	MSH activity U/g
ACTH-(1–10)-OH	0.0022	3.6×10^5
ACTH-(1–23)-OH	75	2.0×10^8
ACTH-(1–24)-OH	110	1.2×10^8
α_p-ACTH (synthetic)	90	1.0×10^8
[Ac-Ser^1]-ACTH-(1–24)-OH	7.3	1.2×10^9

Compared also with the data shown in Table X, it is again evident that corticotropic activity on a weight basis reaches its maximum at about the N-terminal half of the natural corticotropin molecule, as neither of the

higher homologues exhibit higher activity than the octadecapeptide amide. It is remarkable that even such a small molecule as the N-terminal decapeptide shows some steroidogenic activity, indicating that this part of the corticotropins can be considered as the active centre of the hormone. At the same time N-terminal acetylation of the tetracosapeptide brings about a marked decrease in corticotropic activity with simultaneous enhancement of the melanotropic effect. N-terminal modifications of natural corticotropins led to similar results (p. 180).

The octadecapeptide with a free carboxyl group on the C-terminus, a missing link from the investigations of Ramachandran *et al.* [271], was synthesized and tested along with the corresponding amide by Otsuka *et al.* [259]. Their results are somewhat controversial, as they found increase only in the *in vivo* activity when the terminal amino acid was amidated, while both peptides were equally active *in vitro* or when assayed in isolated adrenal cells [244]. Table XII shows the steroidogenic and lipid mobilizing activities of these derivatives.

Table XII

Biological Activity of the N-Terminal Octadecapeptide and Octadecapeptide Amide

Peptide	Steroidogenesis U/mg		Lipolysis, minimal eff. dose, μg	
	in vivo	*in vitro*	Rabbit	Rat
ACTH-(1–18)-OH	17.1	9.35	0.000075	0.0045
ACTH-(1–18)-NH_2	39.6, 58.0	4.7, 11.5	0.000004	0.0027

In accordance with the principle that meaningful correlations between chain length and biological activity can only be obtained if the homologues are assayed by the same test and under identical conditions, some of the N-terminal polypeptide amides synthesized by Bajusz and Medzihradszky were also investigated for corticotropic and lipolytic activity[7](Table XIII). Although these values of corticotropic activity cannot be directly compared with the data obtained by the *in vivo* steroidogenesis assay, the importance of the growing basic core is clearly seen. The appearance and enhancement of the lipolytic activity is very instructive; it reaches its maximum, corresponding to the activity of the natural hormone, with the heptadecapeptide, increasing by one order of magnitude following the incorporation of each basic amino acid. The activity of the octadecapeptide amide is in good agreement with the value found by Otsuka *et al.* [259] (Table XII).

Biological assays of corticotropin fragments synthesized thereafter brought no considerable changes in the picture of structure–activity rela-

Table XIII

Biological Activity of N-Terminal Corticotropin Fragments

Peptide	Ascorbic acid depletion U/mg	Lipolysis minimal eff. dose, μg (rat)
ACTH-(1–15)-NH_2	0.2	0.15
ACTH-(1–16)-NH_2	1.4	0.027
ACTH-(1–17)-NH_2	10.0	0.0033
ACTH-(1–18)-NH_2	27.5	0.0038
ACTH-(1–19)-NH_2	47 [63]	0.0060 [48]
ACTH-(1–20)-NH_2		0.0027 [48]
ACTH-(1–21)-NH_2		0.0055 [48]
α_p-ACTH-(1–28)-OH	100, 107 [327]	

tionships. These will be mentioned only for the sake of completeness. Geiger *et al.* [85] found for the ACTH-(1–23)-tricosapeptide amide some 100 U/mg in the Sayers intravenous test; full activity was exhibited also by the N-terminal peptides containing twenty and twenty-two amino acids and by the peptide amides comprising the 1–20, 1–21 and 1–24 sequences. The 1–23 amide prepared by Fujino *et al.* [69] possessed an activity of 80 U/mg when tested by the *in vivo* steroidogenesis method.

Enhancement of the molar activity with the growing peptide chain was demonstrated by the biological assay of larger fragments of human ACTH [63, 327, 329]. These measurements were carried out using the ascorbic acid depletion assay with intravenous and subcutaneous application, the *in vitro* method of Saffran and Schally [287] and blood corticosterone examinations according to Purves and Sirett [269]. The Sayers' intravenous test proved to be inadequate, as the dose response curves of the international standard and of the synthetic peptides did not run parallel. For comparison Table XIV includes also the corticotropic activities of the N-terminal tetracosapeptide (Synacthen, Ciba) and of the synthetic human corticotropin [10]. Corticotropic activities on a weight basis of the human ACTH fragments are not shown in Table XIV; they were, however, described in the papers by Bajusz *et al.* [11], Szporny *et al.* [327] and Fekete [63].

It is somewhat surprising that the heptacosapeptide corresponding to the human corticotropin 1–27 sequence [262] was found to possess an adrenal stimulating activity of 263 U/mg in the *in vivo* steroidogenesis assay [221] using the Third International Standard for comparison. The authors gave

Table XIV

Molar Activity of Human ACTH and of its N-Terminal Fragments

Peptide	Ascorbic acid depletion, U/μmole	*In vitro* steroidogenesis, U/μmole	Blood corticosterone, U/μmole	
			i.v.	s.c.
ACTH-(1–24)-OH	129	282	276	252
α_h-ACTH-(1–28)-OH	304	301	397	241
α_h-ACTH-(1–32)-OH	385.5	326	486	336
α_h-ACTH (synth.)	495	486	481	489

in their short communication no explanation for this unusually high specific activity.

Although several peptides prepared in various laboratories lacking one or more amino acids from the N-terminus cannot be considered as true N-terminal fragments, their biological effects should be dealt with rather here than among corticotropin analogues. Thus, corticotropic activity of the ACTH-(2–23)-docosapeptide amide (Geiger *et al.* [87]), ACTH-(4–23)-eicosapeptide amide, ACTH-(5–23)-nonadecapeptide amide, ACTH-(6–24)-nonadecapeptide and ACTH-(7–23)-heptadecapeptide amide (all prepared by Fujino *et al.* [71]) are summarized in Table XV.

Table XV

Corticotropic Activity of ACTH Fragments Lacking One or More Amino Acids from the N-Terminus

Peptide	Activity U/mg
ACTH-(2–23)-docosapeptide amide	51
ACTH-(4–23)-eicosapeptide amide	15–20
ACTH-(5–23)-nonadecapeptide amide	1
ACTH-(6–24)-nonadecapeptide	0.1
ACTH-(7–23)-heptadecapeptide amide	<0.001

2. BIOLOGICAL ACTIVITY OF CORTICOTROPIN ANALOGUES

(*i*) *Structural Alterations in the N-Terminal Sequence*

The investigations of Bell *et al.* [14] led to the discovery that, by limited peptic digestion, the ACTH molecule can be shortened by eleven of its thirty-nine amino acid residues without alteration of its corticotropic activ-

ity. Moreover, mild acid hydrolysis readily cleaved the peptide bond between amino acids 24 and 25, resulting in the N-terminal tetracosapeptide still possessing the full biological activity [316]. At the same time enzymic degradation effected by trypsin and chymotrypsin rapidly inactivated the hormone [316] in consequence of extensive cleavage of the molecule in the relatively basic amino terminal portion. Again, treatment of corticotropin with leucine aminopeptidase for 4.5 hours was found to be associated with the removal of more than half of both the first serine and tyrosine residues, leading to a 64% loss of the adrenocorticotropic activity [344]. All these early observations indicated that the adrenal-stimulating property of the corticotropins was unaffected by extensive structural changes involving the C-terminal part of the molecule, while an intact N-terminal sequence seemed to be necessary for eliciting the full biological activity.

Some further data are also available to show the sensitivity of the N-terminal sequence. Periodate oxidation of corticotropin, employed in order to identify the N-terminal serine residue of the hormone [89], was reported to eliminate its ability to deplete adrenal ascorbic acid [55], whereas the melanocyte-stimulating action was increased [56]. The observations of Geschwind and Li [92] confirmed these findings: injection of native corticotropin into hypophysectomized rats maintained the adrenal weights, whereas the weight of the glands of control animals, as well as that of animals injected with a tenfold amount of periodate-treated preparation decreased. In an investigation to determine whether periodate-treated corticotropin would antagonize or inhibit the response to native corticotropin, both preparations were injected simultaneously. The adrenal weights of these animals were not significantly different from the values found for animals receiving the native preparation alone.

Although it has not been proved, periodate-treated corticotropin very likely contains a glyoxylyl residue on the N-terminus, which can be either reduced by borohydride into the glycollyl derivative [56], or converted to a terminal glycine residue by transamination [57]. Boright *et al.* [31] established that the glycollyl derivative when assayed by the *in vivo* plasma corticosterone method elicited a potency of about 1 U/mg. In contrast, when tested on adipose tissue *in vitro*, periodate-borohydride-treated corticotropin retained at least 10% of its original activity. The N-terminal serine thus appeared to be necessary for the full corticotropic activity *in vivo*, and to possess a highly specific function with respect to the adrenal cortex, suggesting that it might be responsible for the specificity of corticotropin for its target organ. At the same time this terminal serine residue is not essential for the action of corticotropin on extra-adrenal tissues; the struc-

tural requirements for adrenocorticotropic activity are not identical with those for the extra-adrenal actions. The glycollyl-corticotropin represented the first evidence for a dissociation between the adrenal and extra-adrenal actions of corticotropin.

When the glyoxylyl compound was not reduced but converted to the glycyl derivative by transamination with copper glutamate [57], both the *in vitro* and *in vivo* corticotropic activity as well as the extra-adrenal activities remained unaltered in comparison with native corticotropin. According to the conclusion drawn by Dixon and Westkamp, retention of the corticotropic activity shows that its great loss on conversion of the terminal serine into a glycollyl group must be due to the loss of the positively charged amino group rather than to the destruction of the hydroxymethyl side chain.

The importance of the free terminal amino group was indicated by the experiments of Waller and Dixon [337], who wanted to establish whether selective N-acetylation of the N-terminal serine residue would diminish the corticotropic activity. They found that the N-acetyl compound possessed less than 10% of the adrenal-stimulating activity of ACTH, whereas it exhibited 5–10 times the melanocyte-stimulating potency of corticotropins when assayed on isolated frog skin, that is 4.4×10^8 U/g compared with the value of 0.7×10^8 U/g shown by the native corticotropin. This finding was not surprising in the light of the enhanced resemblance between acetylated corticotropin and α-melanotropin, although the latter compound is still far more potent with its 2×10^{10} U/g activity.

Acetylation of the synthetic ACTH-(1–24)-tetracosapeptide was also found to increase the MSH potency by an order of magnitude according to the *in vitro* assay, and a similar enhancement of activity could be observed when tested on the *in vivo* route. Corresponding to the modification of the natural hormone, acetylation of the tetracosapeptide led to a product with only 7 U/mg corticotropic activity, compared with 110 U/mg for the parent compound [247]. Thus blocking of the N-terminal amino group has a diverse effect on the melanotropic and corticotropic properties. The role of the acyl groups in the melanocyte-stimulating activity will be discussed later in greater detail.

The effect of substitution of the N-terminal amino acids in the biologically fully active 1–23-amide was investigated by Geiger *et al.* [87]. They synthesized hormone analogues where the first serine residue was replaced by glycine ([Gly^1]-ACTH-(1–23)-tricosapeptide amide), the tyrosine by phenylalanine ([Phe^2]-ACTH-(1–23)-tricosapeptide amide), and the third serine by alanine ([Ala^3]-ACTH-(1–23)-tricosapeptide amide), in addition to a

docosapeptide lacking the N-terminal serine residue (ACTH-(2–23)-docosapeptide amide). All these compounds showed somewhat diminished but still high corticotropic activity, except the glycine derivative, which proved to be fully active in the ascorbic acid depletion test [289] (Table XVI). This

Table XVI

Biological Activity of Corticotropin Analogues Modified on the N-Terminus

Peptide	Ascorbic acid depletion U/mg
ACTH-(1–23)-tricosapeptide amide	100 [85]
[Gly^1]-ACTH-(1–23)-tricosapeptide amide	104
[Phe^2]-ACTH-(1–23)-tricosapeptide amide	66
[Ala^3]-ACTH-(1–23)-tricosapeptide amide	50
ACTH-(2–23)-docosapeptide amide	51

is in agreement with the high activity of the glycyl-corticotropin obtained by the transamination of the glyoxylyl derivative [57]. Similarly, replacement by glycine of the amino terminal serine residue in the octadecapeptide amide [261] caused no remarkable change in the adrenal-stimulating activity. Table XVII shows the biological potencies of these compounds com-

Table XVII

Biological Activity of N-Terminal Octadecapeptides

Peptide	Ascorbic acid depletion, s.c.	Steroidogenesis	
		in vitro [287]	*in vivo* [221]
	U/mg		
α_S-ACTH	120	120	100–180
ACTH-(1–18)-NH_2	45.4	13.7	92
[Gly^1]-ACTH-(1–18)-NH_2	25.6	20.8	167

pared with those of natural sheep ACTH [153]. All these results allow the conclusion that although the alcoholic and phenolic hydroxyl groups contribute to the biological potency, they cannot be regarded as essential functional groups.

The full *in vivo* steroidogenic activity (142 U/g) of the [Ala^1]-ACTH-(1–20)-eicosapeptide amide synthesized recently by Blake and Li [21] again indicates that the side chain of the terminal serine is not involved in this biological process.

A glycine-containing fragment analogue ([$Gly^{1,3}$]-ACTH-(1–24)-tetracosapeptide) was patented by Ciba [42], but its biological properties were not reported. An N-terminal tetradecapeptide of ACTH containing glycine as N-terminal amino acid has been investigated by Nakamura [244], and found to possess a steroidogenic property on isolated adrenal cells, corresponding to 0.02% of that of the porcine ACTH. As this value is about the same as determined for α-MSH and ACTH-(1–13)-tridecapeptide amide [247] (p. 165), it can be concluded that even in such a small fragment the serine side chain does not play any role in eliciting the adrenocorticotropic potency.

In order to obtain more information about the role of the N-terminal functional group, Geiger *et al.* [83] synthesized two new analogues containing proline and β-alanine in the place of the terminal serine residue. [Pro^1]-ACTH-(1–23)-tricosapeptide amide, which contains no primary amino group on the N-terminus, was about half as active as the parent compound in the Sayers test, [β-Ala^1]-ACTH-(1–23)-tricosapeptide amide showed, however, a surprisingly high adrenal-stimulating potency; it was 5 times more active than the tricosapeptide amide with the natural sequence (Table XVIII).

Table XVIII

Biological Activity of Tricosapeptide Analogues

Peptide	Ascorbic acid depletion, U/mg
ACTH-(1–23)-NH_2	100 [85]
[Pro^1]-ACTH-(1–23)-NH_2	48
[β-Ala^1]-ACTH-(1–23)-NH_2	478

The same β-alanine analogue was also prepared by Fujino *et al.* [69]. It possessed an *in vivo* steroidogenic activity of 90 U/mg when assayed 15 min after administration, but when assays were done after 30–40 minutes, the analogue showed at least 3 times the activity of natural corticotropin.

Inouye *et al.* [153, 154] described the synthesis of an octadecapeptide amide corresponding to the first eighteen amino acid residues of corticotropin similarly substituted by β-alanine ([β-Ala^1]-ACTH-(1–18)-octadecapeptide amide). This peptide also proved to be highly active, exhibiting 135 U/mg activity when tested for adrenal ascorbic acid depletion, and 237 U/mg in the *in vitro* steroidogenesis assay. It also resembled natural corticotropin, giving nearly the ratio of unity when compared in intra-

venous and subcutaneous applications. It was also shown that the β-alanine analogue remained active for a longer period of time than the glycine derivative.

Inouye *et al.* also determined the lipolytic activity of this octadecapeptide analogue, and compared it with that of [Gly^1]-ACTH-(1–18)-octadecapeptide amide, ACTH-(1–18)-octadecapeptide amide and natural ACTH. The results are summarized in Table XIX.

Table XIX

Lipolytic Activity of N-Terminal Octadecapeptides

Peptide	Minimal effective dose 10^{-6} mg/5 mg tissue		Rat/rabbit ratio
	Rat	Rabbit	
α_S-ACTH	6.0	7.1	1
ACTH-(1–18)-NH_2	3.0	0.004	750
[Gly^1]-ACTH-(1–18)-NH_2	6.3	0.35	20
[β-Ala^1]-ACTH-(1–18)-NH_2	0.62	0.065	10

It is very likely that the increased activity of analogues containing the non-protein-constituting β-alanine residue is a consequence of increased stability against aminopeptidases rather than the effect of the β-positioned amino group. This is substantiated by the finding that the octadecapeptide amide substituted with α-aminoisobutyric acid in position 1 ([Ibu^1]-ACTH-(1–18)-octadecapeptide amide) exhibits surprisingly high adrenal-stimulating activity (100–700 U/mg depending on the different test methods) [154], although this peptide contains an α-amino group corresponding to the natural sequence.

Using the N-terminal tetracosapeptide as a reference substance, Fujino *et al.* [70] extended the investigations on the role of the terminal functional groups in evoking corticotropic activity, by synthesizing tetracosapeptide analogues with different amino acids in the first position. These compounds as well as their biological potencies determined by the *in vivo* steroidogenesis assay are shown in Table XX.

The activity data of the β-Ala-, γ-Abu- and Sar-derivatives were based on comparison of the blood steroid levels 15 minutes after administration. When assays were done 10–25 minutes later, these analogues showed very high levels of activity and the order of their potencies was as follows:

$$\text{Sar}^1 \geq \beta\text{-Ala}^1 > \gamma\text{-Abu}^1 > \text{Ser}^1 \text{ (natural sequence)}$$

Table XX

Steroidogenic Activity of Tetracosapeptide Analogues

Peptide	Steroidogenesis *in vivo* U/mg
ACTH-(1–24)-OH	90
[β-Ala^1]-ACTH-(1–24)-OH	100–160
[γ-Abu^1]-ACTH-(1–24)-OH	100–160
[Sar^1]-ACTH-(1–24)-OH	100–160
[Pro^1]-ACTH-(1–24)-OH	50–65
[Lys^1]-ACTH-(1–24)-OH	30–50

This again indicates that the presence of an α-amino group is not essential for the stimulation of the adrenals, and that replacement of the first amino acid by an aminopeptidase-resistant residue results in apparently enhanced biological activity. Furthermore, the high activity of the sarcosyl-peptide in comparison with the corresponding prolyl-analogue is remarkable.

As a further clarification of the role of the N-terminal serine in steroidogenic activity, Blake *et al.* [22] reported the synthesis of ACTH N-terminal nonadecapeptides by the solid-phase method, where the chain was lengthened on the serine residue by alanine and proline to give the [Ala-Ser^1]--ACTH-(1–19)-eicosapeptide and the [Pro-Ser^1]-ACTH-(1–19)-eicosapeptide. The *in vivo* steroidogenic activities [336] of these analogues compared with that of the parent nonadecapeptide and with sheep ACTH are shown in Table XXI. In agreement with earlier observations, retention of the biological activity again indicates that the position of the amino-terminal group relative to the remainder of the molecule is not critical for the corticotropic effect.

Table XXI

Steroidogenic Activity of Nonadecapeptide Analogues

Peptide	Steroidogenesis *in vivo* U/mg
α_s-ACTH	100
ACTH-(1–19)-OH	92
[Ala-Ser^1]-ACTH-(1–19)-OH	59
[Pro-Ser^1]-ACTH-(1–19)-OH	35

Table XXII

Corticotropin Analogues with Modified N-Terminal Sequence

Peptide	References
[CHO–CO^1]-α_p-ACTH	[55, 89]
[$HOCH_2$–CO^1]-α_p-ACTH	[56]
[Ac-Ser^1]-α_p-ACTH	[337]
[Ac-Ser^1]-ACTH-(1–24)-tetracosapeptide	[166]
[Gly^1]-α_p-ACTH	[57]
[Gly^1]-ACTH-(1–18)-octadecapeptide amide	[261]
[Gly^1]-ACTH-(1–23)-tricosapeptide amide	[87]
[$Gly^{1,3}$]-ACTH-(1–24)-tetracosapeptide	[42]
[Ala^1]-ACTH-(1–20)-eicosapeptide amide	[21]
[Ala^3]-ACTH-(1–23)-tricosapeptide amide	[87]
[β-Ala^1]-ACTH-(1–18)-octadecapeptide amide	[153, 154]
[β-Ala^1]-ACTH-(1–23)-tricosapeptide amide	[69, 83]
[β-Ala^1]-ACTH-(1–24)-tetracosapeptide	[70]
[Ibu^1]-ACTH-(1–18)-octadecapeptide amide	[154]
[Ibu^1]-ACTH-(1–19)-nonadecapeptide	[72]
[Ibu^1]-ACTH-(1–20)-eicosapeptide	[72]
[Ibu^1]-ACTH-(1–21)-heneicosapeptide	[72]
[Ibu^1]-ACTH-(1–22)-docosapeptide	[72]
[Ibu^1]-ACTH-(1–23)-tricosapeptide	[72]
[Ibu^1]-ACTH-(1–24)-tetracosapeptide	[72]
[Ibu^1]-ACTH-(1–24)-tetracosapeptide methyl ester	[252]
[γ-Abu^1]-ACTH-(1–24)-tetracosapeptide	[70]
[Lys^1]-ACTH-(1–24)-tetracosapeptide	[70]
[Phe^2]-ACTH-(1–23)-tricosapeptide amide	[87]
[Pro^1]-ACTH-(1–23)-tricosapeptide amide	[83]
[Pro^1]-ACTH-(1–24)-tetracosapeptide	[70]
[Sar^1]-ACTH-(1–19)-nonadecapeptide	[72]
[Sar^1]-ACTH-(1–20)-eicosapeptide	[72]
[Sar^1]-ACTH-(1–21)-heneicosapeptide	[72]
[Sar^1]-ACTH-(1–22)-docosapeptide	[72]
[Sar^1]-ACTH-(1–23)-tricosapeptide	[72]
[Sar^1]-ACTH-(1–24)-tetracosapeptide	[70, 72]
[Ala-Ser^1]-ACTH-(1–19)-eicosapeptide	[22]
[Pro-Ser^1]-ACTH-(1–19)-eicosapeptide	[22]

A whole series of hormone analogues of different molecular weight, substituted on the N-terminal serine residue is known from the patent literature [72, 252], without interpretation of their biological properties. These are compiled in Table XXII along with the corticotropin analogues discussed so far.

As a possible means of protecting the peptide against enzymic degradation, Kisfaludy *et al.* [171, 172, 226] have worked out a method for the incorporation of α-aminooxy-acids ($H_2N-O-CH(R)-COOH$) into the ACTH sequence. The synthesized products were derivatives of the [Asp^{25}, Ala^{26}, Gly^{27}]-α_h-ACTH-(1–28)-octacosapeptide, containing OGly, L- and D-OAla, L- and D-OSer in the N-terminal position, the abbreviations standing for the α-aminooxy analogues of glycine, alanine and serine. The D-$OSer^1$ analogue of the α_h-ACTH-(1–32)-dotriacontapeptide has also been synthesized. Some of these derivatives have full or even enhanced biological activity compared with that of the parent compounds.

As an N-terminal acyl group to render the peptide resistant to enzymic hydrolysis, Guttman *et al.* [107, 108] used the β-hydroxypropionyl (deaminoseryl, DeamSer) substituent for the protection of the N-terminal end of corticotropin fragments. These compounds, as well as their salts and metal complexes have strong adrenocorticotropic activity. These deaminoseryl derivatives containing at the same time some additional modifications are presented in Table XXIII.

Table XXIII

Deaminoseryl Analogues of ACTH

Peptide	References
[$DeamSer^1$,Val^{25}]-ACTH-(1–25)-NH_2	[108]
[$DeamSer^1$,$Glu(NH_2)^5$,Val^{25}]-ACTH-(1–25)-NH_2	[108]
[$DeamSer^1$,Nle^4]-ACTH-(1–24)-OH	[107]
[$DeamSer^1$,Nle^4]-ACTH-(1–24)-NH_2	[107]
[$DeamSer^1$,Nle^4,$Glu(NH_2)^5$]-ACTH-(1–24)-OH	[107]
[$DeamSer^1$,Nle^4,$Glu(NH_2)^5$]-ACTH-(1–24)-NH_2	[107]

(*ii*) *Substitution of the Methionine Residue*

In the clarification of relationships between the side chains of the constituting amino acids and biological activity of the adrenocorticotropic hormone, the methionine residue in position 4 played an outstanding role. In 1953 Kuehl and his co-workers [176] reported that the hormone could

be oxidized to yield a biologically inactive product, which was reactivated by treatment with H_2S. A pure preparation was also oxidized with H_2O_2 by Dedman *et al.* [50, 51] to an inactive material, and the oxidized compound could again be reduced to the active hormone by sulfhydryl compounds. Although for several reasons it was difficult to explain the experimental results on the basis of the known amino acid sequence, Dedman *et al.* [52] later presented evidence that the oxidation-reduction centre of the corticotropin was the thioether grouping of methionine. The oxidized hormone differed in the substitution of methionine S-oxide for the methionine present in the untreated material.

Hydrogen peroxide oxidation of the biologically fully active synthetic tetracosapeptide was accompanied by similar inactivation [302] and synthetic porcine corticotropin also lost its activity on treatment with peroxide [311]. The oxidized products can easily be distinguished chromatographically from the untreated compounds. On the basis of this evidence one might conclude that the methionine sulfur is involved in the physiological function of adrenocorticotropin.

In order to clarify the essential role of methionine in adrenocorticotropic activity, Hofmann *et al.* [131, 136] synthesized the eicosapeptide amide corresponding to the N-terminal part of ACTH, containing α-aminobutyric acid in position 4, instead of the methionine residue ([α-Abu^4]-ACTH-(1–20)-eicosapeptide amide). Surprisingly, this compound exhibited a significant level of biological potency: 30–40 U/mg by the adrenal ascorbic acid depletion method, and 74% of the activity of the natural corticotropin in the steroidogenesis assay. This observation eliminated the methionine residue as an essential functional unit for adrenocorticotropic activity. It is remarkable and has not been explained so far why replacement of the whole methionine by an amino acid containing a shorter side chain and no sulfur atom results in a relatively small change of the biological activity, whereas simple oxidation of a thioether group into the sulfoxide or sulfone brings about practically total inactivation.

As far as the space requirement of the side chains is concerned, a much closer similarity to methionine is presented by the norleucine residue, which occupied position 4 in a tetracosapeptide synthesized by Boissonnas *et al.* [28]. The biological potency of [Nle^4]-ACTH-(1–24)-tetracosapeptide is reported to be similar to that of natural corticotropin, although no detailed data are available. Boissonnas *et al.* also described the preparation of a pentacosapeptide amide with norleucine in position 4, and valine in position 25 ([Nle^4, Val^{25}]-ACTH-(1–25)-pentacosapeptide amide) [29, 109]. Actually, position 25 belongs to the species-characteristic sequence of the cortico-

tropins, but none of these hormones contain valine in this place (see p. 121). The reason for the selection of valine as C-terminal amino acid was the supposed enhanced resistance of the -Pro-Val-NH_2 terminal sequence against carboxypeptidase action [110]. Indeed, this compound exhibited a retarded corticotropic activity [29]. Synthesis of a structurally related peptide containing norvaline in place of methionine (Nva[4], Val[25]-ACTH-(1–25)-pentacosapeptide amide) is also described [109].

A further analogue synthesized by Geiger *et al.* [83], and containing leucine in place of methionine ([Leu[4]-ACTH-(1–23)-tricosapeptide amide) indicated again that this amino acid plays no functional part in the biological properties, as this derivative still possessed 66 U/mg activity in the Sayers test. However, contribution of the amino acid side chains to the biological activity must be evaluated carefully before general conclusions are drawn: the same substitution of the methionine residue in a tricosapeptide containing β-alanine in the N-terminal position ([β-Ala[1], Leu[4]-ACTH-(1–23)-tricosapeptide amide) was accompanied by a decrease of activity from about 500 to 73 U/mg [83]. In other words, replacement by β-alanine of the N-terminal serine does not enhance the biological potency, once the methionine has been substituted by the leucine residue.

Finally, the synthesis and biological activities of two additional corticotropin fragment analogues were described by Fujino *et al.* [75]. Both preparations ([Leu[4]-ACTH-(1–24)-tetracosapeptide and [Ile[4]-ACTH-(1–24)-tetracosapeptide) exhibited 55–85 % of the steroidogenic activity of the corresponding fragment with the natural sequence. According to the authors it may be assumed on the basis of this result that the decrease of the biological activity by oxidation of ACTH is in part due to the change of polarity at the position of the methionine residue.

In the literature numerous other derivatives substituted with different amino acids in position 4 are described which contain at the same time additional modifications in the basic core or D-amino acids near the N-terminus. These will be discussed in the following paragraph. The compounds mentioned in this Chapter are summarized in Table XXIV.

(*iii*) *Modifications in the Basic Core*

As it has been shown previously, the basic amino acid core in positions 15–18 of ACTH plays a decisive role in the corticotropic activity. Gradual lengthening of the peptide chain in this region converts the practically inactive N-terminal tetradecapeptide into the almost fully potent octadecapeptide amide. On closer examination of this effect the question

Table XXIV

Corticotropin Analogues Modified in Position 4

Peptide	References
[Met(O)^4]-ACTH	[50, 51, 311]
[Met(O)^4]-ACTH-(1–24)-tetracosapeptide	[302]
[Abu^4]-ACTH-(1–20)-eicosapeptide amide	[131, 136]
[Leu^4]-ACTH-(1–23)-tricosapeptide amide	[83]
[Leu^4]-ACTH-(1–24)-tetracosapeptide	[75]
[Ile^4]-ACTH-(1–24)-tetracosapeptide	[75]
[Nle^4]-ACTH-(1–24)-tetracosapeptide	[75]
[β-Ala^1,Leu^4]-ACTH-(1–23)-tricosapeptide amide	[83]
[Nle^4,Val^{25}]-ACTH-(1–25)-pentacosapeptide amide	[29, 109]
[Nva^4,Val^{25}]-ACTH-(1–25)-pentacosapeptide amide	[109]

arises, whether the guanidino groups of the arginine residues or only the growing number of positively charged groups is responsible for the increased activity. There are examples for both cases among polypeptide hormones. Thus the eighth amino acid in vasopressin can be either lysine or arginine; both compounds are native hormones with similar biological activities ([295] p. 366). On the other hand, replacing either of the terminal arginine residues by lysine in bradykinin results in a sharp decrease of the activity, and substitution by lysine of both arginine residues gives a practically inactive derivative ([295] pp. 114–118).

Similar experiments were carried out with the corticotropin peptides. The 17,18-arginine residues of the N-terminal tetracosapeptide were substituted with ornithine by Tesser and Schwyzer ([Orn^{17,18}]-ACTH-(1–24)-tetracosapeptide) [335]. This analogue exhibited the same corticotropic activity as the parent compound both *in vivo* and *in vitro* and had an even higher melanocyte-stimulating potency, only its lipolytic activity was found to be somewhat lower. It was therefore obvious that the guanidino groups in this sequential part are not important for the biological functions. From the patent literature the synthesis of the corresponding lysine analogue is also known ([Lys^{17,18}]-ACTH-(1–24)-tetracosapeptide) [43], and although no numerical data of biological activity are given, the compound possesses therapeutically valuable corticotropic properties.

As the nature of the basic amino acids in positions 17 and 18 appears to be non-critical, Tesser and Buis [331] extended the investigation to detect the influence of further substitutions, where the lysine residues in positions

15 and 16, and even in 11 were also replaced by ornithine. Supposed that the intrinsic biological potency is not altered by the presence of the shorter-chain primary amino groups, such a modified compound might even have possessed a prolonged effect, as the susceptibility of basic amino acid derivatives to tryptic hydrolysis strongly depends on the length of the side chain bearing the positive charge, and hydrolysis of ornithyl derivatives by trypsin is very slow in comparison with the corresponding arginyl or lysyl derivatives [1, 331]. The two analogues synthesized ([$Orn^{15,16,17,18}$]-ACTH-(1–18)-octadecapeptide amide and [$Orn^{11,15,16,17,18}$]-ACTH-(1–18)-octadecapeptide amide) had similar biological activity as the N-terminal tetracosapeptide used for reference substance, except that the plasma corticosterone concentration after subcutaneous administration [53] showed a relatively more rapid decrease. The absence of the expected prolonged activity can be explained by a metabolic degradation due to non-trypsin-like enzymes. As it will be shown later, the prolonged effect can be attained by ornithine analogues containing at the same time D-serine residue on the N-terminus.

Otsuka *et al.* prepared some N-terminal fragments with altered amino acids in the basic core and on the N-terminus. The [Gly^1, $Arg^{15,16}$]-ACTH-(1–16)-hexadecapeptide [256] and [Gly^1, Arg^{16}]-ACTH-(1–17)-heptadecapeptide amide [257] showed limited activity in the steroidogenesis assay (Table XXV), the heptadecapeptide being the more active compound; this

Table XXV

Biological Activity of Some ACTH Analogues Prepared by Otsuka *et al.* [256, 257]

Peptide	Steroidogenesis U/mg		Lipolysis minimal effective dose μg	
	In vivo	*In vitro*	Rabbit	Rat
[Gly^1,$Arg^{15,16}$]-ACTH-(1–16)-OH	0.124	0.134	0.00093	0.031
[Gly^1,Arg^{16}]-ACTH-(1–17)-NH_2	2.72	1.26	0.00042	0.0037

can be explained by the higher net positive charge of this analogue. These data should be compared with those presented for the N-terminal octadecapeptide and octadecapeptide amide in Table XII.

It is remarkable that the [β-Ala^1,Lys^{17}]-ACTH-(1–17)-heptadecapeptide amide [77] has a corticotropic activity of 100 U/mg [76] in spite of its relatively short basic sequence, which again points to enhanced resistance against proteolytic enzymes. The corresponding octadecapeptide ([β-Ala^1, $Lys^{17,18}$]-ACTH-(1–18)-octadecapeptide amide) known from the patent liter-

ature has a prolonged corticotropic action [282]. The same peptide described by Geiger and Schröder [81] exhibited a steroidogenic activity of 516 U/mg. When reducing the number of charges near the carboxylic terminus by replacing one of the lysine residues by leucine, they found a decrease of the corticotropic activity to one half of that of the parent compound (Table XXVI). If lysine in position 11 was substituted by glycine in a hepta-

Table XXVI

Corticotropic Activity of Some ACTH Fragments Modified in the Basic Core [81]

Peptide	Steroidogenesis, U/mg
[β-Ala^1,Lys^{17,18}]-ACTH-(1–18)-NH_2	516
[β-Ala^1,Lys^{17},Leu^{18}]-ACTH-(1–18)-NH_2	275
[β-Ala^1,Leu^{17},Lys^{18}]-ACTH-(1–18)-NH_2	244
[β-Ala^1,Gly^{11},Lys^{17}]-ACTH-(1–17)-NH-$(CH_2)_4$-NH_2	< 25

decapeptide amide, the activity was drastically lowered, indicating that Lys^{11} contributes to a high degree to the ACTH potency, presumably as a binding site (*cf.* p. 226). The octadecapeptide amide containing β-alanine on the N-terminus and ornithine in position 15 ([β-Ala^1,Orn^{15}]-ACTH-(1–18)-octadecapeptide amide) showed a steroidogenic activity on isolated cells corresponding to about 30% of that of porcine ACTH [244]. Finally, Guttmann *et al.* [109] reported on the synthesis of some pentacosapeptides containing altered amino acids in positions 4 and 25, in addition to arginine substitutions in the basic region. The derivatives discussed in this section are listed in Table XXVII.

(*iv*) *Substitutions in the Active Centre*

It can be seen from the biological activity of the synthetic analogues discussed thus far that replacement of the amino acid residues in the N-terminal sequence does not influence the biological properties to a great extent, the only exception being the oxidation of the methionine residue to the sulfoxide. Substitutions in the basic core also show that the molecule retains its activity if the number of its original basic charges has not been altered. There is, however, a remarkable change in the biological properties when alterations take place in positions 5 to 10. Actually, the sensitivity against modifications of this sequence offered motives for calling this group of amino acids the active centre of the molecule.

Table XXVII

Corticotropin Analogues Modified in the Basic Core

Peptide	References
[$Orn^{17,18}$]-ACTH-(1–24)-tetracosapeptide	[335]
[$Lys^{17,18}$]-ACTH-(1–24)-tetracosapeptide	[43]
[$Orn^{15,16,17,18}$]-ACTH-(1–18)-octadecapeptide amide	[331]
[$Orn^{11,15,16,17,18}$]-ACTH-(1–18)-octadecapeptide amide	[331]
[Gly^{1},$Arg^{15,16}$]-ACTH-(1–16)-hexadecapeptide	[256]
[Gly^{1},Arg^{16}]-ACTH-(1–17)-heptadecapeptide amide	[257]
[β-Ala^{1},Lys^{17}]-ACTH-(1–17)-heptadecapeptide amide	[77]
[β-Ala^{1},$Lys^{17,18}$]-ACTH-(1–18)-octadecapeptide amide	[81, 282]
[Nle^{4},$Lys^{17,18}$,Val^{25}]-ACTH-(1–25)-pentacosapeptide amide	[109]
[Nle^{4},$Lys^{17,18}$,D-Val^{25}]-ACTH-(1–25)-pentacosapeptide amide	[109]
[β-Ala^{1},Orn^{15}]-ACTH-(1–18)-octadecapeptide amide	[244]
[β-Ala^{1},Leu^{17},Lys^{18}]-ACTH-(1–18)-octadecapeptide amide	[81]
[β-Ala^{1},Lys^{17},Leu^{18}]-ACTH-(1–18)-octadecapeptide amide	[81]
[β-Ala^{1},Gly^{11},Lys^{17}]-ACTH-(1–17)-heptadecapeptide 4-amino-butylamide	[81]

It was shown that replacement of lysine and arginine residues by other basic amino acids could be extended to the lysine in position 11, without significant decrease in the biological potency [331]. Replacement of the arginine in position 8 led, however, to products with greatly diminished activity. Chung and Li [39] described the synthesis of the $lysine^{8}$ analogue of ACTH-(1–17)-heptadecapeptide amide, whose *in vitro* corticotropic activity was determined by the ascorbic acid depletion method and found to be less than 1.0 U/mg. The parent peptide, ACTH-(1–17)-heptadecapeptide amide has about 44 U/mg activity under the same conditions [271]. Similarly, the *in vitro* melanocyte-stimulating activity was also markedly reduced when lysine was substituted for arginine in position 8; 1×10^{7} U/g as compared with 2×10^{8} U/g shown by the heptadecapeptide amide containing the natural sequence. In the explanation of the authors it is very likely that the lysine side chain, although bearing the necessary positive charge, does not fit spatially into the hypothetical receptor site.

Replacement of the $arginine^{8}$ by ornithine in a tetracosapeptide analogue ([$Orn^{8,17,18}$]-ACTH-(1–24)-tetracosapeptide) also resulted in a marked decrease in the biological potency, and even when this substitution in position 8 was the only modification in the N-terminal tetracosapeptide

([Orn^8]-ACTH-(1–24)-tetracosapeptide) [334] the mono-ornithine derivative exhibited only 1% activity of the parent compound *in vivo* [53] and *in vitro* [287]. A similar change in the lipolytic and melanotropic activity could also be observed, the latter being 10^7 U/g shown by the mono-, and 10^6 U/g by the triornithine derivative. It could be concluded that the presence of an amidine function in position 8 is essential for the biological activity, or at least much more important than in positions 17 and 18.

In order to clarify the role of the arginine side chain in position 8 in eliciting the biological activity, Tesser *et al.* [332, 333] extended their investigations to further analogues. In addition to the mono-ornithine derivative, they prepared the corresponding homoarginine (Har) compound ([Har^8]-ACTH-(1–24)-tetracosapeptide), the lysine analogue ([Lys^8]-ACTH-(1–24)-tetracosapeptide) and an octadecapeptide amide containing homoarginine in the 8, and lysine residues in the 17 and 18 positions ([Har^8, Lys^{17,18}]-ACTH-(1–18)-octadecapeptide amide). Their biological activities compared with those of some other peptides known previously are shown in Table XXVIII; the activity of the tetracosapeptide is set arbitrarily at 1. From these data the importance of the guanidino group in position 8 is clearly seen.

Table XXVIII

Biological Activity of ACTH Analogues Modified in Position 8

Peptide	Steroidogenesis		Lipolysis	
	In vitro	*In vivo*	*In vitro*	*In vivo*
ACTH-(1–24)-OH	1	1	1	1
[Har^8]-ACTH-(1–24)-OH	0.25	0.3	0.1	0.5
[Orn^8]-ACTH-(1–24)-OH	0.06	not determined	0.003	inactive
[Lys^8]-ACTH-(1–24)-OH	0.04	not determined	0.003	inactive
[Lys^{17,18}]-ACTH-(1–18)-NH_2	1	1	1	1
[Har^8,Lys^{17,18}]-ACTH-(1–18)-NH_2	0.25	1	0.1	1

Geiger and Schröder [82] examined the contribution of the lysine residue in position 11 of the corticotropins to their biological activity. As it has been shown by Tesser and Buis [331], replacement of this amino acid by ornithine does not influence the biological potency, indicating that only the basicity is necessary in this place. The same observation was made by the former authors: the biological activity remained unchanged when lysine was replaced by arginine in a heptadecapeptide analogue, where β-alanine had been substituted for the N-terminal serine residue, lysine for

the arginine in position 17, and the C-terminal carboxyl group was amidated with 1,4-diaminobutane ([β-Ala[1],Arg[11],Lys[17]]-ACTH-(1–17)-heptadecapeptide 4-aminobutylamide). On the other hand, on substituting the basic amino acid in position 11 by neutral ones, the biological activity practically disappeared (Table XXIX).

Table XXIX

Corticotropic Activity of ACTH Analogues Modified in Position 11

Peptide	Ascorbic acid depletion U/mg
[β-Ala[1],Lys[17]]-ACTH-(1–17)-NH-$(CH_2)_4$-NH_2	800
[β-Ala[1],Arg[11],Lys[17]]-ACTH-(1–17)-NH-$(CH_2)_4$-NH_2	780
[β-Ala[1],Nle[11],Lys[17]]-ACTH-(1–17)-NH-$(CH_2)_4$-NH_2	5.6
[β-Ala[1],Gly[11],Lys[17]]-ACTH-(1–17)-NH-$(CH_2)_4$-NH_2	~1

Investigating the functional role of further amino acids in the active centre, Hofmann *et al.* [123] replaced the histidine residue in position 6 of a corticotropin analogue by an unnatural amino acid containing pyrazole side chain instead of the imidazole function: L-β-(pyrazolyl-3)-alanine (Pyr(3)Ala). The resulting compound, [Glu(NH_2)[5],Pyr(3)Ala[6]]-ACTH-(1–20)-eicosapeptide amide, exhibited an activity of about 50 U/mg in the adrenal ascorbic depletion assay, clearly eliminating the acid-base properties of the imidazole ring as essential for the biological functions. The *in vitro* melanocyte-stimulating activity of this analogue (8.4×10^7 U/g) was practically unchanged compared with that of the corresponding eicosapeptide amide containing histidine.

The phenylalanine[9] derivative ([Glu(NH_2)[5],Phe[9]]-ACTH-(1–20)-eicosapeptide amide) has also been prepared [123] and found to possess a biological potency, which was about 2–3% of that of porcine corticotropin, irrespective of whether the activity was determined by steroidogenesis or ascorbic acid depletion. The *in vitro* MSH-activity was again in the range of 10^7 U/g. Although the greatly diminished corticotropic activity of this analogue points to the importance of the tryptophan residue in position 9, the observed biological effect excludes the possibility of a direct functional involvement of the indole side chain. Similar conclusions can be drawn from the experiments of Fujino *et al.* [73], who found an *in vivo* steroidogenic activity of 0.05–1 U/mg for a tetracosapeptide analogue containing N^{α}-methyltryptophan (Metrp) in the place of the tryptophan residue

([Metrp^9]-ACTH-(1–24)-tetracosapeptide), and 15–25 U/mg activity for the [Leu^7]-ACTH-(1–24)-tetracosapeptide analogue.

Corticotropin analogues containing D-amino acids in the active centre have been investigated by Kappeler *et al.* [161] ([D-Glu^5,D-His^6,D-Phe^7, D-Arg^8, D-Trp^9]-ACTH-(1–24)-tetracosapeptide) and by Nakamura [244] ([β-Ala^1,D-Phe^7,Orn^{15}]-ACTH-(1–18)-octadecapeptide amide). The biological properties of the first analogue will be mentioned later (p. 196), and Nakamura's experiments are briefly discussed in Chapters V and VII.

(*v*) *Amino Acids of* D-*Configuration in the ACTH Fragments*

The configurations of the constituting amino acids play a very important part in the biological activity of polypeptide hormones. Depending on the position of the amino acid in question within the polypeptide chain, the configurational effect can be quite different. Change of the natural configuration of the amino acids in the active centre usually leads to inactivation of the hormone, while similar changes in farther located sequences are either not accompanied by any reduction of the biological activity, or, on the contrary, give rise to hormone derivatives with surprisingly high potencies. In any case, change in the configuration of the individual amino acids gives valuable information regarding the structure–activity relationships; therefore the synthesis and biological evaluation of polypeptide hormone analogues containing D-amino acid residues is a useful tool for such research.

In addition to the pure scientific interest in exploring the mechanism of hormone action, the synthesis of hormone analogues bearing D-amino acids has also been stimulated by practical points of view. Because of the inescapable enzymic degradation in the living organism, polypeptide hormones generally have a very limited life-span. When applied therapeutically, this makes frequently repeated administration necessary. Although in most cases some retardation of the hormonal effect can be attained with the aid of various additives, enhancement of the resistance against proteolytic degradation through the incorporation of non-natural amino acid residues also seems to be promising.

Syntheses of corticotropin fragments containing D-amino acids were independently carried out by two research groups in Basel. Kappeler and his co-workers [161] prepared five different analogues of the tetracosapeptide prepared previously at Ciba. Four of them contained D-amino acids in the N-terminal sequence, and the fifth in the active centre of the molecule. In addition to the configurational changes, one analogue had an altered amino acid, D-alanine having been substituted for the N-terminal serine

Peptide	*Structure*
	1 2 3 4 5 6 7 8 9 10 24
ACTH-(1–24)-OH	H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly.....Pro-OH
I	H-ser.....Pro-OH
II	H-ser-tyr.....Pro-OH
III	H-ser-tyr-ser-met.....Pro-OH
IV	H.....glu-his-phe-arg-trp.....Pro-OH
V	H-ala.....Pro-OH

Fig. 41. N-terminal tetracosapeptide analogues of ACTH containing D-amino acids

residue. The structure of these analogues are shown in Fig. 41; the small initial letters in the amino acid abbreviations indicate their D-configuration. Biological evaluation of these compounds led to surprising results. Peptides I and V possessed an at least fivefold potency compared with the tetracosapeptide with natural sequence, not only as regards the corticotropic but also the lipolytic and melanocyte-stimulating activities (Table XXX). The

Table XXX

Biological Activities of the Peptides Published in [161] Related to the Activity of ACTH-(1–24)-tetracosapeptide

Peptide	Ascorbic acid depletion U/mg	Steroidogenesis *in vivo*	Lipolysis (rat)	MSH activity
ACTH-(1–24)-OH	100	1	1	1
[D-Ser^1]-ACTH-(1–24)-OH	750	3–10	5–10	10
[D-Ser^1,D-Tyr^2]-ACTH-(1–24)-OH		1	0.8	10–30
[D-Ser^{1,3},D-Tyr^2,D-Met^4]-ACTH-(1–24)-OH		1	0.3	10
[D-Glu^5,D-His^6,D-Phe^7,D-Arg^8,D-Trp^9]-ACTH-(1–24)-OH		0	0	0.01
[D-Ala^1]-ACTH-(1–24)-OH	500–1000	3	5–10	100

analogues II and III possessed considerable, although not enhanced activity. In view of the known sensitivity of the N-terminal part of the corticotropins, this result may be explained by the increased *in vivo* stability of these peptides, which counterbalances the expected inactivation due to the incorporation of non-natural amino acids. Compound IV proved to be entirely inactive, but exhibited no inhibiting activity in lipolysis even when added in large excess to the parent tetracosapeptide. This finding was later

contradicted by the experiments of Birnbaumer and Rodbell [15], who found this analogue to possess a slight but significant stimulatory effect on lipolysis in fat cells. Also, this compound proved to be a competitive inhibitor of the native hormone, as the adenyl cyclase activity stimulated by a 0.2 μg per ml solution of ACTH was inhibited by 80% at a concentration of 20 μg per ml of the analogue containing D-amino acids in the active centre.

It is to be mentioned here that a corticotropin analogue similar to compound V ([D-Ala^1]-ACTH-(1–20)-eicosapeptide amide; 153 U/mg) showed no potentiation but rather a significant prolongation of the steroidogenic activity [21].

In order to investigate whether replacement by D-amino acids of the natural ones in corticotropin fragments, which were much less active than natural corticotropin, was capable of increasing the biological activity, Rittel [280] achieved the synthesis of D-seryl-corticotropin in addition to three shorter-chain analogues bearing D-serine on the N-terminus:

[D-Ser^1]-ACTH-(1–13)-tridecapeptide amide
[D-Ser^1]-ACTH-(1–16)-hexadecapeptide amide
[D-Ser^1]-ACTH-(1–19)-nonadecapeptide
[D-Ser^1]-ACTH.

When assayed *in vivo* by reference to adrenal steroidogenesis or adrenal ascorbic acid depletion, all four peptides were found to possess levels of activity, which were 5–10 times higher than those of the corresponding all-L-peptides.

Enhanced enzyme resistance, and thus the apparent increase of activity of corticotropin fragments possessing D-amino acids on the N-terminus can be extended to analogues which had previously been subjected to other alterations with the intention to make the products more stable and their synthesis more economical, and which alterations themselves do not influence the biological activity (e.g. exchange of methionine, simplification of the basic core). This explains the vast number of analogues which have been synthesized in recent years with the intention of therapeutical application. On account of the various structural variations applied simultaneously, the biological activities of these compounds do not allow unambiguous and useful conclusions regarding the structure–activity relationships. For this reason, these compounds will be mentioned only briefly in the following.

One of the most important representatives of this group is the compound denoted as DW-75, which has been synthesized by Boissonnas *et al.* [27, 110]. Besides substituting the N-terminal serine by its D-antipode, this compound contains norleucine in place of the methionine residue, and valine at posi-

tion 25 ([D-Ser^1,Nle^4,Val^{25}]-ACTH-(1–25)-pentacosapeptide amide). As expected, DW-75 was resistent to aminopeptidase or carboxypeptidase digestion for 24 hours. According to the biological investigations by Doepfner [60] and Jenny *et al.* [159], this compound had an activity of 625 U/mg in the s.c. adrenal ascorbic acid depletion assay, 275 U/mg in the *in vitro* corticosterone release, and showed a somewhat enhanced lipolytic activity as compared with natural corticotropin. The high potency of this peptide was also confirmed in human experiments.

Syntheses of further analogues have been reported by the Sandoz group, their main structural feature being the common N-terminal pentacosapeptide sequence of corticotropins with valine residue in the C-terminal position ([Val^{25}]-ACTH-(1–25)-pentacosapeptide amide) [26, 109]. To this series belong the [D-Ser^1,Val^{25}]-ACTH-(1–25)-pentacosapeptide amide and the [D-Ser^1,Glu(NH$_2$)^5,Val^{25}]-ACTH-(1–25)-pentacosapeptide amide [106]; these polypeptides possess a high adrenocorticotropic activity without the antigenic properties of natural ACTH.

Pless *et al.* also prepared the N-terminal tricosapeptides, containing norleucine in place of the methionine residue and D-serine on the N-terminus. Amide groups in different positions represent further variations [265]:

[D-Ser^1,Nle^4]-ACTH-(1–23)-tricosapeptide
[D-Ser^1,Nle^4]-ACTH-(1–23)-tricosapeptide amide
[D-Ser^1,Nle^4,Glu(NH$_2$)^5]-ACTH-(1–23)-tricosapeptide
[D-Ser^1,Nle^4,Glu(NH$_2$)^5]-ACTH-(1–23)-tricosapeptide amide.

Compounds of the same type but containing 24 amino acids were the following [266]:

[D-Ser^1,Nle^4]-ACTH-(1–24)-tetracosapeptide
[D-Ser^1,Nle^4]-ACTH-(1–24)-tetracosapeptide amide
[D-Ser^1,Nle^4,Glu(NH$_2$)^5]-ACTH-(1–24)-tetracosapeptide
[D-Ser^1,Nle^4,Glu(NH$_2$)^5]-ACTH-(1–24)-tetracosapeptide amide.

Beyond variations in the first and fourth positions, amino acids altered in the basic sequence can be found among the pentacosapeptides patented in 1970 [111, 267]:

[D-Ser^1,Nle^4,Orn^{17},Val^{25}]-ACTH-(1–25)-pentacosapeptide amide
[D-Ser^1,Nle^4,Glu(NH$_2$)^5,Orn^{17},Val^{25}]-ACTH-(1–25)-pentacosapeptide amide
[D-Ser^1,Nle^4,Orn^{15,16,17},Val^{25}]-ACTH-(1–25)-pentacosapeptide amide
[D-Ser^1,Nle^4,Glu(NH$_2$)^5,Orn^{15,16,17},Val^{25}]-ACTH-(1–25)-pentacosapeptide amide.

All these compounds can be used therapeutically instead of natural corticotropin.

The D-seryl analogues of the corticotropin fragments simultaneously containing altered basic amino acids at positions 17 and 18, exhibited a significantly prolonged effect in addition to the increased biological activity.

The [D-Ser^1,Lys^{17,18}]-ACTH-(1–18)-octadecapeptide amide [277] proved to be particularly suitable in human therapy [338, 339]. In animal experiments it revealed a more than 30-fold corticotropic potency related to the octadecapeptide amide with the natural structure [271]. Compared with the N-terminal tetracosapeptide at threshold levels this compound (also called Ciba 41.795-Ba) proved to be 10 times more active, but when the doses producing equal areas under the time curves were compared, it was 100 times more potent than the tetracosapeptide, while the duration of action was 4 times longer [227]. At least three compounds are known in which the arginine, and even the lysine residues have been replaced by ornithine: [D-Ser^1, Orn^{17,18}]-ACTH-(1–24)-tetracosapeptide [53], [D-Ser^1, Orn^{15,16,17,18}]-ACTH-(1–18)-octadecapeptide amide [331] and [D-Ser^1, Orn^{11,15,16,17,18}]-ACTH-(1–18)-octadecapeptide amide [331]. All these compounds show a pronounced prolongation of activity.

A series of substances of similar structure have been synthesized at Ciba; these are listed in Table XXXI.

Table XXXI

Some Corticotropin Analogues Containing N-Terminal D-Amino Acids

Peptide	References
[D-Ser^1,Lys^{17,18}]-ACTH-(1–19)-nonadecapeptide	[34]
[D-Ser^1,Lys^{17,18}]-ACTH-(1–19)-nonadecapeptide amide	[34]
[D-Ser^1,Lys^{17,18}]-ACTH-(1–24)-tetracosapeptide	[44]
[D-Ser^1,Gly^3,Lys^{17,18}]-ACTH-(1–18)-octadecapeptide amide	[156]
[D-Ser^1,Gly^3,Lys^{17,18}]-ACTH-(1–24)-tetracosapeptide	[156]
[D-Ser^1,Nle^4,Lys^{17,18}]-ACTH-(1–18)-octadecapeptide amide	[46, 284]
[D-Ser^1,Nle^4, Lys^{17,18}]-ACTH-(1–24)-tetracosapeptide	[47, 155]
[D-Ser^1,Lys^{17,18},Val^{19}]-ACTH-(1–19)-nonadecapeptide amide	[45]
[D-Ser^1,Nle^4,Lys^{17,18}],Val^{19}]-ACTH-(1–19)-nonadecapeptide amide	[45]
[D-Ser^1,Nle^4,Glu(NH$_2$)^5, Lys^{17,18}]-ACTH-(1–18)-octadecapeptide amide	[46]
[D-Ser^1,Nle^4,Glu(NH$_2$)^5,Lys^{17,18}]-ACTH-(1–24)-tetracosapeptide	[47]
[D-α-Phg^1,Lys^{17,18}]-ACTH-(1–18)-octadecapeptide amide*	[283]
[D-α-Phg^1,Lys^{17,18}]-ACTH-(1–24)-tetracosapeptide*	[283]

* Phg = phenylglycine

We have seen in the previous paragraph that replacement of the strongly basic arginine residues in positions 17 and 18 by the less basic ornithine or lysine did not influence the biological potency of the corticotropin fragments.

In order to assess the effect of a further decrease in basicity in the same positions, Brugger *et al.* [35] synthesized two nonadecapeptides, containing D-serine on the N-terminus and norvaline and norleucine residues, respectively, at positions 17 and 18 ([D-Ser^1, Nva^{17,18}]-ACTH-(1–19)-nonadecapeptide amide and [D-Ser^1, Nle^{17,18}]-ACTH-(1–19)-nonadecapeptide amide). Both compounds displayed less activity with regard to plasma corticosterone concentration than the two analogues synthesized by Rittel [280] and used for comparison: [D-Ser^1]-ACTH-(1–16)-hexadecapeptide amide and [D-Ser^1]-ACTH-(1–19)-nonadecapeptide (p. 197).

(*vi*) *Miscellaneous Substitutions*

As enzymic degradation is believed to be responsible for the limited lifespan of polypeptide fragments, several efforts have been made to protect the polypeptide chain on both terminal parts. Non-natural amino acids such as D-serine or β-alanine served as potent protecting units for the N-terminus, and the -Pro-Val-NH_2 sequence provided a remarkable stability to some of the analogues synthesized by Boissonnas *et al.* [106, 110]. However, since specific enzymes are capable of hydrolyzing the C-terminal amide group [93], it seemed advisable to use substituted amides to obtain more efficient protection. For this purpose Geiger [76] synthesized a heptadecapeptide analogue containing 4-aminobutylamide group on the C-terminus ([β-Ala^1,Lys^{17}]-ACTH-(1–17)-heptadecapeptide 4-aminobutylamide); the amino group served to increase the biologically important basicity of the terminal sequence. As expected, this substitution led to a product with significantly enhanced biological activity, as tested by the method of Sayers *et al.* [289] (Table XXXII).

Table XXXII

Comparison of the Corticotropic Activity of Heptadecapeptide Analogues

Peptide	Ascorbic acid depletion U/mg
ACTH-(1–17)-NH_2	10 [7]
[β-Ala^1,Lys^{17}]-ACTH-(1–17)-NH_2	100
[β-Ala^1,Lys^{17}]-ACTH-(1–17)-NH-$(CH_2)_4$-NH_2	800

Syntheses and biological study of similarly substituted corticotropin fragments were reported on the Second American Peptide Symposium in 1970 by Geiger and Schröder [81]. In consequence of their specific substi-

tutions especially on the C-terminal amide group, these compounds retained a surprisingly high biological potency in spite of the elimination of some of their N-terminal amino acids. As it is seen from Table XXXIII, the terminal amino group was protected with various acyl groups; some of them can be regarded as acyl radicals derived from desamino amino acids.

Table XXXIII

Corticotropic Activity of ACTH Fragments Containing ω-Amino Alkylamides

Peptide	Ascorbic acid depletion U/mg
[β-Ala^1,Lys^{17}]-ACTH-(1–17)-NH-$(CH_2)_4$-NH_2	807
[β-Ala^1]-ACTH-(1–16)-NH-$(CH_2)_5$-NH_2	136
[HO-C_6H_4-CH_2CH_2-CO-Ser^3,Lys^{17}]-ACTH-(3–17)-NH-$(CH_2)_4$-NH_2	149
[CH_3-$(CH_2)_3$-OCO-Glu^5,Lys^{17}]-ACTH-(5–17)-NH-$(CH_2)_4$-NH_2	92
[CH_3CH_2-OCO-His^6]-ACTH-(6–16)-NH-$(CH_2)_5$-NH_2	7
[β-Ala^1]-ACTH-(1–10)-NH-$(CH_2)_4$-NH_2	0.4

These studies clearly show that the presence of the N-terminal pentapeptide is not necessary for corticotropic activity, provided that sufficient binding groups are located on the C-terminal part. It is remarkable that a peptide of such a low molecular weight as the decapeptide derivative is still capable of bringing about biological response. This again demonstrates that the actual hormonal information is situated in the sequence 6–10 of corticotropin [310].

Besides the compounds mentioned above, the analogues of this type shown in Table XXXIV are known from the patent literature.

In an attempt at rendering these polypeptides orally active, Brugger [34] synthesized a series of corticotropin analogues bearing lipophylic substituents on the C-terminal carboxyl or carboxamide groups. These compounds are derivatives of the common [D-Ser^1,Lys^{17,18}]-ACTH-(1–19)-nonadecapeptide, containing alkyl groups with up to 24 C-atoms in amide or ester bonding. The expected improved activity failed to come about; these compounds were not more active when administered orally than the corresponding nonadecapeptide containing no lipophylic substituent [34, 281]. The fact that the parent compound also showed corticotropic activity on oral administration indicates that corticotropin peptides are resorbed from the gastrointestinal tract in such a way which enables polar compounds to pass through the intestinal walls. Obviously this route is not influenced by intro-

Table XXXIV

Some Corticotropin Analogues Containing ω-Amino Alkylamides Published in the Patent Literature

Peptide	References
[D-Ser[1],Lys[17]]-ACTH-(1–17)-heptadecapeptide 4-aminobutylamide	[78]
[γ-Abu[1],Lys[17]]-ACTH-(1–17)-heptadecapeptide 4-aminobutylamide	[78]
[β-Ala[1],Orn[17]]-ACTH-(1–17)-heptadecapeptide 2-aminoethylamide	[78]
[β-Ala[1],Lys[17]]-ACTH-(1–17)-heptadecapeptide 6-aminohexylamide	[78]
[HO-$(CH_2)_2$-CO-Tyr[2],Lys[17]]-ACTH-(2–17)-hexadecapeptide 4-aminobutylamide	[78]
[CH_3CH_2OCO-Tyr[2],Lys[17]]-ACTH-(2–17)-hexadecapeptide 4-aminobutylamide	[78]
[HO-C_6H_4-$(CH_2)_2$-CO-Ser[3],Lys[17]]-ACTH-(3–17)-pentadecapeptide 6-aminohexylamide	[79]
[HO-C_6H_4-$(CH_2)_2$-CO-Ser[3],Orn[17]]-ACTH-(3–17)-pentadecapeptide 2-aminoethylamide	[79]
[CH_3-$(CH_2)_3$-OCO-Glu[5],Lys[17]]-ACTH-(5–17)-tridecapeptide 6-aminohexylamide	[80]
[CH_3-$(CH_2)_3$-OCO-Glu[5],Orn[17]]-ACTH-(5–17)-tridecapeptide 2-aminoethylamide	[80]

ducing lipophylic substituents into the molecule. In addition to these compounds several orally active corticotropin preparations containing various acyl groups on the N-terminus have been described [36]:

[Ac-D-Ser[1],Lys[17,18]]-ACTH-(1–24)-tetracosapeptide
[n-$C_{13}H_{27}$CO-D-Ser[1],Lys[17,18]]-ACTH-(1–24)-tetracosapeptide
[Ac-D-Ser[1],Lys[17,18]]-ACTH-(1–18)-octadecapeptide amide
[C_4H_9CO-D-Ser[1],Lys[17,18]]-ACTH-(1–18)-octadecapeptide amide
[n-$C_{13}H_{27}$CO-D-Ser[1],Lys[17,18]]-ACTH-(1–18)-octadecapeptide amide
[n-C_8H_{17}CO-D-Ser[1],Lys[17,18]]-ACTH-(1–18)-octadecapeptide amide.

It is very likely that enzyme resistance is responsible for the remarkably high activity of a nonadecapeptide alcohol corresponding to the N-terminal sequence of ACTH, containing prolinol in place of the proline residue occupying position 19 in the native hormone [250]. [Prolinol[19]]-ACTH-(1–19)-nonadecapeptide was found to possess 338 U/μmole *in vitro* steroidogenic [287] activity compared with 122 U/μmole shown by the nonadecapeptide amide.

Diminished susceptibility toward aminopeptidase action could be expected in the case of the nonadecapeptide analogue containing N-methylphenylalanine (Mephe) in the position 2 ([Mephe[2]]-ACTH-(1–19)-nonadecapeptide) [20], as the tripeptide Arg-Mephe-Ala proved to be fully resistant to this

enzyme. Since the *in vivo* steroidogenic activity of this analogue was only 45 U/mg, replacement of the tyrosine by N-methylphenylalanine seems to affect interaction with the cellular receptor unfavourably.

V. STRUCTURE–FUNCTION RELATIONSHIPS OF THE MELANOTROPINS

1. BIOLOGICAL ACTIVITY OF MELANOTROPIN FRAGMENTS

Both the corticotropins and the melanocyte-stimulating hormones contain the common amino acid sequence Met-Glu-His-Phe-Arg-Trp-Gly. Since all these substances stimulate melanocytes, it seemed likely that the structural requirements for this biological property reside in this heptapeptide sequence. Hofmann and co-workers initiated investigations to gain insight into the relationships between peptide structure and melanotropic activity. In 1957 they were the first to publish the synthesis and biological activity of an octapeptide fragment containing this common amino acid sequence [133], and this synthesis was later followed by the preparation of further fragments [134, 138, 139], some of them also possessing melanocyte-stimulating potency (Table XXXV, compounds I–VIII). The pentapeptide His--Phe-Arg-Trp-Gly (Table XXXV, compound III) has also been synthesized by Schwyzer and Li [305] and found to possess an activity of 3×10^4 U/g. The biological activity (2×10^5 U/g) of the Glu-His-Phe-Arg-Trp-Gly hexapeptide, synthesized by Kappeler, (Table XXXV, compound IX) was also given in this paper. The synthesis and biological examination of the pentapeptide was reported again much later by Hano *et al.* [114] who found an activity of 7×10^3 U/g, although in a different biological test. It is remarkable that even the tetrapeptide His-Phe-Arg-Trp proved to be active [253, 254], its melanocyte-expanding activity was, however, only one-millionth of that of the natural hormone. This is the smallest peptide fragment thus far described which has melanotropic activity, suggesting that this compound (Table XXXV, compound X) contains the structural elements which are concerned with the biological functions.

The addition of amino acids to either terminus of the His-Phe-Arg-Trp-Gly pentapeptide results in a stepwise increase in the biological activity. Thus, synthetic and partially protected C-terminal fragments of α-MSH, which served as intermediates in the synthesis of the native hormone, exhibited enhanced melanocyte-stimulating activity compared with the effect of the pentapeptide. These compounds, prepared by Hofmann, Yajima and their co-workers, as well as the biological potency of the derivatives are listed in

Table XXXV

In vitro Melanocyte-stimulating Activity of α-Melanotropin Fragments and Fragment Analogues

	Peptide	Activity MSH U/g	References
I	H-Ser-Met-Glu(NH_2)-His-Phe-Arg-Trp-Gly-OH	0.7×10^6	[133, 134]
II	Z-Ser-Met-Glu(NH_2)-His-Phe-Arg-Trp-Gly-OH	0.7×10^6	[133, 134]
III	H-His-Phe-Arg-Trp-Gly-OH	1.5×10^4	[138, 139]
		3×10^4	[305]
		7×10^4	[114]
		4×10^3	[178]
IV	H-His-Phe-Arg-Trp-Gly-Lys(Tos)-Pro-Val-NH_2	0.5×10^6	[134, 139]
V	H-Met-Glu(NH_2)-His-Phe-Arg-OH	inactive	[134]
VI	Z-Met-Glu(NH_2)-His-Phe-Arg-OH	inactive	[134]
VII	Z-Ser-Tyr-Ser-Met-Glu(NH_2)-His-Phe-Arg-OH	inactive	[134]
VIII	H-Ser-Tyr-Ser-Met-Glu(NH_2)-His-Phe-Arg-OH	inactive	[134]
IX	H-Glu-His-Phe-Arg-Trp-Gly-OH	2×10^5	[305]
X	H-His-Phe-Arg-Trp-OH	3.6×10^4	[253, 254]
XI	H-His-Phe-Arg(NO_2)-Trp-Gly-Lys(For)-Pro-Val-NH_2	0.5×10^6	[132, 141]
XII	H-His-Phe-Arg-Trp-Gly-Lys(For)-Pro-Val-NH_2	8.0×10^6	[132, 141]
		2.0×10^4	[351]
		2.0×10^6	[348]
XIII	H-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	8.0×10^6	[140, 141]
XIV	H-Met-Glu(NH_2)-His-Phe-Arg-Trp-Gly-OH	2.8×10^5	[162, 163]
XV	H-Glu(NH_2)-His-Phe-Arg-Trp-Gly-OH	2×10^5	[160, 163]
XVI	H-Met-Glu-His-Phe-Arg-Trp-Gly-OH	1.4×10^6	[219]
XVII	H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-OH	2.9×10^6	[140, 141]
		1×10^6	[212]
		3.6×10^5	[247]
XVIII	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-OH	7.0×10^4	[347]
XIX	Ac-Ser-Tyr-Ser-Met-Glu(NH_2)-His-Phe-Arg-Trp-Gly-OH	3.6×10^6	[140, 141]
XX	H-Glu-His-Phe-Arg-Trp-Gly-Lys(For)-Pro-Val-NH_2	1.4×10^7	[351]
XXI	H-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	$\sim 2 \times 10^8$	[105]
XXII	Ac-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	$\sim 5 \times 10^9$	[105]
XXIII	H-Phe-Arg-Trp-Gly-Lys(For)-Pro-Val-NH_2	inactive	[177]
		$1\text{–}2 \times 10^4$	[178]
XXIV	H-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	inactive	[346]
		$1 \times 10^6, 2 \times 10^4$	[178]
		4×10^5	[247]

Table XXXV (*cont.*)

	Peptide	Activity MSH U/g	References
XXV	H-Gly-His-Phe-Arg-Trp-Gly-OH	2.3×10^5	[292]
XXVI	H-His-Phe-Orn-Trp-Gly-OH	inactive	[357, 358]
		$2.4–2.9 \times 10^4$	[218, 293]
XXVII	H-His-Phe-Lys-Trp-Gly-OH	1×10^4	[357, 358]
		inactive	[39]
XXVIII	H-His-Phe-Cit-Trp-Gly-OH	inhibitor	[23]
XXIX	H-Ser-Tyr-Ser-Met-Glu-His-Phe-Lys-Trp-Gly-OH	2×10^4	[39]
XXX	H-Ser-Tyr-Ser-Met-Glu(NH_2)-His-Phe-Arg-Trp-Gly--N_2H_3	1×10^6	[212]
XXXI	BOC-Ser-Tyr-Ser-Met-Glu(NH_2)-His-Phe-Arg-Trp--Gly-N_2H_3	1×10^7	[212]

Table XXXV (compounds IV, XI, XII, XIII). The activity of 2.0×10^4 U/g given by Yajima [351] for compound XII must be a misprint, as later Yajima himself cited an activity of 2.0×10^6 U/g for the same compound [348], and the higher activity corresponds better with the value found by Hofmann [132, 141].

Lengthening of the pentapeptide on the N-terminus is accompanied by a limited increase in the biological potency, as shown by compounds I, IX, XIV, XV, XVI, and even the N-terminal decapeptide of α-melanotropin exhibits an activity which is only moderately higher than that of the His--Phe-Arg-Trp-Gly pentapeptide, although biological measurements show some deviation at this point (Table XXXV, compounds XVII, XVIII, XIX). A significant increase of the biological potency takes place on simultaneous lengthening the pentapeptide on both termini (Table XXXV, peptides XX, XXI, XXII), gradually reaching its maximum at the tridecapeptide stage (see Table XXXVI, compound I).

From the biological activity of the fragments listed in Table XXXV some cautious conclusions can be drawn. According to the definition used so far, the His-Phe-Arg-Trp tetrapeptide sequence can be regarded as the functional active centre of α-melanotropin. Any peptides lacking the histidine or the tryptophan residue are inactive even if they contain the whole sequence either on the amino, or on the carboxyl end (e.g. compounds VII and VIII or XXIII and XXIV in Table XXXV). In contrast to these obser-

vations indicating the required presence of histidine for melanocyte-stimulating activity, recently Gros and Leygues found an *in vitro* activity of 10^6 U/g for the heptapeptide XXIV, which has been isolated from porcine pituitary [99]. Synthetic XXIV was also found to be active by Liddle and Schally ([247], and cited in [178]), although its activity was lower by two orders of magnitude (2×10^4 U/g). The partially protected heptapeptide (compound XXIII) also had some activity according to the latter authors. It is remarkable that in this series of measurements even the key pentapeptide proved to be less active than described previously (4×10^3 U/g).

As lengthening of the chain on both ends of the pentapeptide results only in enhancement (though by up to a million) of the biological activity, it is very likely that the most important role of the functional groups present in these terminal sequences is to increase the affinity between the peptide and its receptor unit. It is impossible to evaluate the effect of acetylation of the terminal amino group of the melanotropin fragments: according to Yajima [347] acetylation led to products with diminished activity (compounds XVII and XVIII in Table XXXV), while Guttmann and Boissonnas [105] observed an enhancement of the biological potency (compounds XXI, XXII). The positive effect of the acetyl group on the biological activity observed by the latter authors, was explained by the possible structural requirement of an acetyl-seryl moiety being present for the appearance of a strong melanotropic activity. It is also possible that the presence of an acetyl group on the N-terminal amino group of the peptide results in resistance against degradation by aminopeptidases, thus the apparent biological activity of the acetylated molecule is increased by lengthening its life. Formylation of the ε-amino group of the lysine residue is not accompanied by any change in the biological activity (Table XXXV, compounds XII and XIII), whereas substitution with bulky acyl groups results in significant loss of activity (compound IV). Amide substitution of the glutamic acid γ-carboxyl group does not influence the biological effect (compounds IX and XV). The insignificance of the free carboxylic group is also shown by the fact that although the hexapeptide Glu-His-Phe-Arg-Trp-Gly has an activity almost 10 times higher than that of the pentapeptide from which the glutamyl residue is absent, this increase is merely due to the added length of the peptide chain, and not specifically to the presence of the glutamyl residue. Schnabel and Li [292] synthesized the corresponding glycine derivative (Table XXXV, compound XXV) and found a melanocyte-stimulating activity (2.3×10^5 U/g), which was the same as that of the glutamyl compound (Table XXXV, compound IX). In contrast, the heptapeptide XVI prepared by Li *et al.* [219] had an MSH potency of 1.4×10^6 U/g,

which is considerably higher than the activity reported for the glutamine analogue (2.8×10^5 U/g [162, 163]).

Investigations on the essential role of the histidine and tryptophan residues were extended also to the study of the importance of the arginine residue. The fragment analogues synthesized (Table XXXV, compounds XXVI, XXVII, XXVIII, XXIX) contained ornithine, lysine or citrulline in place of the arginine. The basic amino acids seem to be biologically equivalent, according to the experiments of Yajima *et al.*, whereas the results of Chung and Li [39] point to the significance of the guanidino group. The citrulline derivative turned out to be a non-melanotropic agent, possessing at the same time some inhibitory effect on the melanocyte-stimulating activity.

2. BIOLOGICAL ACTIVITY OF α-MELANOTROPIN ANALOGUES

Apart from the result that the activity gradually increases with the elongation of the amino acid sequence in the active centre, investigation of the melanotropin fragments did not provide significant information concerning the role of the individual amino acids. From this point of view it seems to be more promising to study the biological properties of melanotropin analogues which can be regarded as derivatives of the whole molecule. Obviously, the preparation of such compounds requires considerable synthetic effort, and α-melanotropin, containing only 13 amino acids may be the limit for such experiments, where the synthetic work involved is still in proportion with the scientific results to be expected. This is the reason why only a limited number of α-melanotropin analogues with small structural alterations are known, where, for example, only one amino acid has been modified or replaced by another residue, or only one amino acid has been omitted from the sequence. A number of these compounds served as intermediates in the synthesis of melanotropic substances. In the following the melanocyte-stimulating activity of these analogues will be discussed, and the possible contribution of these investigations to the understanding of the structure–activity relationships in this field will be analyzed.

In the course of the synthesis of the α-melanotropin sequence Hofmann *et al.* prepared some derivatives, in which the lysine ε-amino group was acylated and the glutamic acid γ-carboxyl group was blocked by amidation. If acylation was accomplished by introducing a formyl group (Table XXXVI, compound II), the peptide retained full biological potency, but elicited only 10% activity when the lysine side chain was acylated with the large tosyl substituent (Table XXXVI, compound III) [144, 145].

Table XXXVI

In vitro Melanocyte-stimulating Activity of α-Melanotropin Analogues

	Peptide	Activity MSH U/g	References
	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 (α-MSH)	2×10^{10}	
I	H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	1.9×10^{9} $\sim1\times10^{9}$	[140, 141] [105]
II	Ac-Ser-Tyr-Ser-Met-Glu(NH_2)-His-Phe-Arg-Trp-Gly-Lys(For)-Pro-Val-NH_2	2.2×10^{10}	[145]
III	Ac-Ser-Tyr-Ser-Met-Glu(NH)$_2$-His-Phe-Arg-Trp-Gly-Lys(Tos)-Pro-Val-NH_2	2.0×10^{9}	[144, 145]
IV	Z-Ser-Tyr-Ser-Met-Glu(NH_2)-His-Phe-Arg-Trp-Gly-Lys(Tos)-Pro-Val-NH_2	$0.8–1.5\times10^{8}$	[139, 145]
V	Ac-Ser-Tyr-Ser-Met-Glu(NH_2)-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	1×10^{9}	[16]
VI	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys(For)-Pro-Val-NH_2	$0.25–2\times10^{10}$	[351]
VII	Bz-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	$4–8\times10^{9}$	[298]
VIII	Ac-Ser-Tyr-Ser(Ac)-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	$\sim4\times10^{9}$	[67]
IX	Ac-Ser(Ac)-Tyr-Ser(Ac)-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	$\sim4\times10^{9}$	[67]
X	Ac-Ser-Tyr-Ser-Met(O)-Glu(NH_2)-His-Phe-Arg-Trp-Gly-Lys(For)-Pro-Val-NH_2	10^{7}	[131, 141]
XI	Ac-Ser-Tyr-Ser-Met(O)-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	6×10^{7}	[222]
XII	Ac-Ser-Tyr-Ser-Abu-Glu(NH_2)-His-Phe-Arg-Trp-Gly-Lys(For)-Pro-Val-NH_2	2×10^{8}	[131, 141]
XIII	Ac-Ser-Tyr-Ser-Leu-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	1×10^{10}	[249]
XIV	Ac-Ser-Tyr-Ser-Ile-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	1×10^{10}	[249]
XV	Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	$\sim5\times10^{10}$	[238]
XVI	Ac-Ser-Tyr-Ser——Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	1×10^{9}	[237]
XVII	Ac-Ser-Tyr-Ser-β-Ala-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	$\sim5\times10^{9}$	[237]
XVIII	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp——Lys-Pro-Val-NH_2	1.5×10^{8}	[237]
XIX	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-β-Ala-Lys-Pro-Val-NH_2	1.3×10^{10}	[237]
XX	Z-Ser-Tyr-Ser-Met-Glu(OBz)-His-Phe-Arg-Trp-Gly-Lys(Z)-Pro-Val-NH_2	$\sim10^{8}$	[67]

A similarly protected tridecapeptide amide bearing a benzyloxycarbonyl group on the N-terminal amino group instead of the acetyl substituent was also active, but this activity was by two orders of magnitude lower than that of the natural product [139, 145] (Table XXXVI, compound IV). Hofmann has pointed out that when blocking groups are attached to peptides of high biological activity, the effects of this substitution are to be interpreted cautiously, as the observable biological activity may be due to the blocked peptide, or may merely reflect the ability of the test object to remove the blocking group with the formation of the active parent compound. Since it is not likely that animal tissue is capable of removing ε-amino-formyl or tosyl substituents from the α-melanotropin sequence [122], the significant activity of compounds II, III and IV eliminates this amino group as an element essential for function. The space requirement of the tosyl and the terminal benzyloxycarbonyl groups seems to be responsible for the lower biological activity of compounds III and IV. A similar phenomenon is shown by melanotropin analogues where the only modification is the replacement of the terminal acetyl group by the benzoyl substituent [298] (Table XXXVI, compound VII), or both amino groups are substituted by benzyloxycarbonyl groups in addition to the benzyl ester substitution of the glutamic acid residue [67] (Table XXXVI, compound XX). As compound II shows full biological potency, the γ-carboxyl of the glutamyl residue also appears to be insignificant in evoking melanocyte-stimulating activity, although the diminished activity of the glutamine analogue of α-MSH (Table XXXVI, compound V) synthesized much later by Blake *et al.* [16] is in contrast with the above finding. This result has not been explained so far. One may speculate that the simultaneous blocking of the glutamic acid and lysine side chains does not interfere with the original charge distribution of the hormone molecule, while the sole protection of the glutamic acid renders the peptide more basic and thus less potent. It is known, however, that an α-melanotropin derivative with blocked lysine side chain as the only modification in the molecule is still capable of eliciting the full biological potency [351] (Table XXXVI, compound VI) in spite of the altered net charge. On the other hand, there are observations which again point to the possible importance of the charge relations in the molecule: the tridecapeptide bearing free amino terminus possesses only 10% activity of the native hormone (compound I in Table XXXVI). As it could be concluded previously from the biological activity of the smaller fragments (p. 206), the validity of this phenomenon is questionable among these compounds. The effect of acetylation on the melanocyte-stimulating activity appears to be positive in the corticotropin molecule (see p. 180), and among N-ter-

minal ACTH fragments (compare the activities of the ACTH-(1–24)-tetracosapeptide and of its N^{α}-acetyl analogue in Table XI). Additional acetylation of the serine side chains results in some decrease in the biological potency [67] (Table XXXVI, compounds VIII, IX).

As it has been discussed before (p. 187), oxidation of the methionine thioether group in the corticotropin molecule results in a total loss of the hormonal activity. Exposure to hydrogen peroxide greatly reduced the melanocyte-stimulating potency of an α-melanotropin derivative (compound II in Table XXXVI), yielding the corresponding methionine S-oxide compound, X, with a biological activity of about 10^7 U/g [131, 141]. Lo *et al.* [222] demonstrated the same phenomenon in connection with the oxidation–reduction behaviour of natural α-MSH from bovine pituitary glands. The methionine S-oxide analogue of α-melanotropin exhibited an activity of 6×10^7 U/g by the *in vitro* frog skin assay procedure (compound XI in Table XXXVI). It could not be stated with certainty, whether this analogue was actually present in the glands under the natural conditions, or was a product formed during the isolation process. In spite of this inactivation by oxidation, similarly to the corticotropin series, the methionine sulfur is not an essential element for melanotropic activity, since a derivative containing α-aminobutyric acid in the place of methionine still exhibits a significant level of biological potency (2×10^8 U/g) [131, 141] (compound XII in Table XXXVI). Exclusion of the thioether grouping as being essential for the function does not mean that the methionine side chain is unimportant in this respect. The diminished activity of the analogue XII must be due to the replacement of methionine by the α-aminobutyric acid residue, as the two additional modifications in compound XII (glutamine in position 5 and ε-formyllysine in position 11) do not interfere with the melanotropic property (*cf.* compound II in Table XXXVI).

As far as the role of the methionine side chain is concerned, a better picture can be obtained by the biological testing of derivatives containing modifications in position 4 only. Nishimura and Fujino [249] reported the synthesis of [Leu^4]-α-melanotropin and [Ile^4]-α-melanotropin, and Medzihradszky *et al.* synthesized the corresponding norleucine derivative [238] (compounds XIII, XIV and XV, Table XXXVI), all peptides having the same or somewhat higher biological potency than natural α-MSH. These findings indicate that in this position an apolar side chain with appropriate spacefilling is required.

With the general assumption that only minimal alteration of the native sequence will allow meaningful conclusions concerning the details of interaction between hormone and receptor molecules, the Hungarian researchers

synthesized a series of systematically selected analogues of the intact α-melanotropin [237, 238].* From the comparison of the activities of α-melanotropin fragments of different chain length, it seemed likely that the affinity or binding of the active centre to the hypothetical receptor molecule is greatly enhanced by the two terminal sequences. Correspondingly, the modification of these regions may provide information about the chemical properties and/or about the conformational features of the binding groups of the receptor molecule. To decide whether or not the distance between the active centre and binding sequence of α-melanotropin has any role in placing the functional groups into a position suitable for optimal binding, the synthesis and biological testing of analogues were undertaken, where the two amino acids lying outside the active centre, namely methionine in position 4 and glycine in position 10, were omitted, or replaced by β-alanine [237] (compounds XVI, XVII, XVIII and XIX in Table XXXVI). It appears from the activity data presented in Table XXXVI that the glycine residue, although bearing no functional side chain whatsoever, must play quite an important role in connecting the active centre with the C-terminal part of the molecule. Its function is therefore to contribute to the precise conformation needed for the biological effect, or simply to assure the optimal distance between the active centre and binding sites, the latter possibility being the more probable one, since β-alanine is capable of taking over this function. The full activity displayed by the β-alanine analogue indicates that the elongation of the peptide backbone by one methylene group does not increase the distance between these subunits to such an extent which would affect the interaction between the hormone and receptor molecules, supposed that the preserved activity is a reflection of identical binding.

The similar role of the methionine residue is less significant; [desMet^4]-α-melanotropin is a fairly active analogue. This fact is in accordance with the observed moderate influence of the N-terminal variations on the biological properties of α-melanotropin.

The investigations of Nakamura *et al.* [245, 246] on the melanocyte-stimulating activity of synthetic polypeptides related to ACTH should be mentioned here, rather than among the corticotropin derivatives, as these results add some new aspects to the understanding of structure–activity relationships in this field. Using *in vivo* measurements and taking in account also the duration of action for the evaluation of melanotropic properties, these authors determined the minimal effective dose for each analogue and com-

* Some of these results were presented at the Symposium on Peptides and Proteins, 19 April 1974, Kiev, USSR, and on the 9th Meeting of the Federation of European Biochemical Societies, 27 August 1974, Budapest, Hungary.

pared them with that of α-melanotropin and other melanotropic substances. These data are listed in Table XXXVII (*cf.* Table XL).

Table XXXVII

Melanocyte-stimulating Activity of Some Corticotropin Fragment Analogues [245, 246]

Peptide	MSH activity	
	Minimal effective dose (μg/frog)	Relative potency
α-MSH	0.0021	1
β_h-MSH	0.0049	0.4
α_p-ACTH	0.021	0.1
ACTH-(1–24)-OH	0.014	0.2
[Gly^1]-ACTH-(1–18)-NH_2	0.098	0.02
[β-Ala^1]-ACTH-(1–18)-NH_2	0.0032	0.66
[Ibu^1]-ACTH-(1–18)-NH_2	0.0052	0.40
[β-Ala^1,Orn^{15}]-ACTH-(1–18)-NH_2	0.0080	0.3
[β-Ala^1,D-Phe^7,Orn^{15}]-ACTH-(1–18)-NH_2	0.000047	41.7
[Gly^1]-ACTH-(1–10)-OH	2.04	0.001
[β-Ala^1]-ACTH-(1–10)-OH	2.42	0.00087
[Ibu^1]-ACTH-(1–10)-OH	2.00	0.001
[Gly^1]-ACTH-(1–15)-OH	1.35	0.0015

The observed differences in the biological activity of these derivatives can be explained by structure-dependent binding to the receptor and by different susceptibility to peptidases, the two effects cannot be separated from each other in the *in vivo* conditions used. [β-Ala^1, D-Phe^7, Orn^{15}]-ACTH-(1–18)-octadecapeptide amide is the first substance described possessing higher melanotropic activity than α-MSH. Some α-MSH analogues, among them [D-Ser^1]-α-melanotropin were reported to have growth-hormone regulating activity [54].

3. CONFIGURATIONAL CHANGES IN THE ACTIVE CENTRE OF α-MELANOTROPIN

Early observations made by Landgrebe and Mitchell [181] on the effect of alkali treatment of melanocyte-stimulating hormone indicate that heating pituitary extracts in an alkaline solution caused potentiation, that is an increase of the melanotropic activity, as well as prolongation (increased time of darkening of frog skin) of the action in an *in vivo* system. Lee *et al.*

[187, 193] observed only the prolongation effect on treating pure α-melanotropin with alkali: a markedly longer time was required for a maximum response in *in vitro* experiments, which phenomenon actually should be called rather retardation than prolongation. The activities of alkali-treated α-MSH and untreated material measured after 180 minutes were essentially the same.

Prolongation of the melanocyte-stimulating effect of alkali-treated α-melanotropin was also observed by Lerner *et al.* [195]. When fully darkened, the MSH-treated frog skin usually rapidly lightens on washing with fresh buffer solutions, owing to hormone inactivation or dissociation from the skin. When darkening was effected by alkali-treated melanotropin, it was not possible to wash the skin back to its original light state within a practical time limit [187].

Synthetic peptides containing only the amino acids of the active centre of corticotropins and melanotropins exhibit similar properties on treatment with hot alkali. The pentapeptide L-His-L-Phe-L-Arg-L-Trp-Gly showed a greatly prolonged activity in darkening the skin of hypophysectomized frogs when kept in 0.1 M NaOH solution in a boiling water-bath for 15 minutes [202, 305]. Since it seemed reasonable to assume that either the conversion of arginine to ornithine, or the racemization of L-phenylalanine, or both are responsible for the prolongation effect, Li *et al.* [218] synthesized the ornithine-containing pentapeptides with L- and D-phenylalanine. Their melanocyte-stimulating activities were similar ($2.6–2.9 \times 10^4$ U/g) and identical with that of the pentapeptide of natural sequence (3×10^4 [305]), except that the D-Phe derivative exhibited a remarkable prolongation effect, which was also shown by the L-Phe compound after alkali–heat treatment. This result pointed to the significance of the configuration of the phenylalanine residue, confirmed later by the synthesis of the L-His-D-Phe-L-Arg-L-Trp-Gly pentapeptide, which exerted an enhanced specific activity of 3.3×10^5 U/g [293]. It is noteworthy that these peptides react in a different way depending on the biological test used; the ornithine analogue containing D-phenylalanine showed an increased potency when tested on lizard skin compared with its activity measured by the frog skin assay. Nevertheless, lizard skin is less sensitive towards the ornithine analogues than is the frog skin. These results are summarized in Table XXXVIII.

Yajima and his co-workers [114] repeated the experiments of Schwyzer and Li, and heated the synthetic L-His-L-Phe-L-Arg-L-Trp-Gly pentapeptide with sodium hydroxide. The alkali-treated sample exhibited considerable potentiation; an about 40-fold increase in the melanocyte-stimulating activity was observed. It is, however, somewhat confusing that no prolongation

Table XXXVIII

Melanocyte-stimulating Activity of Some MSH Analogues Measured on Different Test Animals

Peptide	MSH activity	
	Frog skin (U/g)	Lizard skin (min. eff. dose, μg/ml)
L-His-L-Phe-L-Arg-L-Trp-Gly	3.1×10^4	0.2
L-His-D-Phe-L-Arg-L-Trp-Gly	3.3×10^5	0.02
L-His-L-Phe-L-Orn-L-Trp-Gly	2.9×10^4	8.5
L-His-D-Phe-L-Orn-L-Trp-Gly	2.6×10^4	1.2

or retardation phenomenon could be demonstrated. A detailed examination of the alkali-treated all-L-pentapeptide showed that no cleavage occurred in the peptide chain, but a significant part of the arginine was converted to ornithine. By using an enzymic technique it could be established that the constituent amino acids had been racemized to different extents.

Lee *et al.* [187, 193] could later demonstrate that the observed prolongation–retardation of the melanocyte-stimulating effect of α-melanotropin was indeed brought about by the racemization of phenylalanyl and arginyl residues: the Phe-Arg and Arg-Trp bonds in the alkali-treated hormone proved to be resistant to chymotryptic and tryptic hydrolysis. Seeking minimal conditions for inducing prolongation of the melanocyte-stimulating activity, Lande and Lerner [179] observed that prolongation increased with time, reaching a maximum in 40 minutes. Analysis of the treated material showed an overall racemization of the constituent amino acids, the conversion of serine, histidine and phenylalanine being the greatest (60–70%). The changes in biological activity cannot be ascribed unequivocally to the D-configuration of any particular amino acid, since partial racemization has been sufficient for the attainment of total resistance to lightening. It should be mentioned here that true potentiation of the melanocyte-stimulating activity of pure natural hormones was observed only in the case of the alkali-treated ACTH [264, 317]. The most obvious explanation is that under these conditions the corticotropin molecule undergoes partial degradation resulting in N-terminal peptides with higher melanotropic potency.

In order to delineate the structural elements responsible for the prolongation and potentiation effects, a great number of D-amino acid-containing analogues of the pentapeptide representing the active centre have been synthesized and tested for melanotropic activity. These compounds and their biological activities are shown in Table XXXIX.

Table XXXIX

Melanocyte-stimulating Activity of Melanotropin Fragments and Analogues Containing D-Amino Acids

	Peptide	Activity MSH U/g	References
I	L-His-L-Phe-L-Orn-L-Trp-Gly	2.9×10^4	[218]
II	L-His-D-Phe-L-Orn-L-Trp-Gly	2.6×10^4	[218]
III	L-His-L-Phe-L-Arg-L-Trp-Gly	3.1×10^4	[305]
IV	L D L L	3.3×10^5	[293]
		1×10^6	[360]
V	D D D D	inhibitor	[113, 355]
VI	D L L L	inactive	[354, 356]
VII	L L L D	1×10^5	[354, 356]
VIII	L D D L	2.1×10^4	[359]
IX	D D D L	inactive	[359]
X	L D D D	inactive	[359]
XI	D L L D	inactive	[360]
XII	D D L L	5.5×10^4	[360]
XIII	L L D L	inactive	[357, 358]
		3×10^4	[196]
XIV	D L D L	inactive	[357, 358]
XV	L D L D	1×10^6	[357, 358]
XVI	D-Phe-D-Arg-D-Trp-Gly-Lys(For)-Pro-Val-NH_2	inhibitor	[348]
XVII	Glu-D-His-D-Phe-D-Arg-D-Trp-Gly-Lys(For)-Pro--Val-NH_2	1.5×10^4	[348]
XVIII	Ac-Ser-Tyr-Ser-Met-Glu-D-His-D-Phe-D-Arg--D-Trp-Gly-Lys-Pro-Val-NH_2	3×10^5	[348]

Hano *et al.* [113] synthesized D-His-D-Phe-D-Arg-D-Trp-Gly, the antipode of the active fragment. This substance had no darkening activity by itself, but lightened the skin which had been previously darkened with melanocyte-stimulating peptides. In addition, the all-D-peptide had the ability to prevent the darkening activity of similar amounts of the L-compound, thus it behaved like a competitive inhibitor specific to peptides which embody the active sequence of the melanotropin molecule, since it had no influence on the darkening action of caffeine. As the inhibitor property of this compound was not in conformity with the prolongation effect observed on the partial racemization of α-melanotropin, further variants of the penta-

peptide have been synthesized by Yajima and co-workers [354, 356]. The pentapeptide containing D-histidine residue was completely inactive (Table XXXIX, compound VI), but when tryptophan was replaced by the D-isomer, the synthetic pentapeptide (compound VII) possessed a melanocyte-stimulating activity slightly higher than that of the parent compound (Table XXXIX, compound III). Thus, in the case of the pentapeptide, incorporation of D-tryptophan resulted in some potentiation, but no retardation or prolongation of the biological effect. From the observation that both compounds possessing D-histidine residue (compounds V and VI) are inactive, it could be concluded that L-histidine plays an important part in eliciting melanocyte-stimulating activity. In order to clarify this point, Yajima *et al.* [359] prepared three further stereoisomers, the L-D-D-L, D-D-D-L and L-D-D-D analogues (Table XXXIX, compounds VIII, IX, X). Compound VIII was as active as the all-L-pentapeptide, but IX and X did not possess MSH-activity; in fact a weak inhibitory action toward α-melanotropin activity was noted. These results indicate that the presence of a single L-histidine or L-tryptophan residue is not sufficient for the melanotropic activity, when the rest of the amino acid residues possess D-configuration.

The synthesis and biological investigation of further pentapeptide isomers seem to have shed some light on the role of the configuration of the individual amino acids in the melanocyte-stimulating activity, at least until new developments in this field will demand new working hypothesis. These isomers and their biological potencies are also shown in Table XXXIX (compounds IV, XI, XII, XIII, XIV, XV) [357, 358, 360]. From the biological measurements of these derivatives it is most interesting that, in accordance with the observations of Schnabel and Li [293], the analogue containing D-phenylalanine but otherwise L-amino acid residues is more active than the all-L-pentapeptide, supporting the view that the receptor site does not exhibit a high degree of stereospecificity, as long as small peptides are concerned. It is remarkable that contrary to the results discussed so far, a peptide containing D-histidine can also be active if the phenylalanine is of the D-configuration but the second basic amino acid, arginine, has the natural configuration (compound XII). In the interpretation of Yajima *et al.* [358], aromatic residues with D-configuration exert a stronger binding force to cell receptors than the L-isomers do. When these binding forces are at the lowest value, as in peptides containing L-phenylalanine and L-tryptophan, both basic residues must be in the L-form to elicit a detectable cellular response. When the stronger binding D-phenylalanine or D-tryptophan is present, only one of the basic residues has to be of L-configuration in order

to stimulate the melanocytes. Whether this speculation is true for the analogues of the whole melanotropin sequence remains to be established. It is interesting to note that the all-D-retro-pentapeptide D-Trp-D-Arg-D-Phe--D-His-D-Glu synthesized by Chung and Li [40] proved to be essentially inactive.

Finally, the synthesis of α-melanotropin and some of its larger fragments containing D-amino acids in the active centre has also been described [348] (Table XXXIX, compounds XVI, XVII, XVIII). Two of them showed a very small melanocyte-stimulating activity (compounds XVII and XVIII). Investigations of this kind have, however, their limitations. Since extremely small quantities of contaminating L-peptides possessing high biological potency may influence the evaluation of the melanocyte-stimulating properties of the stereoisomers, the estimated activities cannot serve without reservation as a basis for conclusions concerning configuration–activity relationships.

Table XL

Comparison of the *in vitro* and *in vivo* Activities of Peptides Related to Melanotropin and Corticotropin

Peptide	MSH activity (U/g)	
	In vivo	*In vitro*
His-Phe-Arg-Trp-Gly	0.3×10^5	0.1×10^5
L D L L	2.5×10^6	2.3×10^6
D D L L	2.1×10^6	0.3×10^6
L L L D	7.0×10^5	0.8×10^5
L D D L	6.6×10^5	1.0×10^5
D D D L	1.0×10^5	0.4×10^5
L D D D	0.7×10^5	0.3×10^5
D L L D	0.4×10^5	0.1×10^5
D L L L	0.1×10^5	0.1×10^5
D D D D	$< 0.1 \times 10^5$	0.4×10^4
[Ac-Ser[1]]-ACTH-(1–24)-OH	1.6×10^9	0.5×10^9
ACTH-(1–16)-OMe	5.8×10^7	1.7×10^8
ACTH-(1–24)-N_2H_3	5.2×10^7	5.6×10^7
ACTH-(7–13)-NH_2	7.1×10^5	6.7×10^5
ACTH-(1–10)-OH	4.3×10^5	1.9×10^5
ACTH-(5–10)-OH	0.4×10^5	0.3×10^5
ACTH-(4–10)-OH	0.3×10^5	0.2×10^5
ACTH-(11–24)-OH	$< 0.02 \times 10^5$	0.02×10^5

Most of the activity data given in this Chapter were based on *in vitro* measurements following the original or modified procedure of Shizume *et al.* [319]. In this respect a comparison of the activities of stereoisomeric melanotropin fragments and some peptides related to ACTH by the *in vitro* and *in vivo* [165] method reported by Kastin *et al.* [166] seems to be worthwhile to mention here (Table XL).

4. BIOLOGICAL ACTIVITY OF DERIVATIVES RELATED TO THE β-MELANOTROPINS

No systematic investigations have been reported concerning structure–function relationships in β-melanotropins. However, the melanocyte-stimulating potency of some synthetic fragments and intermediates is known and these values are summarized in Table XLI.

Table XLI

Melanocyte-stimulating Activity of Peptides Related to the β-Melanotropins

Peptide	Activity MSH U/g	References
[Z-Asp(NH_2)^1,Lys(Tos)^6,Glu(NH_2)^8,Lys(Tos)^{17}]-β_b-MSH dimethyl ester	1.4×10^7	[303]
[Z-Glu(NH_2)^8,Lys(Tos)^{17}]-β_b-MSH-(8–18)-undecapeptide dimethyl ester	6.8×10^4	[303]
[Glu(NH_2)^8,Lys(Tos)^{17}]-β_b-MSH-(8–18)-undecapeptide dimethyl ester	8.4×10^4	[303]
H-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys(For)-Asp-OH	1×10^6	[346, 364]
	3×10^6	[361]
H-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH	2.4×10^6	[346, 364]
H-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys(For)-Asp-OH	6.0×10^6	[346, 361]
H-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys(For)--Asp-OH	1.9×10^6	[346, 361]
	2.3×10^7	[361]
H-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys(For)--Asp-OH	1.8×10^8	[346, 361]
H-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro--Lys(For)-Asp-OH	2.2×10^{12}	[346, 361]

Similarly to the α-melanotropin derivatives, the free ε-amino group of the lysine residue does not appear to be essential for the melanocyte-stimulating activity of β-MSH fragments. The same holds true of the ε-formyllysine derivatives of the monkey and human β-melanotropins, as both compounds possess a biological potency ([Lys(For)^{17}]-β_m-MSH: 2.0×10^9 U/g

[363], [Lys(For)^{21}]-β_h-MSH: 3.4×10^9 U/g [349]) equal to those of the corresponding natural hormones.

In view of the considerations concerning activities found in the range of 10^{11}–10^{12} U/g (p. 165), it seems dubious to interpret the surprisingly high potency of the pentadecapeptide (10^{12} U/g) as compared with the natural hormone (10^9 U/g), as a result of the shortened sequence.

VI. LABELLED DERIVATIVES OF ADRENOCORTICOTROPIC AND MELANOTROPIC HORMONES

In the foregoing the synthesis and biological activity of hormone fragments and hormone analogues have been discussed, which, beyond their therapeutic applicability, have been prepared with the aim to throw light on the main features of the relationships between chemical structure and biological functions. Recently the investigation of further important physiological properties of the polypeptide hormones has been initiated. In the living organism, between their biosynthesis and metabolism, these hormones participate in various, mostly specific biological processes, among which the hormone release, the active transport and the interaction with specific receptors deserve special emphasis.

Under physiological circumstances, in the natural environment these processes take place involving extremely small (10^{-12}–10^{-13} moles) amounts of the hormone. Since a considerably higher hormone concentration could exceed the capacity of the sites taking normally part in the biological processes, it is likely that the application of unphysiologically high doses would result in aspecific transport, binding and metabolism of the polypeptide hormone. For this reason, to obtain insight into the normal hormone mechanism, one should possibly work at physiological concentrations. It is, however, obvious that such small amounts of peptide material cannot be detected by chemical methods, and biological evaluations are applicable only in special cases. Therefore, in recent years a new trend can be observed, consisting in the modification of the polypeptide hormones by labelling, which makes possible the determination and quantitative estimation of the peptide in physiological amounts, by chemical or physicochemical means.

Leading to minimal, or even to no structural alteration, labelling with radioisotopes seems to be the most promising method. Without going into details, some aspects of selecting the suitable labelling will be briefly discussed below.

In general, it is advisable to label specifically in a particular amino acid, which is possibly situated in the active centre. Otherwise it might occur

that partial enzymic degradation separates biologically active sequences from the radioactive label, thus localization and measurement of the radioactivity would give misleading results. Any amino acid can be labelled with carbon-14, and the labelled amino acid can be used as starting material for the synthesis of polypeptides. Low specific activities and laborious synthetic work with expensive radioactive substances are the drawbacks of this procedure. High specific activity can be attained by the incorporation of tritium atoms: if the molecule contains precursor amino acids with suitable chemical properties (for example an aromatic halogen atom, unsaturated bonds) catalytic tritiation can be effected in one of the last steps of the synthesis, saving uncomfortable and tedious work with highly radioactive material. Finally, in special cases, radioiodination can be performed with satisfactory selectivity; this substitution implies, however, significant structural modifications of the molecule, which is often accompanied by changes in the biological properties.

In the first paper of a series Medzihradszky *et al.* [239] reported the synthesis of the N-terminal decapeptide of the adrenocorticotropic hormone labelled with carbon-14 on the C-terminal glycine residue. This peptide — which also possesses biological activities of its own [97, 140, 247, 268] — served in its protected form as starting material in the synthesis of the ^{14}C-labelled N-terminal heptadecapeptide amide of adrenocorticotropin ([^{14}C-Gly^{10}]-ACTH-(1–17)-heptadecapeptide amide) [240]. ^{14}C-Glycine was also used for labelling α-melanotropin, with a specific activity of 34 mCi/mmole [66]. Moroder and Hofmann [242] synthesized a labelled analogue of the N-terminal eicosapeptide amide of ACTH, containing ^{14}C-phenylalanine in the active centre ([Glu(NH$_2$)^5,^{14}C-Phe^7]-ACTH-(1–20)-eicosapeptide amide). Another ^{14}C-labelled analogue of the eicosapeptide ([^{14}C-Ala^1]-ACTH-(1–20)-eicosapeptide amide) has recently been synthesized by Blake and Li [21]. In spite of their relatively low specific radioactivity (0.1–0.4 mCi/mmole), these hormone analogues can be successfully applied to the investigation of the hypothetical corticotropin receptor in the adrenal [137]. Hofmann *et al.* were able to show that a particulate fraction prepared from beef adrenal cortical tissue, specifically bound this labelled eicosapeptide analogue, which, on the other hand, exhibited little activity for similarly prepared particulates from other organs. At the same time the [Glu(NH$_2$)^5, ^{14}C-Phe^7]-ACTH-(1–10)-decapeptide had very poor affinity for the particulate fraction, and the biologically inactive [Ac-Ser^1, Glu(NH$_2$)^5, Lys(For)^{11,15,16,21}]-ACTH-(1–23)-tricosapeptide amide also failed to displace radioactivity from the labelled eicosapeptide amide–particulate complex. These results verified the hypothesis that the basic sequence in positions

15–18 is an important binding site of the ACTH molecule. In addition, Hofmann *et al.* [137] observed that the α-melanotropin analogue [Glu(NH_2)^5, Lys(For)^{11}]-α-MSH, which is fully active as far as melanocyte-expansion is concerned, was also ineffective in displacing the radioactivity from the above mentioned complex. Correspondingly, the conclusion could be drawn that although α-MSH has the functionally important features of the ACTH molecule, it fails to stimulate the adrenal cortex, because it lacks the essential binding sites. On the other hand, both ACTH and melanotropin must possess common attachment sites for melanocytes. The same holds true of the structurally related [Gly^1]-ACTH-(1–14)-tetradecapeptide, which has been shown to be unable to displace ^{125}I-labelled human ACTH bound to isolated adrenal cells, while ACTH-(11–18)-octapeptide amide, containing the binding portion of the corticotropins, was highly effective in this respect [244].

Similar experiments have been conducted with the ^{14}C-labelled ACTH analogue using a plasma membrane fraction from bovine adrenal cortex [64]. The specificity of this reaction was again demonstrated by competitive binding studies with adrenocorticotropic hormone and corticotropin analogues. The labelled peptide-membrane complex could be used for the investigation of the relationships between binding properties and the apparent corticotropic activity of various corticotropin derivatives. For example [D-Ser^1, Lys^{17,18}]-ACTH-(1–18)-octadecapeptide amide [277] and [β-Ala^1, Lys^{17}]-ACTH-(1–17)-heptadecapeptide 4-aminobutylamide [76] were as effective as ACTH-(1–23)-tricosapeptide in displacing radioactivity from the membrane fraction, although they are much more potent than the natural sequence. It was suggested that their apparently higher biological potency is not the result of greater affinity for the binding sites, rather it reflects increased stability to enzymic cleavage. Finn *et al.* [64] also tried to establish the importance for binding of the individual lysine residues in the peptide fragment ACTH-(1–18)-octadecapeptide by alternatively substituting the lysine side chains with formyl groups. They concluded — although without presenting the experimental data — that all the lysine residues are important, especially the one in position 11.

These results clearly show that labelled compounds of low specific activity can be successfully used for binding studies between polypeptide hormones and receptor systems. Investigations of the biological reactions of polypeptide hormones are, however, greatly facilitated by increasing the specific activity, simultaneously widening the application possibilities.

Synthesis of the ACTH-(1–10)-decapeptide containing tyrosine labelled with tritium in the 3,5-positions of the benzene ring was described by Medzihradszky and co-workers [234, 240]. 3,5-^{3}H-Tyrosine with a specific

activity of about 3 Ci/mmole served as the starting material, and after eight synthetic steps the free decapeptide still contained 2 Ci/mmole radioactivity, some loss of activity having been observed in reactions where partial hydrogen–tritium exchange could not be avoided.

Synthesis of α-melanotropin labelled with tritium on the tyrosine residue has been reported by the same research group [235, 240]. Similarly to other polypeptide hormones, this compound exerts its biological function in very small amounts; as little as 0.02 ng of α-MSH per ml gives a positive effect in the skin darkening reaction, and melanocyte-stimulating substances can be found in the blood at a concentration of 1–2 units per ml [318]. Transport processes, metabolism and the mechanism of action of α-MSH can be studied by the use of this labelled hormone preparation possessing about 3 Ci/mmole specific activity. The tritiated hormone has also been synthesized in an alternative way [248]; this time 3,5-dibromotyrosine served as the precursor amino acid, and the synthetic hormone containing dibromotyrosine was transformed into the labelled derivative in the last step by catalytic tritiation. By this procedure, under proper tritiation conditions, the highest theoretically possible activity (50–60 Ci/mmole) can be attained. By the use of these precursor peptides the danger of autoradiolysis which may occur during storage can be minimized, since the required quantity of labelled product can always be prepared immediately before application.

Brundish and Wade [37] described the synthesis *via* the diiodo derivative of an ACTH-(1–24)-tetracosapeptide labelled with tritium in the tyrosine residue at position 23, possessing a specific radioactivity of 46 Ci/mmole. Starting from the partially protected 11–24 fragment [302], iodination of the tyrosine residue was effected by means of iodine monochloride, and the product was coupled with the protected N-terminal decapeptide. Deblocking with trifluoroacetic acid afforded the free iodinated peptide, which, in turn, was tritiated in the presence of palladium–rhodium catalyst. Incorporation of the tritium atom can thus be achieved in a highly specific way; neither the histidine or methionine, nor the second tyrosine residue near the N-terminus interfere with the labelling of the tyrosine[23]. The relatively great distance of the labelled site from the active centre should be mentioned as the only drawback of this procedure, as enzymic splitting of the C-terminal dipeptide would lead to the loss of the radioactivity in a biologically still fully active peptide. It is noteworthy that the free iodinated tetracosapeptide differed from the parent tetracosapeptide in its behaviour on ion-exchange chromatography and in its potency in an isolated adrenal cell bioassay, indicating that experimental results obtained by using polypeptide hormones labelled with radioiodine should be interpreted very carefully.

A tritiated ACTH fragment analogue, [Phe^2,4,5-^{3}H-Nva^4]-ACTH-(1–24)-tetracosapeptide, along with the non-labelled derivative, has been synthesized by Schwyzer and Karlaganis [304] with a specific radioactivity of 7.4 Ci/mmole. The protected precursor peptide contained an allylglycine residue in position 4; tritiation was accomplished in the presence of palladium–charcoal catalyst. The product, which had about 5% of its radioactivity in the histidine residue, possessed only 10% of the biological activity of the tetracosapeptide with the natural sequence [183, 304]. In sufficient dosage the labelled analogue proved to be a full hormonal agonist (*cf.* [244, 296]) in isolated rat lipocytes and adrenal cortical cells. The compound found application in the investigation of binding to cellular receptors.

Although radioiodination may produce labelled peptides with more or less altered biological properties, this relatively simple labelling technique is often used for preparing compounds with high specific radioactivity. Using a ^{125}I-labelled synthetic human ACTH with a specific activity of 600 Ci/mmole, Nakamura [244] was able to prove that peptides containing the 15–18 basic sequence (ACTH-(11–18)-octapeptide amide, [Gly^1]-ACTH-(1–18)-octadecapeptide amide) decreased the binding of the labelled hormone to isolated adrenal cells, whereas the [Gly^1]-ACTH-(1–14)-tetradecapeptide did not. This result, which is in agreement with the findings of Hofmann *et al.* again demonstrates the importance of the basic sequence of corticotropins in binding to the cell receptors.

As an example for another type of labelling, Schwyzer and Schiller [309] substituted the lysine ε-amino group in position 21 by the dansyl (1-dimethylaminonaphthalene-5-sulfonyl) group, supposing that this substitution, situated outside the active centre and the binding sites, would not interfere with the biological properties of the analogue. The resulting compound, [N$^\varepsilon$-dansyl-Lys^{21}]-ACTH-(1–24)-tetracosapeptide, could be used for investigations of interactions between hormones and their potential receptor molecules, as the dansyl substituent allows studies by fluorescence polarization, and measurements of the intramolecular and intermolecular distances by means of energy transfer. Thus it was established that glucose-6-phosphate dehydrogenase derived from the adrenal cortex specifically binds the hormone analogue. As far as conformational features are concerned, a fairly stable distance of 20–26 Å between the dansyl substituent and the indole side chain of the tryptophan in position 9 was established. The dansyl-labelled analogue exerts a similar but significantly lower activity than the parent peptide when tested on the receptors of isolated fat cell and adrenal cortical cell membrane preparations.

VII. CONCLUSIONS

The biological investigations on corticotropin and melanotropin fragments and analogues, discussed in the foregoing, have revealed several connections leading to obvious inferences. Such conclusions concerning the structure–activity relationships are all the more important as they have in many cases provided the basis for further structural modifications. In this last Chapter it seems reasonable to attempt giving a brief summing up of these results, along with the treatment of some relevant problems whose insertion into the earlier discussion might have broken the train of thought.

The hypothesis concerning the mode of action of polypeptide hormones presented by Hofmann [121] seems to be valid even today, and is supported by the study of a remarkable number of newly synthesized hormone derivatives. These investigations have led to a detailed knowledge of the role of the constituting amino acids in the biological action, and prompted speculations on the possible analogy between hormone–receptor interaction on the one hand, and the behaviour of the ribonuclease S-peptide S-protein system on the other [130].

It has become clear in the corticotropin–melanotropin series that small fragments of the original hormone molecule can be effective in evoking biological responses, as demonstrated by the corticotropic activity of the ACTH-(5–10)-hexapeptide [310], or by the melanocyte-stimulating property of the His-Phe-Arg-Trp tetrapeptide of melanotropins [254]. Thus, these peptides correspond to the active centre or active site of the hormone molecules. They are "full agonists", according to the terminology of Schwyzer [296] containing both the "address" portion of the molecule which enables the specific receptor to recognize the hormone, and the "message" part, capable of giving the receptor the necessary stimulus to display the characteristic biological activity.

The active site contains the amino acids whose side groups are functionally involved in the chemical events bringing about the specific physiological response. By definition, elimination of these groups, such as replacement of these amino acids by residues bearing other functional groups, should then result in complete loss of the biological activity. For a long time histidine and tryptophan had been regarded as amino acids "essential" for the melanocyte-stimulating activity (p. 205), until the limited but reproducible activity of the Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 heptapeptide amide (Table XXXV) was discovered. The essential role of the arginine residue can also be excluded, since its replacement by ornithine does not alter significantly the biological potency of the His-Phe-Arg-Trp-Gly pentapeptide.

Finally, there is no indication for the statement that phenylalanine or tryptophan would be indispensable for the melanotropic property; indeed, the activity of the L-His-D-Phe-L-Arg-D-Trp-Gly pentapeptide (Table XXXIX) points to the fact that at least the natural configuration is not necessary at these positions. On the basis of the available experimental evidence it is tempting to believe that none of the amino acids in the active site is of critical importance *per se* in eliciting melanocyte-stimulating activity, as far as the relatively small melanotropin fragments are concerned. It is then very likely that not a single "essential" side chain, but rather a proper combination of functional groups must be responsible for the productive interaction with the receptor unit. Consequently, it has to be assumed that contrary to the established mechanism of enzyme action, no chemical process attached to a particular amino acid residue is required in the hormone–receptor reaction. However, it should be borne in mind that systematic replacement of the individual amino acids in the active centre has only been performed with the 6–10-pentapeptide fragment possessing a very low degree of biological potency. A more secure basis for the confirmation or rejection, or even for the generalization, of the above hypothesis could be obtained by effecting similar alterations in the whole α-melanotropin molecule.

Similar conclusions can be reached also in the case of the adrenocorticotropic hormone. All amino acids in the active centre can be replaced by other residues, sometimes containing functional groups with markedly different chemical properties. Thus it appears likely that the free γ-carboxyl group of the glutamic acid residue is not essential for the stimulation of the adrenal cortex: ACTH-(1–20)-eicosapeptide amide and [$Glu(NH_2)^5$]-ACTH-(1–20)-eicosapeptide amide are approximately equipotent, both *in vivo* and *in vitro* [242]. The amphoteric character of the histidine-imidazole side chain was excluded as an essential condition for the biological activity by the experiment when this residue was replaced by the corresponding pyrazolyl-alanine [123] (p. 194). As far as the role of the arginine side-chain is concerned, Tesser found it to be necessary for the receptor stimulation [332], but its replacement by other basic amino acids resulted only in a decrease of the activity (p. 193), but not in full inactivation, indicating that though the guanidino group largely contributes to the biological function, it cannot be regarded as essential. The same can be said about the role of the tryptophan residue (p. 194), although experimental data point to the importance of this amino acid. ACTH substituted on the tryptophan residue by the *o*-nitrophenyl sulfenyl group ([Trp(Nps)^9]-ACTH) proved to be a specific and competitive inhibitor of ACTH-induced lipolysis in isolated

fat cells and fat cell ghosts [272, 273] as well as in other biological functions in isolated adrenal cells [243, 314]. The fairly high adrenal-stimulating potency of the [Leu^7]-ACTH-(1–24)-tetracosapeptide [73] points to the limited significance of the phenylalanine residue in position 7, although conversion of the L-phenylalanine into the D-derivative, as in the [β-Ala^1, D-Phe^7,Orn^{15}]-ACTH-(1–18)-octadecapeptide amide reduced the steroidogenic action by two orders of magnitude [244].

All data point to the possible main role of the amino acid sequence lying outside the active site in securing a favourable binding to the specific receptor unit. In the case of α-melanotropin, the lengthening of the peptide chain on both termini of the active centre brings about a million-fold rise in the biological potency (p. 206). The two binding sequences in the adrenocorticotropic hormone (the N-terminal pentapeptide and the basic core in positions 15–18) contribute to the binding to the ACTH-receptor to a different extent, the latter being more important than the first [81]. The experimental results have led to the conclusion that in these latter positions only the basicity is important: the constituting amino acids can be substituted by other basic ones without any significant change in the biological activity. From studies with a series of synthetic peptides related to ACTH a correlation seems to emerge between the corticotropic activity and the net positive charge of the basic core [215] (Table XLII).

This part of the molecule by interaction with the negatively charged portion of the receptor unit, may get the hormone active site into the pref-

Table XLII

Correlation between the Net Positive Charge and Biological Activity of Corticotropin Fragments

Peptide	Net positive charge on the C-terminus	Corticotropic activity U/mg
ACTH-(1–16)-OH	+1	<1
ACTH-(1–16)-OMe ACTH-(1–17)-OH	+2	1–10
ACTH-(1–17)-NH_2 ACTH-(1–18)-OH ACTH-(1–19)-OH	+3	10–70
ACTH-(1–18)-NH_2 ACTH-(1–19)-NH_2 ACTH-(1–20)-NH_2	+4	>70

erable contact with the receptor surface, necessary to evoke a series of specific physiological events. The observations of Brugger *et al.* [35] on the decreased potency of the nonadecapeptides containing neutral amino acids instead of the arginine residues in positions 17–18, is somewhat inconsistent with the net charge hypothesis. These analogues were less potent than the hexadecapeptide amide with the same number of net positive charges. Brugger *et al.* explained this result by a suppressed binding capacity of the hormone to the receptor caused by the added neutral residues.

The importance of an optimal steric arrangement of binding and active sites in a hormone molecule for productive interaction with the receptor unit is shown by examples both in the adrenocorticotropic and melanotropic hormones. Omission of the Lys-Pro-Val-Gly tetrapeptide sequence occupying positions 11 to 14 from the ACTH-(1–19)-nonadecapeptide, (ACTH-(1–10 + 15–19)-pentadecapeptide) (p. 173), led to a sharp decrease in the corticotropic activity, although this peptide contains both the amino acid region believed to form the active centre and the positively charged core required for binding to the receptor. Omission of the glycine residue in position 10 had the same effect for the melanocyte-stimulating activity of [desGly^{10}]-α-melanotropin [237]. While in the latter case only the optimal distance between the two sequences with different functions has been changed, Ramachandran *et al.* [271] suggested the possibility that in the corticotropin analogue the missing proline residue could significantly influence conformational relations.

In the course of the investigation of structure–activity relationships of the adrenocorticotropic hormone, efforts were directed towards a possible differentiation between the structural elements responsible for the various biological properties. It was shown in the foregoing that active centre and binding sites for the corticotropic behaviour could be distinguished with certainty. There are also data available for the more or less successful structural separation of the different biological functions. As an example, the periodate-borohydride treated corticotropin can be mentioned, which had lost most of its *in vivo* adrenal-stimulating activity, retaining a great part of its extra-adrenal properties. Boright *et al.* [31] explained this observation by the specific binding of the N-terminal serine to the adrenal cortex, which is known today to be rather an increased binding of the terminal amino group [57, 87, 184, 261]. Selective binding specific to the different tissues ("address") is followed by a relatively nonspecific stimulus, which is capable of influencing biological processes in a variety of tissues without a specific discrimination, the active site in corticotropins responsible for the corticotropic and melanotropic activity being the same.

A further example for selective binding and thus for a structural separation of the corticotropic and melanocyte-stimulating activity is acetylation of the N-terminal serine residue of corticotropin derivatives, resulting in enhancement of the melanotropic and decrease in the corticotropic potency [247, 276] (p. 180). The synthetic ACTH-(1–10 + 15–19)-pentadecapeptide (p. 173) retained the full lipid-mobilizing potency, but possessed only a greatly diminished melanocyte-stimulating and corticotropic activity as compared with the natural hormone, thus providing another good example for the structural separation of different biological features. Obviously, combination of the N-terminal part and the basic core represent sufficient binding force for the fat cell receptor, while the Lys-Pro-Val-Gly tetrapeptide sequence lying between them serves only to recognize melanotropin and corticotropin receptors. The importance for melanocyte-stimulation of the tripeptide sequence in positions 11–13 is shown by the much higher activity of the tridecapeptide in comparison with the decapeptide (10^{10} *vs.* 10^6 U/g, Tables XXXV and XXXVI, p. 204, 208). To determine whether the amino acid side chains of this tripeptide sequence are important for the biological activity, or the only role of this part of the molecule is to secure optimal distance between the active centre and basic core of corticotropins, Blake and Li [17] synthesized analogues in which the lysine, proline and valine residues have been replaced by glycine. Corticotropic and melanocyte-stimulating activities of these analogues are shown in Table XLIII, indicating that the biological properties greatly depend on the specific character of the amino acids in positions 11–13.

Little can be said at present about the role of amino acid residues occupying positions which lie outside the active centre and which, according to our knowledge, do not necessarily possess binding properties. These may be involved in the manifestation of the various physiological features of the hormone, such as the transport of the polypeptide from the producing organ, or from the site of administration, to the receptor unit. It has been reported that the intact molecule remains longer in circulation than its fragments, and its half life in various tissues was found to be higher than that of its analogues possessing enhanced corticotropic potency [154]. The steroidogenic activity of the native hormone is, on molar basis, higher than that exhibited by the synthetic fragments [247, 271, 327] (but see [186] for somewhat contradictory results). Studies on the interaction of ACTH and ACTH fragments with serum proteins, although giving no unequivocal results, point to some differences in binding and biological activity, in favour of the intact hormone [323]. Comparison of the molar activities of natural corticotropins [8, 247] shows significant divergency, which seems

Table XLIII

Biological Activity of Corticotropin Fragments Modified in the Sequence 11–13

Peptide	Steroidogenesis U/μmole	MSH activity	
		in vivo μmole	*in vitro* U/μmole
α_s-ACTH	617	4.4×10^{-5}	2.9×10^{5}
ACTH-(1–19)-nonadecapeptide	82	8.7×10^{-5}	3.3×10^{5}
ACTH-(1–17)-heptadecapeptide amide	88	9.6×10^{-5}	4.2×10^{5}
ACTH-(1–10+15–19)-pentadecapeptide	0.8	1×10^{-2}	3.7×10^{3}
[$Gly^{11,12,13}$]-ACTH-(1–19)-nonadecapeptide	0.3	2.5×10^{-3}	6×10^{3}
[$Gly^{12,13}$]-ACTH-(1–17)-heptadecapeptide amide	1.0	5×10^{-3}	4×10^{3}
[$Gly^{11,13}$]-ACTH-(1–17)-heptadecapeptide amide	<0.5	5×10^{-3}	6×10^{3}
[Gly^{13}]-ACTH-(1–17)-heptadecapeptide amide	2.8	2.5×10^{-4}	8×10^{4}

to be a consequence of structural differences in the C-terminal region. The immunological property of corticotropins is also associated with the C-terminal sequences possessing somewhat different structures in positions 25–33 [151, 220]. Terminal sequences may be responsible for the diminished susceptibility of the native hormones towards the action of intracellular and peripheral peptidases. In addition to the increased basicity, this can be the reason for the enhanced biological activity of peptides terminating in prolinol [250] or simply bearing an amide group, compared with the corresponding acids. The increased potency of peptide analogues containing unnatural amino acids has also been explained by enzyme resistance. It should be mentioned, however, that although the theory of the diminished susceptibility of some hormone derivatives towards digestion by exopeptidases seems to be quite obvious and is widely accepted, there is no direct evidence for this hypothesis, and even the enzymes, which may be responsible for the breakdown of polypeptide hormones, are mostly unknown.

No conclusions have been drawn thus far concerning the structural basis for the lipolytic activity of corticotropins and corticotropin fragments. Many data are compiled in Tables IV, IX, XII, XIII, XIX, XXV, XXVIII, so it is sufficient here to briefly summarize the available results. The *in vitro* adipokinetic activity is greatly dependent on the species of the test animal. When rat adipose tissue is used, a positive correlation between adipokinetic and steroidogenic activity can be found, while lipolysis in the rabbit tissue is independent of the corticotropic potency [270, 330]. The difference

between the mode of action of corticotropin fragments on rat adipose tissue as compared with rabbit, is not clear at present. The lack of a hypothetical enzyme system capable of degrading corticotropin peptides in the rabbit tissue may be responsible for its enhanced sensitivity to ACTH [286].

The natural corticotropin molecules exhibit a nearly identical activity on both tissues (Table IV, p. 155). ACTH fragments containing at least 17 amino acids from the N-terminus retain their full lipolytic activity, which decreases by one order of magnitude on the stepwise shortening of the peptide chain from the carboxyl end (Table XIII, p. 177). This finding, along with the observation that even the 4–10 sequence and its Arg^5-analogue exhibit lipolytic activity [61] again indicates a common active site for adipokinetic, melanotropic and corticotropic activity. As far as separation of the lipolytic and adrenocorticotropic properties is concerned, Rittel [281] was unable to find analogues where the effects could be dissociated in any significant degree. On the other hand, the ACTH-(1–10 + 15–19)-pentadecapeptide investigated by Li *et al.* [216] exhibited an adipokinetic activity as high as that of the natural corticotropins (p. 173), while losing almost all of its melanocyte-stimulating and corticotropic potency. It should be noted, however, that this measurement was made on rabbit adipose tissue, which is, as mentioned above, about 100 times more sensitive than the rat, when tested for the activity of corticotropin fragments (Table XII, p. 176).

It is not within the scope of this review to give a detailed account on further biological effects of corticotropins. It is worth mentioning, however, that structure–function studies are not limited to the investigations of corticotropic, melanotropic and adipokinetic properties; structure-dependent are, for example, also the psychopharmacological effects of corticotropin fragments. Extinction of the conditioned avoidance behaviour, or in general, acceleration of the extinction rate of inhibition reactions have been studied by deWied and Greven, in relation to the length of the peptide chain [97, 98]. A similar dependence of the adrenal and ovarian blood flow on the chain length of various corticotropin fragments has been found by Stark *et al.* [321]: natural porcine and synthetic human ACTH augmented blood flow much less than did the N-terminal peptides. Changing the length of ACTH fragments influenced the blood-flow raising adrenal effect to an extent different from the extra-adrenal one, offering evidence to show that alteration of the chain length may separate the adrenal and extra-adrenal effects. Finally, the corticotropin-releasing activity, a specific property of short-chain peptides derived from the N-terminal portion of the adrenocorticotropic hormone should be mentioned here [162, 219, 268] (*cf.* [122], p. 239).

Synthesis and biological testing of hormone fragments and analogues are undoubtedly the most promising approach toward assessing structural requirements for biological activity. This type of experimentation has, however, certain limitations, which have to be kept in view when planning further research work in this field. Selection of the most suitable hormone analogue for a given purpose appears to be important. Unequivocal identification of side chain groups necessary for biological functions can be successful only when hormone analogues with minimal modifications are used. More complex alteration of the amino acid residues can result in change of the biological activity either in consequence of the omission of the original side chain, or owing to the newly introduced functional group. It seems to be much better to exchange amino acids closely related to each other, such as residues possessing different alkyl side chains, to study the effects of changing apolarity and stereochemical features. Replacement of glycine possessing no side chain by any other residue, or *vice versa*, may also provide a clear picture on the role of functional groups in the biological action. Substitutions such as Lys-Orn, Arg-Cit, His-Pyr(3)Ala, Met-Met(O) or Tyr-Phe can be regarded as other good approaches, since these modifications involve changes of a single structural feature. It is rather difficult to evaluate for example, a carboxyl–carboxamide replacement, as extinction of the acidic function is accompanied by the formation of a possible hydrogen bond system; similar difficulty may be encountered when amino acids other than glycine are eliminated from the sequence, since the steric arrangement of the remaining parts of the peptide and functional groups will change simultaneously. Drastic structural modifications are justified only in localizing the smallest, still biologically active, fragments of the hormone molecule.

It is evident that well-established conclusions can only be based on reliable biological test methods. Measurements, where even that is questionable, whether intravenous or subcutaneous applications produce higher biological potency, may be the source of interesting observations, yet fully inadequate as a basis of clearing up structure–activity relationships. It should also be borne in mind that biological evaluation of peptide hormone derivatives generally involves comparison with the natural hormone as standard, and this comparison will be in accordance with reality only when both the natural hormone and the tested compound are similarly influenced by the various factors prevailing under the circumstances of the biological measurement [142]. Little is known, however, about the mode of action of peptide hormones and hormone derivatives; the results obtained in such a way have therefore to be interpreted with reservation.

Investigation of structure–activity relationships requires hormone analogues of maximal purity. The inescapable degree of contaminations, such as L-isomer under 0.1% in an analogue containing D-amino acids, or small amount of methionine in a methionine-S-oxide derivative set limits to the validity of conclusions, especially when the parent molecule is much more active than its modified derivative. Similar considerations are valid for impurities possessing inhibitor properties, although this is a rather rare situation.

Caution is advisable in the generalization of the results. Modification of an analogue often has quite an other effect than the same alteration of another derivative. A very good example for this possibility can be found in the oxytocin field; deamino-oxytocin [150] is about twice as active as oxytocin, yet deamino-[Thr^4]-oxytocin is less potent, although [Thr^4]-oxytocin itself is much more active than the native hormone [228].

Looking back upon the data and evaluation of the results summarized in the present review, the question may arise whether the information gained about the structure–activity relationships come up to the expectations, and if they are commensurable with the vast amount of experimental work involved in the synthesis and biological examination of hormone fragments and analogues. It can be established that investigations of corticotropin and melanotropin derivatives greatly contributed to the localization of active sites and sequential portions responsible for binding to the receptor. Conclusions could be drawn concerning physiologically important stereochemical features of the hormones, and possible chemical properties of the receptor unit were proposed, which are necessary to recognize the hormone molecule. Use of hormone analogues labelled with radioactive isotopes made possible the discovery and isolation of hormone receptors and the kinetic study of hormone–receptor interactions. As these results could not have been reached in any other way, this kind of experimentation seems to have been justified in retrospect, and will most probably find application also in the future.

REFERENCES

1. Baines, N. J., Baird, J. B., Elmore, D. T., "The kinetics of hydrolysis of derivatives of arginine, homoarginine and ornithine by trypsin." *Biochem. J.*, *90*, 470 (1964).
2. Bajusz, S., Kisfaludy, L., Medzihradszky, K., Paulay, Z., Lőw, M., "Synthesis of the N-terminal octacosapeptide of porcine corticotropin." *Acta Chim. (Budapest)*, in preparation.
3. Bajusz, S., Lázár, T., "Synthesis of peptides related to the C-terminal 25–39 sequences of corticotropins." *Acta Chim. Acad. Sci. Hung.*, *48*, 111 (1966).

4. Bajusz, S., Lénárd, K., "Synthesis of the dodecapeptide sequence 10–21 of β-corticotropin." *Coll. Czech. Chem. Comm., 27,* 2257 (1962).
5. Bajusz, S., Lénárd, K., Kisfaludy, L., Medzihradszky, K., Bruckner, V., "Synthese eines Dodecapeptid-Derivats für den Aufbau corticotrop wirksamer Polypeptide." *Acta Chim. Acad. Sci. Hung., 30,* 239 (1962).
6. Bajusz, S., Medzihradszky, K., "Erfahrungen über das Verhalten geschützter Peptide bei der Reaktion mit Natrium in flüssigem Ammoniak." in "Peptides. Proc. 5th European Peptide Symp., Oxford, 1962", (Ed. Young, G. T.) p. 49, MacMillan, London, 1963.
7. Bajusz, S., Medzihradszky, K., "Synthesis and biological activity of corticotropin fragments." in "Peptides. Proc. 8th European Peptide Symp., Noordwijk, 1966" (Eds. Beyerman, H. C., van de Linde, A., Maassen van den Brink, W.) p. 209, North Holland, Amsterdam, 1967.
8. Bajusz, S., Medzihradszky, K., Kisfaludy, L., Lőw, M., Paulay, Z., Láng, Zs., "Synthesis of human ACTH and its fragments." in "Proc. of the Vth Conf. Hung. Therap. Pharmacol.", p. 47 (1968).
9. Bajusz, S., Medzihradszky, K., Kisfaludy,L., Lőw, M., Paulay, Z., Láng, Zs., Szporny, L., "Eljárás emberi corticotropin szintézisére." (Process for the synthesis of human corticotropin.) Hung. Pat., 155.254; *Chem. Abstr., 71,* 70936 (1969).
10. Bajusz, S., Medzihradszky, K., Paulay, Z., Láng, Zs., "Totalsynthese des menschlichen Corticotropins (α_h-ACTH)." *Acta Chim. Acad. Sci. Hung., 52,* 335 (1967).
11. Bajusz, S., Paulay, Z., Láng, Zs., Medzihradszky, K., Kisfaludy, L., Lőw, M., "Synthesis and biological properties of human corticotropin and its fragments." in "Peptides. Proc. 9th European Peptide Symp., Paris, 1968" (Ed. Bricas, E.) p. 237, North Holland, Amsterdam, 1968.
12. Barthe, P., Desaulles, P. A., Schär, B., Staehelin, M., "Biological activity of synthetic β-corticotropin." *Nature, 202,* 908 (1964).
13. Bell, P. H., "Purification and structure of β-corticotropin." *J. Am. Chem. Soc., 76,* 5565 (1954).
14. Bell, P. H., Howard, K. S., Shepherd, R. G., Finn, B. M., Meisenhelder, J. H., "Studies with corticotropin. II. Pepsin degradation of β-corticotropin." *J. Am. Chem. Soc., 78,* 5059 (1956).
15. Birnbaumer, L., Rodbell, M., "Adenyl cyclase in fat cells. II. Hormone receptors." *J. Biol. Chem., 244,* 3477 (1969).
16. Blake, J., Crooks, R. W., Li, C. H. "Solid-phase synthesis of [5-glutamine]-α-melanotropin." *Biochemistry, 9,* 2071 (1970).
17. Blake, J., Li, C. H., "The syntheses and biological activities of [$Gly^{11,12,13}$]-α-ACTH-(1–19)-nonadecapeptide, [$Gly^{12,13}$]-α-ACTH-(1–17)-heptadecapeptide amide, [$Gly^{11,13}$]-α-ACTH-(1–17)-heptadecapeptide amide and [Gly^{13}]-α-ACTH-(1–17)-heptadecapeptide amide." *Biochim. Biophys. Acta, 147,* 386 (1967).
18. Blake, J., Li, C. H., "Adrenocorticotropins. 39. The solid-phase synthesis of methionylglutamylhistidylphenylalanylarginyltryptophylglycine." *J. Am. Chem. Soc., 90,* 5882 (1968).
19. Blake, J., Li, C. H., "The solid-phase synthesis of alpha-melanotropin." *Int. J. Protein Res., 3,* 185 (1971).
20. Blake, J. Li, C. H., "Adrenocorticotropin. 43. The synthesis and steroidogenic activity of [2-N-methylphenylalanine]-adrenocorticotropin-(1–19)." *Int. J. Peptide Protein Res., 4,* 343 (1972).
21. Blake, J., Li, C. H., "Adrenocorticotropin. 45. Synthesis and steroidogenic activity of [1-alanine]-adrenocorticotropin-(1–20)-amide, [1-D-alanine]-adrenocorticotropin-(1–20)-amide and [1-^{14}C-alanine]-adrenocorticotropin-(1–20)-amide." *J. Med. Chem., 17,* 233 (1974).
22. Blake, J., Wang, K.-T., Li, C. H. "Adrenocorticotropin. Solid-phase synthesis of α^{1-19}-adrenocorticotropic hormone, alanyl-α^{1-19}-adrenocorticotropic hormone and prolyl-α^{1-19}-adrenocorticotropic hormone and their adrenocorticotropic activity." *Biochemistry, 11,* 438 (1972).
23. Bodánszky, M., Ondetti, M. A., Rubin, B., Piala, J. J., Fried, J., Sheehan, J. T., Birkhimer, C. A., "Biologically active citrulline peptides." *Nature, 194,* 482 (1962).

24. Boissonnas, R. A., Guttmann, St., Huguenin, R. L., Jacquenoud, P.-A., Sandrin, Ed., "Synthèse de la L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophanyl-glycyl-ε-CBO-L-lysyl-L-prolyl-L-valyl-amide." *Helv. Chim. Acta, 41*, 1867 (1958).
25. Boissonnas, R. A., Guttmann, St., Jacquenoud, P.-A., Sandrin, Ed., Waller, J. -P., "Synthèse du glycyl-N$^{\varepsilon}$CBO-L-lysyl-L-prolyl-L-valyl-glycyl-N$^{\varepsilon}$-CBO-L-lysyl-N$^{\varepsilon}$-CBO-L-lysyl-L-arginyl-L-arginyl-L-prolyl-L-valinate de méthyle, un peptide représentant la séquence 10–20 de l'ACTH." *Helv. Chim. Acta, 44*, 123 (1961).
26. Boissonnas, R. A., Guttmann, St., Pless, J., "Preparation of a pentacosapeptide." Belg. Pat., 636.667; *Chem. Abstr., 62*, 633c (1965).
27. Boissonnas, R. A., Guttmann, St., Pless, J., "Synthesis of D-Ser^{1}-Nle^{4}-(Val-NH$_2$)^{25}-β-corticotropin-(1–25), a highly potent analogue of ACTH." *Experientia, 22*, 526 (1966).
28. Boissonnas, R. A., Guttmann, St., Pless, J., "A new polypeptide." Belg. Pat., 653.017; *Chem. Abstr. 64*, 12792c (1966).
29. Boissonnas, R. A., Guttmann, St., Pless, J., Doepfner, W., "ACTH-like pentacosapeptide injection preparation." Swiss Pat., 456.849; *Chem. Abstr., 70*, 38100 (1969).
30. Boissonnas, R. A., Guttmann, St., Waller, J.-P., Jacquenoud, P.-A., "Synthesis of a polypeptide with ACTH-like structure." *Experientia, 12*, 446 (1956).
31. Boright, H. A., Engel, F. L., Lebovitz, H. E., Kostyo J. L., White, J. E., "A dissociation between the adrenal and extra-adrenal actions of corticotrophine by periodate oxidation." *Biochem. J., 83*, 95 (1962).
32. Bruckner, V., Bajusz, S., Kisfaludy, L., Medzihradszky, K., Lőw, M., Paulay, Z., Szporny, L., Hajós, Gy., "Biológiailag aktív polipeptidek." (Biologically active polypeptides.) Hung. Pat., 155.880; *Chem. Abstr., 72*, 3777 (1970).
33. Bruckner, V., Medzihradszky, K., Bajusz, S., Kisfaludy, L., "Biológiailag aktív polipeptidek." (Biologically active polypeptides.) Hung. Pat., 151.214 (1962).
34. Brugger, M., "Synthese von lipophil substituierten ACTH-Peptiden." *Helv. Chim. Acta, 54*, 1261 (1971).
35. Brugger, M., Barthe, P., Desaulles, P. A., "Structure and activity of corticotrophic peptides: Synthesis and biological activity of two corticotrophic peptides with neutral amino acids in position 17 and 18." *Experientia, 26*, 1050 (1970).
36. Brugger, M., Iselin, B., Rittel, W., "D-Serine containing peptides with ACTH activity." Ger. Offen., 2,027.346; *Chem. Abstr., 74*, 142357 (1971).
37. Brundish, D. E., Wade, R., "Synthesis of [3,5-^{3}H$_2$-Tyr^{23}]-β-corticotrophin-(1–24)-tetracosapeptide." *J. Chem. Soc. (Perkin I), 1973*, 2875.
38. Burton, J., Lande, S., "Semisynthetic polypeptides. Transformation of native porcine β-melanotropin into the lysine-10 analogue of the human hormone." *J. Am. Chem. Soc., 92*, 3746 (1970).
39. Chung, D., Li, C. H., "Adrenocorticotropins. XXXVII. The synthesis of lysine^{8}-α-$^{1-17}$-ACTH-NH$_2$ and its biological properties." *J. Am. Chem. Soc., 89*, 4208 (1967).
40. Chung, D., Li, C. H., "The synthesis of D-tryptophyl-D-arginyl-D-phenylalanyl-D-histidyl-D-glutamic acid and its effect on melanotropic activity." *Biochim. Biophys. Acta, 136*, 570 (1967).
41. Ciba Ltd., "ACTH 1–19 and its therapeutically valuable salts." French Pat., M. 1561; *Chem. Abstr., 59*, 8872e (1963).
42. Ciba Ltd. "New peptides with adrenocorticotropin activity." Neth. Appl., 6,602.538; *Chem. Abstr., 67*, 22144 (1967).
43. Ciba Ltd., "Peptides with ACTH activity." French Pat., 1,506.515; *Chem. Abstr., 70*, 106889 (1969).
44. Ciba Ltd., "Peptides in which the first amino acid has the D-configuration." Fr. Addn., 92.368; Addn. to Fr. Pat., 1,512.342; *Chem. Abstr., 72*, 3776 (1970).
45. Ciba Ltd., "ACTH-active peptide amides." Brit. Pat., 1,231.494; *Chem. Abstr., 75*, 36698 (1971).
46. Ciba Ltd., "Peptides with the first amino acid having the D-configuration and with ACTH effect." Fr. Addn., 94.933; *Chem. Abstr., 77*, 20022 (1972).
47. Ciba Ltd., "Peptides with the first amino acid having the D-configuration and with ACTH effect." Fr. Addn., 94.938; *Chem. Abstr., 77*, 102249 (1972).
48. Cseh, G., unpublished results.

49. Cseh, Gy., Fövényi, J., Góth, E., Gráf, L., Tamási, Gy., "Lipotróp hormon (β-lipotropin) kimutatása emberi hypophysisben." (Detection of a lipotropic hormone (β-lipotropin) in human hypophysis.) *Orvosi Hetilap, 110,* 1546 (1969).
50. Dedman, M. L., Farmer, T. H., Morris, C. J. O. R., "Oxidation–reduction properties of adrenocorticotrophic hormone." *Biochem. J., 59,* XII (1955).
51. Dedman, M. L., Farmer, T. H., Morris, C. J. O. R., "Studies on pituitary adrenocorticotrophin. 2. Oxidation–reduction properties of the hormone." *Biochem. J., 66,* 166 (1957).
52. Dedman, M. L., Farmer, T. H., Morris, C. J. O. R., "Studies on pituitary adrenocorticotrophin. 3. Identification of the oxidation–reduction centre." *Biochem. J., 78,* 348 (1961).
53. Desaulles, P. A., Rittel, W., "Investigation of hypothalamic pituitary-adrenal function." *Memoirs of the Soc. Endocr.,* No. *17,* 125 (1968).
54. Desaulles, P. A., Zahnd, G. R., "β^{1-13}Corticotropin derivatives." Ger. Offen., 2,158.123; *Chem. Abstr., 77,* 75489 (1972).
55. Dixon, H. B. F., "The action of periodate on ACTH." *Biochem. J., 62,* 25p. (1956).
56. Dixon, H. B. F., "Treatment of corticotrophin with periodate and borohydride." *Biochem. J., 83,* 91 (1962).
57. Dixon, H. B. F., Weitkamp, L. R., "Conversion of the N-terminal serine residue of corticotrophin into glycine." *Biochem. J., 84,* 462 (1962).
58. Dixon, J. S., Li, C. H., "The isolation and structure of α-melanocyte-stimulating hormone from horse pituitaries." *J. Am. Chem. Soc., 82,* 4568 (1960).
59. Dixon, J. S., Li, C. H., "The isolation and structure of β-melanocyte-stimulating hormone from horse pituitary glands." *Gen. and Comp. Endocrinol., 1,* 161 (1961).
60. Doepfner, W., "Biological characterization of a new and highly potent synthetic analogue of corticotrophin." *Experientia, 22,* 527 (1966).
61. Draper, M. W., Merrifield, R. B., Rizack, M. A., "Lipolytic activity of Met--Arg-His-Phe-Arg-Trp-Gly, a synthetic analog of the ACTH-(4–10) core sequence." *J. Med. Chem., 16,* 1326 (1973).
62. Engel, F. L., "Extra-adrenal actions of adrenocorticotropin." *Vitamins, Hormones, 19,* 189 (1961).
63. Fekete, Gy., "Synthetic ACTH." in "Polypeptide hormones." Proc. Fourth Congr. of the Hungarian Society of Endocrinology and Metabolism, Budapest, 1969, p. 65.
64. Finn, F. M., Widnell, C. C., Hofmann, K., "Localization of an adrenocorticotropic hormone receptor on bovine adrenal cortical membranes." *J. Biol. Chem., 247,* 5695 (1972).
65. Fisher, J. D., "Adrenocorticotropin," in Methods in Hormone Research, *2,* 617 (1962).
66. Fittkau, S., Medzihradszky, K., Seprődi, J., "Synthese von [1-^{14}C] Glycin^{10}-α-melanotropin." *J. Prakt. Chem., 316,* 679 (1974).
67. Flückiger, E., *Acta Endocrinol. Suppl., 51,* 333 (1960); *Arch. Exptl. Pathol. Pharmakol., 245,* 168 (1963); cited in [295] p. 186.
68. Fujino, M., Hatanaka, C., Nishimura, O., "Synthesis of peptides related to corticotropin (ACTH). I. Synthesis of α-ACTH-(1–23)-tricosapeptide amide." *Chem. Pharm. Bull., 17,* 2186 (1969).
69. Fujino, M., Hatanaka, C., Nishimura, O., "Synthesis of peptides related to corticotropin (ACTH). III. Syntheses of α^{1-23}-NH_2-ACTH and β-alanine^1-α^{1-23}-NH_2-ACTH." *Chem. Pharm. Bull., 18,* 771 (1970).
70. Fujino, M., Hatanaka, C., Nishimura, O., "Synthesis of peptides related to corticotropin (ACTH). IV. Syntheses of β-alanine^1-, γ-aminobutyric acid^1-, sarcosine^1-, and lysiproline^1- ne^1-α^{1-24}-ACTH." *Chem. Pharm. Bull., 18,* 1288 (1970).
71. Fujino, M., Hatanaka, C., Nishimura, O., "Synthesis of peptides related to corticotropin (ACTH). VI. Syntheses and biological activity of the peptides corresponding to the amino acid sequences 4–23, 5–23, 6–24, 7–23 in ACTH." *Chem. Pharm. Bull., 19,* 1066 (1971).
72. Fujino, M., Hatanaka, C., Nishimura, O., Sanno, Y., "Polypeptide compounds related to adrenocorticotropic hormones." Ger. Offen., 2,055.151; *Chem. Abstr., 75,* 98824 (1971).

73. FUJINO, M., HATANAKA, C., NISHIMURA, O., SHINAGAWA, S., "Synthesis of peptides related to corticotropin (ACTH). VII. Syntheses and biological activity of leucine^{7}-α^{1-24}-ACTH and N$^{\alpha}$-methyltryptophan^{9}-α^{1-24}-ACTH." *Chem. Pharm. Bull., 19*, 1075 (1971).
74. FUJINO, M., NISHIMURA, O., HATANAKA, C., "Synthesis of peptides related to corticotropin (ACTH). II. Synthesis of the protected peptides corresponding to the sequence 1–3, 4–6, 7–10, 11–14, 15–19 and 20—23 of ACTH." *Chem. Pharm. Bull., 17*, 2135 (1969).
75. FUJINO, M., NISHIMURA, O., HATANAKA, C., "Synthesis of peptides related to corticotropin (ACTH). V. Syntheses of leucine^{4}- and isoleucine^{4}-α^{1-24}-ACTH." *Chem. Pharm. Bull., 18*, 1291 (1970).
76. GEIGER, R., "Synthese eines Heptadecapeptides mit hoher adrenocorticotroper Wirkung. *Ann. 750*, 165 (1971).
77. GEIGER, R., SCHRÖDER, H.-G., Unpublished results, cited in [76].
78. GEIGER, R., SCHRÖDER, H.-G., "Adrenocorticotropic peptides." Ger. Offen., 1,954.794; *Chem. Abstr., 75*, 49596 (1971).
79. GEIGER, R., SCHRÖDER, H.-G., "Pentadecapeptides with adrenocorticotropic effects." Ger. Offen., 1,965.101; *Chem. Abstr., 75*, 64279 (1971).
80. GEIGER, R., SCHRÖDER, H.-G., "Tridecapeptides with adrenocorticotropic effects." Ger. Offen., 1,965.102; *Chem. Abstr., 75*, 64280 (1971).
81. GEIGER, R., SCHRÖDER, H.-G., "New short-chain synthetic corticotrophin analogues with high corticotrophic activity" in "Progress in Peptide Research." (Ed. LANDE, S.) Vol. II. p. 273, Gordon and Breach, New York, 1972.
82. GEIGER, R., SCHRÖDER, H.-G., "Synthetische Analoga des Corticotropins. Der Beitrag von Lysin in Position 11 zur biologischen Wirkung. *H.-S. Z. Physiol. Chem., 354*, 156 (1973).
83. GEIGER, R., SCHRÖDER, H.-G., SIEDEL, W., "Synthetische Analoga des Corticotropins. Veränderung der N-terminalen Amino-Gruppe des Corticotropin-(1–23)-trikosipeptid-amides." *Ann., 726*, 177 (1969).
84. GEIGER, R., STURM, K., SIEDEL, W., "Synthese von Peptiden mit der Aminosäuresequenz 1–10 des Corticotropins." *Chem. Ber., 96*, 1080 (1963).
85. GEIGER, R., STURM, K., SIEDEL, W., "Synthese eines biologisch aktiven Tricosapeptidamides mit der Aminosäuresequenz 1–23 des Corticotropins (ACTH)." *Chem. Ber., 97*, 1207 (1964).
86. GEIGER, R., STURM, K., SIEDEL, W., "Peptide with ACTH activity." Ger. Pat., 1,246.747; *Chem. Abstr., 68*, 30059 (1968).
87. GEIGER, R., STURM, K., VOGEL, G., SIEDEL, W., "Synthetische Analoga des Corticotropins. Zur Bedeutung der aminoterminalen Sequenz Ser-Tyr-Ser für die adrenocorticotrope Wirkung." *Z. für Naturforsch., 19b*, 858 (1964).
88. GESCHWIND, I. I., "Comparative Endocrinology." (Ed. GORBMAN, A.) p. 421. Wiley, New York, 1959.
89. GESCHWIND, I. I., LI, C. H., "Corticotropins (ACTH). V. The application of oxidation with periodate to the determination of the N-terminal serine in α-corticotropin." *Biochim. Biophys. Acta, 15*, 442 (1954).
90. GESCHWIND, I. I., LI, C. H., BARNAFI, L., "Isolation and structure of melanocyte-stimulating hormone from porcine pituitary glands." *J. Am. Chem. Soc., 78*, 4494 (1956).
91. GESCHWIND, I. I., LI, C. H., BARNAFI, L., "The isolation, characterization and amino acid sequence of a melanocyte-stimulating hormone from bovine pituitary glands." *J. Am. Chem. Soc., 79*, 6394 (1957).
92. GESCHWIND, I. I., LI, C. H., "The reaction of several protein hormones with periodate." *Endocrinology, 63*, 449 (1958).
93. GLASS, J. D., SCHWARTZ, I. L., WALTER, R., "Enzymic inactivation of peptide hormones possessing a C-terminal amide group." *Proc. Natl. Acad. Sci. USA, 63*, 1426 (1969).
94. GRÁF, L., "Re-examination of the sequence of the C-terminal tryptic fragment from porcine adrenocorticotropic hormone." *Acta Biochim. Biophys. Acad. Sci. Hung., 7*, 293 (1972).

95. Gráf, L., Bajusz, S., Patthy, A., Barát, E., Cseh, G., "Revised amide location for porcine and human adrenocorticotropic hormone." *Acta Biochim. Biophys. Acad. Sci. Hung., 6,* 415 (1971).
96. Gráf, L., Cseh, G., Nagy, I., Kurcz, M., "An evidence for deamidation of prolactin monomer." *Acta Biochim. Biophys. Acad. Sci. Hung., 5,* 299 (1970).
97. Greven, H. M., De Wied, D., "The active sequence in the ACTH molecule responsible for inhibition of the extinction of conditioned avoidance behaviour in rats." *European J. Pharmacol., 2,* 14 (1967).
98. Greven, H. M., "Psychopharmacologically active peptides and peptide derivatives." Ger Offen., 2,311.099; *Chem. Abstr., 79,* 146853 (1973); Ger. Offen., 2,315.826; *Chem. Abstr., 80,* 15212 (1974) etc.
99. Gros, C., Leygues, C., "Étude des peptides isolés à partir de la posthypophyse de porc." *Bull. Soc. Chim. France,* 2840 (1964).
100. Guillemin, R., "Steroidogenic activities of purified α-, β-, γ-, δ-corticotropins and pepsin degradation products of β-corticotropin." *Endocrinology, 66,* 819 (1960).
101. Guillemin, R., Clayton, G. W., Lipscomb, H. S., Smith, J. D., "Fluorometric measurements of rat plasma and adrenal corticosteron concentration." *J. Lab. Clin. Med., 53,* 830 (1959).
102. Guttmann, St., "On the use of the tosyl group for the protection of basic amino acids." in "Peptides. Proc. 5th European Peptide Symp., Oxford, 1962," (Ed. Young, G. T.) p. 41. MacMillan, London, 1963.
103. Guttmann, St., Boissonnas, R. A., "Synthèse du N-acétyl-L-séryl-L-tyrosyl--D-méthionyl-γ-L-glutamate de benzyle et de peptides apparantés." *Helv. Chim. Acta, 41,* 1852 (1958).
104. Guttmann, St., Boissonnas, R. A., "Synthèse de l'α-mélanotropine (α-MSH) de porc." *Helv. Chim. Acta, 42,* 1257 (1959).
105. Guttmann, St., Boissonnas, R. A., "Influence of the structure of the N-terminal extremity of α-MSH on the melanophore stimulating activity of this hormone." *Experientia, 17,* 265 (1961).
106. Guttmann, St., Boissonnas, R. A., Pless, J., "Adrenocorticotropic pentacosapeptides." Swiss Pat., 459.252; *Chem. Abstr., 70,* 58299 (1969).
107. Guttmann, St., Boissonnas, R. A., Pless, J., "Tetracosapeptides." French Pat., 1,513.943; *Chem. Abstr., 71,* 70934 (1969).
108. Guttmann, St., Boissonnas, R. A., Pless, J., "Pentacosapeptides." French Pat., 1,513.944; *Chem. Abstr., 71,* 81734 (1969).
109. Guttmann, St., Pless, J., Boissonnas, R. A., "Synthesis of peptides related to ACTH." in Proc. 7th European Peptide Symp., Budapest, 1964; *Acta Chim. Acad. Sci. Hung., 44,* 141 (1965).
110. Guttmann, St., Pless, J., Boissonnas, R. A., "Synthesis of a highly active peptide related to corticotropin." in "Peptides. Proc. 8th European Peptide Symp., Noordwijk, 1966" (Eds. Beyerman, H. C., van de Linde, A., Maassen van den Brink, W.) p. 221, North Holland, Amsterdam, 1967.
111. Guttmann, St., Pless, J., Boissonnas, R. A., "Therapeutic pentacosapeptides." Swiss Pat., 494.741; *Chem. Abstr., 74,* 54178 (1971).
112. Hamburger, C., "Assay of corticotrophin with prednisolone pretreatment instead of hypophysectomy in the adrenal ascorbic acid test." *Acta Endocrinol., 35,* 594 (1960).
113. Hano, K., Koida, M., Kubo, K., Yajima, H., "Evaluation of the physiological properties of D-histidyl-D-phenylalanyl-D-arginyl-D-tryptophyl-glycine in frog melanocyte." *Biochim. Biophys. Acta, 90,* 201 (1964).
114. Hano, K., Koida, M., Yajima, H., Kubo, K., Oshima, T., "Studies on peptides. X. Sodium hydroxide treatment of L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine and its potentiated melanocyte-stimulating activity *in vitro*." *Biochim. Biophys. Acta, 115,* 337 (1966).
115. Harris, J. I., "Structure of a melanocyte-stimulating hormone from the human pituitary gland." *Nature, 184,* 167 (1959).
116. Harris, J. I. "Studies on pituitary polypeptide hormones. 3. The structure of α-melanocyte-stimulating hormone from pig pituitary glands." *Biochem. J., 71,* 451 (1959).

117. Harris, J. I., "The chemistry of pituitary polypeptide hormones." *Brit. Med. Bull., 16,* 189 (1960).
118. Harris, J. I., Lerner, A. B., "Amino acid sequence of the α-melanocyte-stimulating hormone." *Nature, 179,* 1346 (1957).
119. Harris, J. I., Roos, P., "Amino acid sequence of a melanophore-stimulating peptide". *Nature, 178,* 90 (1956).
120. Hays, E. E., White, W. F., "The chemistry of the corticotropins." *Recent Progress in Hormone Research, 10,* 265 (1954).
121. Hofmann, K., "Preliminary observations relating structure and function in some pituitary hormones." *Brookhaven Symposia in Biol., 13,* 184 (1960).
122. Hofmann, K., "Chemistry and function of polypeptide hormones." *Ann. Rev. Biochem., 31,* 213 (1963).
123. Hofmann, K., Andreatta, R., Bohn, H., Moroder, L., "Studies on polypeptides. XLV. Structure–function studies in the β-corticotropin series." *J. Med. Chem., 13,* 339 (1970).
124. Hofmann, K., Jöhl, A., Furlenmeier, A. E., Kappeler, H., "Studies on polypeptides. VIII. Synthesis of peptides related to corticotropin." *J. Am. Chem. Soc., 79,* 1636 (1957).
125. Hofmann, K., Kappeler, H., Furlenmeier, A. E., Woolner, M. E., Schwartz, E. T., Thompson, T. A., "Studies on polypeptides, IX. Synthesis of peptides containing basic amino acid residues, related to corticotropin and intermedin." *J. Am. Chem. Soc., 79,* 1641 (1957).
126. Hofmann, K., Katsoyannis, P. G., "Synthesis and function of peptides of biological interest." in "The Proteins." Second edition, Vol. I. (Ed. Neurath, H.) p. 54, Academic Press, New York, 1963.
127. Hofmann, K., Lande, S., "Studies on polypeptides. XIX. Improved routes to His-Phe-Arg-Trp-Gly, a key intermediate in the synthesis of ACTH peptides." *J. Am. Chem. Soc., 83,* 2286 (1961).
128. Hofmann, K., Liu, T.-y. Yajima, H., Yanaihara, N., Lande, S., "Studies on polypeptides. XXI. The synthesis of a partially protected tridecapeptide amide corresponding to positions 11 to 23 in the corticotropin sequence." *J. Am. Chem. Soc., 83,* 2294 (1961).
129. Hofmann, K., Liu, T.-y., Yajima, H., Yanaihara, N., Yanaihara, C., Humes, J. L., "Studies on polypeptides. XXII. High adrenocorticotropic activity in the rat and in man of a synthetic eicosapeptide amide." *J. Am. Chem. Soc., 84,* 1054 (1962).
130. Hofmann, K., Montibeller, J. A., Finn, F. M., "ACTH-antagonists." *Proc. Natl. Acad. Sci. USA, 71,* 80 (1974).
131. Hofmann, K., Rosenthaler, J., Wells, R. D., Yajima, H., "Studies on polypeptides. XXVIII. Elimination of the methionine residue as an essential functional unit for *in vivo* adrenocorticotropic activity." *J. Am. Chem. Soc., 86,* 4991 (1964).
132. Hofmann, K., Stutz, E., Spühler, G., Yajima, H., Schwartz, E. T., "Studies on polypeptides. XVI. The preparation of N^{ε}-formyl-lysine and its application to the synthesis of peptides." *J. Am. Chem. Soc., 82,* 3727 (1960).
133. Hofmann, K., Thompson, T. A., Schwartz, E. T., "Studies on polypeptides. XI. Preparation of an octapeptide possessing melanocyte-stimulating activity." *J. Am. Chem. Soc., 79,* 6087 (1957).
134. Hofmann, K., Thompson, T. A., Woolner, M. E., Spühler, G., Yajima, H., Cipera, J. D., Schwartz, E. T., "Studies on polypeptides. XV. Observations on the relation between structure and melanocyte expanding activity of synthetic peptides." *J. Am. Chem. Soc., 82,* 3721 (1960).
135. Hofmann, K., Thompson, T. A., Yajima, H., Schwartz, E. T., Inouye, H., "Studies on polypeptides. XIV. The synthesis of peptides related to the N-terminus of alpha-MSH and of corticotropins." *J. Am. Chem. Soc., 82,* 3715 (1960).
136. Hofmann, K., Wells, R. D., Yajima, H., Rosenthaler, J., "Studies on polypeptides. XXVII. Elimination of the methionine residue as an essential functional unit for *in vivo* adrenocorticotropic activity." *J. Am. Chem. Soc., 85,* 1546 (1963).

137. HOFMANN, K., WINGENDER, W., FINN, F. M., "Correlation of adrenocorticotropic activity of ACTH analogues with degree of binding to an adrenal cortical particulate preparation." *Proc. Natl. Acad. Sci. USA, 67*, 829 (1970).
138. HOFMANN, K., WOOLNER, M. E., SPÜHLER, G., SCHWARTZ, E. T., "Studies on polypeptides. X. The synthesis of a pentapeptide corresponding to an amino acid sequence present in corticotropin and in melanocyte-stimulating hormones." *J. Am. Chem. Soc., 80*, 1486 (1958).
139. HOFMANN, K., WOOLNER, M. E., YAJIMA, H., SPÜHLER, G., THOMPSON, T. A., SCHWARTZ, E. T., "Studies on polypeptides. XII. The synthesis of a physiologically active blocked tridecapeptide amide possessing the amino acid sequence of alpha-MSH." *J. Am. Chem. Soc., 80*, 6458 (1958).
140. HOFMANN, K., YAJIMA, H., "Studies on polypeptides. XX. Synthesis and corticotropic activity of a peptide amide corresponding to the N-terminal tridecapeptide sequence of the corticotropins." *J. Am. Chem. Soc., 83*, 2289 (1961).
141. HOFMANN, K., YAJIMA, H., "Synthetic pituitary hormones." *Recent Progress in Hormone Research, 18*, 41 (1962).
142. HOFMANN, K., YAJIMA, H., LIU, T.-Y., YANAIHARA, N., "Studies on polypeptides. XXIV. Synthesis and biological evaluation of a tricosapeptide possessing essentially the full biological activity of ACTH." *J. Am. Chem. Soc., 84*, 4475 (1962).
143. HOFMANN, K., YAJIMA, H., LIU, T.-Y., YANAIHARA, N., YANAIHARA, C., HUMES, J. L., "Studies on polypeptides. XXV. The adrenocorticotropic potency of an eicosapeptide amide corresponding to the N-terminal portion of the ACTH molecule; contribution to the relation between peptide chain-length and biological activity." *J. Am. Chem. Soc., 84*, 4481 (1962).
144. HOFMANN, K., YAJIMA, H., SCHWARTZ, E. T., "Studies on polypeptides. XIII. The synthesis of a derivative of α-MSH possessing a high degree of melanocyte-expanding activity *in vitro*." *Biochim. Biophys. Acta, 36*, 252 (1959).
145. HOFMANN, K., YAJIMA, H., SCHWARTZ, E. T., "Studies on polypeptides. XVII. The synthesis of three acyl tridecapeptide amides possessing a high level of melanocyte-expanding activity *in vitro*." *J. Am. Chem. Soc., 82*, 3732 (1960).
146. HOFMANN, K., YAJIMA, H., YANAIHARA, N., LIU, T.-Y., LANDE, S., "Studies on polypeptides. XVIII. The synthesis of a tricosapeptide possessing essentially the full biological activity of natural ACTH." *J. Am. Chem. Soc., 83*, 487 (1961).
147. HOFMANN, K., YANAIHARA, N., LANDE, S., YAJIMA, H., "Studies on polypeptides. XXIII. Synthesis and biological activity of a hexadecapeptide corresponding to the N-terminal sequence of the corticotropins." *J. Am. Chem. Soc., 84*, 4470 (1962).
148. HOGBEN, L. T., SLOME, D., "Pigmentary effector system VI. The dual character of endocrine coordination in amphibian color change." *Proc. Roy. Soc. (London), 108B*, 10 (1931).
149. HOLLENBERG, C. H., RABEN, M. S., ASTWOOD, E. B., "The lipolytic response to corticotropin." *Endocrinology, 68*, 589 (1961).
150. HOPE, D. B., MURTI, V. V. S., DUVIGNEAUD, V., "A highly potent analogue of oxytocin, desamino-oxytocin." *J. Biol. Chem., 237*, 1563 (1962).
151. IMURA, H., SPARKS, L. L., GROTSKY, G. M., FORSHAM, P. H., "Immunologic studies of adrenocorticotropic hormone; dissociation of biologic and immunologic activities". *J. Clin. Endocrinol. Metab., 25*, 1361 (1965).
152. INOUYE, K., "Syntheses of peptides related to the N-terminal structure of corticotropin. V. Synthesis of the peptide fragments, His-Phe-Arg-Trp, His-Phe-Arg-Trp-Gly and Glu-His-Phe-Arg-Trp-Gly." *Bull. Chem. Soc. Japan, 38*, 1148 (1965).
153. INOUYE, K., TANAKA, A., OTSUKA, H., "Synthesis of corticotropin peptides. XI. Synthesis and biological properties of [1-β-alanine]-ACTH-(1–18)-octadecapeptide amide." *Bull. Chem. Soc. Japan, 43*, 1163 (1970).
154. INOUYE, K., WATANABE, K., NAMBA, K., OTSUKA, H., "Synthesis of corticotropin peptides. XII. The effect of amino-terminal substitution in the corticotropin 1–18 peptide." *Bull. Chem. Soc. Japan, 43*, 3873 (1970).
155. ISELIN, B., KAPPELER, H., RINIKER, B., RITTEL, W., "Peptides with ACTH activity." South African Pat., 6,800.178; *Chem. Abstr., 70*, 78367 (1969).

156. Iselin, B., Kappeler, H., Riniker, B., Rittel, W., "Peptides with ACTH activity." South African Pat., 6,705.324; *Chem. Abstr.*, *70*, 106890 (1969).
157. Iselin, B., Schwyzer, R., "Synthese von Peptid-Zwischenprodukten für den Aufbau eines corticotrop wirksamen Nonadecapeptids. II. Derivate des L-Seryl-L-tyrosyl-L-serins." *Helv. Chim. Acta*, *44*, 169 (1961).
158. Iselin, B., Schwyzer, R., "Synthese von Peptid-Zwischenprodukten für den Aufbau des β-Melanophoren-stimulierenden Hormons (β-MSH) des Rindes. I. Geschützte Peptidsequenzen 1–6 und 1–7." *Helv. Chim. Acta*, *45*, 1499 (1962).
159. Jenny, M., Muller, A. F., Mach, R. S., "The adrenocorticotropic action of a new synthetic pentacosapeptide." *Experientia*, *22*, 528 (1966).
160. Kappeler, H., "Synthese von L-Glutaminyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycin." *Helv. Chim. Acta*, *44*, 476 (1961).
161. Kappeler, H., Riniker, B., Rittel, W., Desaulles, P., Maier, R., Schär, B., Staehelin, M., "Synthesis and biological activity of peptides related to ACTH." in "Peptides, Proc. 8th European Peptide Symp., Noordwijk, 1966" (Eds. Beyerman, H. C., van de Linde, A., Maassen van den Brink, W.) p. 209, North Holland, Amsterdam, 1967.
162. Kappeler, H., Schwyzer, R., "Synthese eines Heptapeptids mit starker CRF-Wirkung." *Helv. Chim. Acta*, *43*, 1453 (1960).
163. Kappeler, H., Schwyzer, R., "Synthetic peptides related to the corticotropins (ACTH) and the melanophore-stimulating hormones (MSH) possessing corticotropin releasing activity (CRF-activity)." *Experientia*, *16*, 415 (1960).
164. Kappeler, H., Schwyzer, R., "Die Synthese eines Tetracosapeptides mit der Aminosäuresequenz eines hochaktiven Abbauproduktes des β-Corticotropins (ACTH) aus Schweinehypophysen." *Helv. Chim. Acta*, *44*, 1136 (1961).
165. Kastin, A. J., Ross, G. T., "Modified *in vivo* assay for MSH." *Experientia*, *20*, 461 (1964).
166. Kastin, A. J., Schally, A. V., Yajima, H., Kubo, K., "Melanocyte-stimulating hormone activity of synthetic MSH and ACTH peptides *in vivo* and *in vitro*." *Nature*, *207*, 978 (1965).
167. Kisfaludy, L., Dualszky, S., "*p*-Chlorcarbobenzoxy-Aminosäuren und -Peptide. I." *Acta Chim. Acad. Sci. Hung.*, *24*, 301 (1960).
168. Kisfaludy, L., Dualszky, S., "Synthese der Heptapeptidsequenz 22–28 des β-Corticotropins." *Coll. Czech. Chem. Comm.*, *27*, 2258 (1962).
169. Kisfaludy, L., Dualszky, S., Medzihradszky, K., Bajusz, S., Bruckner, V., "Synthese eines Heptapeptid-Derivats für den Aufbau corticotrop wirksamer Polypeptide." *Acta Chim. Acad. Sci. Hung.*, *30*, 473 (1962).
170. Kisfaludy, L., Lőw, M., "Synthesis of the 1–28 and 1–32 fragments of human corticotropin." *Acta Chim. Acad. Sci. Hung.*, *58*, 231 (1968).
171. Kisfaludy, L., Lőw, M., Dancsi, L., Patthy, A., Nyéki, O., Sárközi, M., "α-Aminooxyacids and derivatives." in "Peptides, Proc. of the 12th European Peptide Symp., Reinhardsbrunn, 1972." (Eds. Hanson, H., Jakubke, H. D.) p. 409, North Holland, Amsterdam, 1973.
172. Kisfaludy, L., Lőw, M., Dancsi, L., Schön, I., Szirtes, T., Nyéki, O., Szirmai, M., Szporny, L., Hajós, Gy., "Peptides containing α-aminooxy acids in N-terminal position, with ACTH action." Ger. Offen., 2,341.775; *Chem. Abstr.*, *80*, 133841 (1974).
173. Kisfaludy, L., Lőw, M., Szirtes, T., Schön, I., Sárközi, M., Bajusz, S., Turán, A., Juhász, A., Beke, R., Gráf, L., Medzihradszky, K., "A novel method for the synthesis of longer peptides including the corrected structure of human ACTH and its fragments" in "Chemistry and Biology of Peptides. Proc. 3rd American Peptide Symposium, 1972." p. 299, Ann Arbor Sci. Publ. (1972).
174. Kovács, J., Kisfaludy, L., Ceprini, M. Q., "On the optical purity of peptide active esters prepared by N,N′-dicyclohexylcarbodiimide and complexes of N,N′-dicyclohexylcarbodiimide-pentachlorophenol and N,N′-dicyclohexylcarbodiimide-pentafluorophenol." *J. Am. Chem. Soc.*, *89*, 183 (1967).
175. Kovács, K., László, F. A., Dursz, F., Szijj, I., Faredin, I., Tóth, I., Czakó, L., Biró, A., Julesz, M., "Effects of synthetic human corticotrophin." *Lancet*, 698 (1968).

176. KUEHL, F. A., MEISINGER, M. A. P., BRINK, N. G., FOLKERS, K., "Pituitary hormones. VI. The purification of corticotropin-B by countercurrent distribution." *J. Am. Chem. Soc., 75,* 1955 (1953).
177. LANDE, S., "The synthesis of peptides related to α-melanocyte-stimulating hormone and the corticotropins." Thesis, University of Pittsburgh, 1960.
178. LANDE, S., LERNER, A. B., "The biochemistry of melanotropic agents." *Pharm. Rev., 19,* 1 (1967).
179. LANDE, S., LERNER, A. B., "Racemization of α-melanotropin." *Biochim. Biophys. Acta, 251,* 246 (1971).
180. LANDE, S., LERNER, A. B., UPTON, G. V., "Pituitary peptides. Isolation of new peptides related to β-melanocyte-stimulating hormone." *J. Biol. Chem., 240,* 4259 (1965).
181. LANDGREBE, F. W., MITCHELL, G. M., "A purified extract of the intermediate lobe of pituitary." *J. Exp. Physiol., 39,* 11 (1954).
182. LANDGREBE, F. W., WARING, H., "Biological assay and standardization of melanophore expanding pituitary hormone." *Quart. J. Exper. Physiol., 33,* 1 (1944).
183. LANG, V., KARLAGANIS, G., SEELIG, S., SAYERS, G., SCHWYZER, R., "Hormone-receptor interactions. Biological activities of [Phenylalanine^2, norvaline^4]-adrenocorticotropin-(1–24)-tetrakosipeptide and its 4,5-dehydro-4,5-ditritio-norvaline^4 analogue in isolated rat lipocytes and adrenal cortex cells: lipolysis, corticosterone and *cyclic* adenosine-3′,5′-monophosphate-production." *Helv. Chim. Acta, 56,* 1069 (1973).
194. LEBOVITZ, H. E., ENGEL, F. L., "Relationships between the structure and biological activities of corticotropin and related peptides; alterations in the N-terminal serine." *Endocrinology, 73,* 573 (1963).
185. LEBOVITZ, H. E., ENGEL, F. L., "Relationships between the structure and biological activities of corticotropin and related peptides." *Metabolism Clin. Exper., 13,* 1230 (1964) (Brook Lodge Conference, Kalamazoo, Mich., 1963).
186. LEBOVITZ, H. E., ENGEL, F. L., "Relations between the structure and biological activities of corticotropin and related peptides. Biological activities of a synthetic eicosapeptide amide." *Endocrinology, 75,* 831 (1964).
187. LEE, T. H., BUETTNER-JANUSCH, V., "On the mechanism of sodium hydroxide modification of α-melanocyte-stimulating hormone." *J. Biol. Chem., 238,* 2012 (1963).
188. LEE, T. H., LEE, M. S., "Studies on human pituitary α-melanocyte-stimulating hormone." *Fed. Proc. Fed. Amer. Soc. Exp. Biol., 32,* Abstr. 490 (1973).
189. LEE, T. H., LERNER, A. B., "Isolation of MSH from hog pituitary gland." *J. Biol. Chem., 221,* 943 (1956).
190. LEE, T. H., LERNER, A. B., BUETTNER-JANUSCH, V., "Isolation and structure of human corticotropin (ACTH)." *J. Am. Chem. Soc., 81,* 6084 (1959).
191. LEE, T. H., LERNER, A. B., BUETTNER-JANUSCH, V., "The isolation and structure of α- and β-melanocyte-stimulating hormones from monkey pituitary glands." *J. Biol. Chem., 236,* 1390 (1961).
192. LEE, T. H., LERNER, A. B., BUETTNER-JANUSCH, V., "On the structure of human corticotropin (adrenocorticotrop hormone)." *J. Biol. Chem., 236,* 2970 (1961).
193. LEE, T. H., LERNER, A. B., BUETTNER-JANUSCH, V., "Species differences and structural requirements for melanocyte-stimulating activity of melanocyte-stimulating hormones." *Ann. N. Y. Acad. Sci., 100,* 658 (1963).
194. LÉONIS, J., LI, C. H., CHUNG, D., "Corticotropins (ACTH). XV. The action of chymotrypsin on α-corticotropin." *J. Am. Chem. Soc., 81,* 419 (1959).
195. LERNER, A. B., LANDE, S., KULOVICH, S., "Biological properties of racemized α-MSH, β-MSH and ACTH." *Excerpta Med. Int. Congr. Ser.,* No. *83,* 392 (1965).
196. LERNER, A. B., LEE, T. H., "The melanocyte-stimulating hormones." *Vitamins, Hormones, 20,* 337 (1962).
197. LERNER, A. B., MCGUIRE, J. S., "Melanocyte-stimulating hormone and adrenocorticotrophic hormone. Their relation to pigmentation." *The New England J. of Med., 270,* 539 (1964).
198. LERNER, A. B., SHIZUME, K., BANDING, I., "The mechanism of endocrine control of melanin pigmentation." *J. Clin. Endocrin. Metab., 14,* 1463 (1954).

199. Lerner, A. B., Wright, M. R., "*In vitro* frog skin assay for agents that darken and lighten melanocytes." *Methods of Biochemical Analysis, 8*, 295 (1960).
200. Li, C. H., "Adrenocorticotropic hormone (ACTH, corticotropin)." *Advances in Protein Chemistry, 11*, 138 (1956).
201. Li, C. H., "Hormones of the anterior pituitary gland. II. Melanocyte-stimulating and lactogenic hormones." *Advances in Protein Chemistry, 12*, 269 (1957).
202. Li, C. H., "The relation of chemical structure to the biologic activity of pituitary hormones." *Lab. Invest., 8*, 574 (1959).
203. Li, C. H., "Synthesis and biological properties of ACTH peptides." *Recent Progress in Hormone Research, 18*, 1 (1962).
204. Li, C. H., "Adrenocorticotropin 45. Revised amino acid sequences for sheep and bovine hormones." *Biochem. Biophys. Res. Comm., 49*, 835 (1972).
205. Li, C. H., Chung, D., Ramachandran, J., "A new synthesis of a biologically active nonadecapeptide corresponding to the first nineteen amino acid residues of adrenocorticotropins." *J. Am. Chem. Soc., 86*, 2715 (1964).
206. Li, C. H., Chung, D., Ramachandran, J., Gorup, B., "The synthesis of a heptadecapeptide possessing adrenocorticotropic, melanotropic and lipolytic activities." *J. Am. Chem. Soc., 84*, 2460 (1962).
207. Li, C. H., Dixon, J. S., Chung, D., "The structure of bovine corticotropin." *J. Am. Chem. Soc., 80*, 2587 (1958).
208. Li, C. H., Geschwind, I. I., Cole, R. D., Raacke, I. D., Harris, J. I., Dixon, J. S., "Amino acid sequence of alpha-corticotropin." *Nature, 176*, 687 (1955).
209. Li, C. H., Geschwind, I. I., Levy, A. L., Harris, J. I., Dixon, J. S., Pon, N. G., Porath, J. O., "Isolation and properties of alpha-corticotropin from sheep pituitary glands." *Nature, 173*, 251 (1954).
210. Li, C. H., Gorup, B., Chung, D., Ramachandran, J., "A new synthesis of Glu-His-Phe-Arg-Trp-Gly." *J. Org. Chem., 28*, 178 (1963).
211. Li, C. H., Hemmasi, B., "Adrenocorticotropin. 40. The synthesis of a protected nonapeptide and of a biologically active nonadecapeptide related to adrenocorticotropic hormone. [5-glutamine]-adrenocorticotropin-(1–19)." *J. Med. Chem., 15*, 217 (1972).
212. Li, C. H., Hemmasi, B., "Solid phase synthesis of seryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine hydrazide and its N^{ε}-*tert*-butyloxycarbonyl derivative and their melanotropic activity." *J. Med. Chem., 15*, 697 (1972).
213. Li, C. H., Meienhofer, J., Schnabel, E., Chung, D., Lo, T. B., Ramachandran, J., "The synthesis of a nonadecapeptide possessing adrenocorticotropic and melanotropic activities." *J. Am. Chem. Soc., 82*, 5760 (1960).
214. Li, C. H., Meienhofer, J., Schnabel, E., Chung, D., Lo, T. B., Ramachandran, J., "Synthesis of a biologically active nonadecapeptide corresponding to the first nineteen amino acid residues of adrenocorticotropins." *J. Am. Chem. Soc., 83*, 4449 (1961).
215. Li, C. H., Oelofsen, W., "The chemistry and biology of ACTH and related peptides." in "The Adrenal Cortex" (Ed. Eisenstein, A. B.), p. 185, Churchill, London, 1967.
216. Li, C. H., Ramachandran, J., Chung, D., "Synthesis of a biologically active pentadecapeptide corresponding to an altered sequence in the adrenocorticotropin (ACTH) structure." *J. Am. Chem. Soc., 85*, 1895 (1963); *86*, 2711 (1964).
217. Li, C. H., Ramachandran, J., Chung, D., Gorup, B., "Synthesis of a biologically active heptadecapeptide related to adrenocorticotropin." *J. Am. Chem. Soc., 86*, 2703 (1964).
218. Li, C. H., Schnabel, E., Chung, D., "The synthesis of L-histidyl-L-phenylalanyl-L-ornithyl-L-tryptophyl-glycine and L-histidyl-D-phenylalanyl-L-ornithyl-L-tryptophyl-glycine and their melanocyte-stimulating activity." *J. Am. Chem. Soc., 82*, 2062 (1960)
219. Li, C. H., Schnabel, E., Chung, D., Lo, T. B., "Synthesis of L-methionyl-L-glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine and its melanocyte-stimulating and corticotropin-releasing activity." *Nature, 189*, 143 (1961).

220. LIDDLE, G. W., GIVENS, J. R., NICHOLSON, W. E., ISLAND, D. F., "The ectopic ACTH syndrome." *Excerpta Medica Found. Int. Congr. Series*, No. *83*, 1063 (1965).
221. LIPSCOMB, H. S., NELSON, D. H., "A sensitive biologic assay for adrenocorticotropin (ACTH)." *Endocrinology*, *71*, 13 (1962).
222. LO, T. B., DIXON, J. S., LI, C. H., "Isolation of methionine sulfoxide analogue of α-melanocyte-stimulating hormone from bovine pituitary glands." *Biochim. Biophys. Acta*, *53*, 584 (1961).
223. LOHMAR, P., LI, C. H., "Biological properties of ovine β-lipotropic hormone." *Endrocrinology*, *82*, 898 (1968).
224. LOWRY, P. A., CHADWICK, A., "Purification and amino acid sequence of melanocyte-stimulating hormone from the dogfish *Squalus acanthias*." *Biochem. J.*, *118*, 713 (1970).
225. LŐW, M., KISFALUDY, L., "Some observations with N-hydroxysuccinimide esters." in "Proc. 7th European Peptide Symp., Budapest, 1964" *Acta Chim. Acad. Sci. Hung. 44*, 61 (1965).
226. LŐW, M., KISFALUDY, L., PATTHY, A., SZIRMAI, M., NYÉKI, O., SZPORNY, L., HAJÓS, GY., "Peptides containing α-aminooxy acids on both ends of the chain, with ACTH action." Ger. Offen., 2,342.862; *Chem. Abstr.*, *80*, 133840 (1974).
227. MAIER, R., BARTHE, P. L., SCHENKEL-HULLIGER, L., DESAULLES, P. A., "The biological activity of [1-D-serine,17-18-dilysine]-β-corticotrophin-(1–18)-octadecapeptide-amide." *Acta Endocrinol.*, *68*, 458 (1970).
228. MANNING, M., SAWYER, W. H., "Solid-phase synthesis and some pharmacological properties of 4-threonine analogues of the neurohypophysial hormones." in "Progress in Peptide Research." Vol. II., "Proc. Second American Peptide Symp., Cleveland, 1970." (Ed. LANDE, S.) p. 79, Gordon and Breach, 1972.
229. MEDZIHRADSZKY, K., BAJUSZ, S., "Biológiailag aktív ACTH fragmensek szintézise." (Synthesis of biologically active ACTH fragments.) *Kémiai Közl.*, *29*, 379 (1968).
230. MEDZIHRADSZKY, K., BAJUSZ, S., KISFALUDY, L., "Adrenokortikotrop hatású polipeptidek és az emberi kortikotropin teljes szintézise." (Total synthesis of the human corticotropin and of polypeptides with adrenocorticotropic activity.) *Kémiai Közl.*, *28*, 219 (1967).
231. MEDZIHRADSZKY, K., BRUCKNER, V., BAJUSZ, S., KISFALUDY, L., PAULAY, Z., LŐW, M., "Synthesis of the N-terminal tetradecapeptide of corticotropins." *Acta Chim. (Budapest)*. In preparation.
232. MEDZIHRADSZKY, K., BRUCKNER, V., KAJTÁR, M., LŐW, M., BAJUSZ, S., KISFALUDY, L., "Synthese eines Nonapeptid-Derivats für den Aufbau corticotrop wirksamer Polypeptide." *Acta Chim. Acad. Sci. Hung.*, *30*, 105 (1962).
233. MEDZIHRADSZKY, K., KAJTÁR, M., LŐW, M., "Synthese des Nonapeptids der Sequenz 1–9 des β-Corticotropins." *Coll. Czech. Chem. Comm.*, *27*, 2256 (1962).
234. MEDZIHRADSZKY, K., NIKOLICS, K., SEPRŐDI, J., "Labelled polypeptides. II. Synthesis of the N-terminal decapeptide of ACTH labelled with tritium on the tyrosine residue." *Ann. Univ. Sci. Eötvös L. nom. Sect. Chim.*, *13*, 25 (1972).
235. MEDZIHRADSZKY, K., NIKOLICZ, K., TEPLÁN, I., SEPRŐDI, J., FITTKAU, S., "Labelled polypeptides. III. Synthesis of α-melanotropin labelled with tritium on the tyrosine residue." *J. Label. Comp.*, *10*, 23 (1974).
236. MEDZIHRADSZKY, K., PONGRÁCZ, K., "Synthesis of an undecapeptide amide and a dodecapeptide amide with the N-terminal sequence of corticotropins." *Acta Chim. (Budapest)*, In the press.
237. MEDZIHRADSZKY, K., SEPRŐDI, J., MEDZIHRADSZKY-SCHWEIGER, H., "Studies on melanotropins. I. Synthesis and biological activity of α-melanotropin analogues modified in positions 4 and 10." *Acta Chim. (Budapest)*. In the press.
238. MEDZIHRADSZKY, K., SEPRŐDI, J., MEDZIHRADSZKY-SCHWEIGER, H., MAGYAR, A., "Studies on melanotropins. II. Effect of the side-chain variations in position 4 of α-melanotropin on the melanocyte-stimulating activity. *Acta Chim. (Budapest)*, In preparation.
239. MEDZIHRADSZKY, K., S.-VARGHA, H., FITTKAU, S., MARQUARDT, I., "Labelled polypeptides. I. Synthesis of the N-terminal decapeptide of ACTH labelled with ^{14}C on the glycine residue." *Acta Chim. (Budapest)*, *65*, 449 (1970).

240. MEDZIHRADSZKY, K., S.-VARGHA, H., FITTKAU, S., TEPLÁN, I., "Synthesis of polypeptide hormones labelled with carbon-14 and tritium." in "Peptides. Proc. 11th European Peptide Symp. Vienna, 1971." (Ed. NESVADBA, H.) p. 306, North Holland, Amsterdam, 1973.
241. MERRIFIELD, R. B. "Solid-phase peptide synthesis. I. The synthesis of a tetrapeptide." *J. Am. Chem. Soc., 85*, 2149 (1963).
242. MORODER, L., HOFMANN, K., "Studies on polypeptides. 46. Synthesis of a stably labelled, biologically active adrenocorticotropin fragment." *J. Med. Chem., 13*, 839 (1970).
243. MOYLE, W. R., KONG, Y. C., RAMACHANDRAN, J., "Steroidogenesis and cyclic adenosine 3,′5′-monophosphate accumulation in rat adrenal cells." *J. Biol. Chem., 248*, 2409 (1973).
244. NAKAMURA, M., "Studies on the role of the basic amino acid residues of ACTH peptide in steroidogenesis by isolated adrenal cells." *J. Biochem., 71*, 1029 (1972).
245. NAKAMURA, M., TANAKA, A., "Some aspects of the relationship between N-terminal structure and MSH activity of three decapeptides related to ACTH: a comparative study of Gly^1-ACTH(1–10)OH, Ibu^1-ACTH(1–10)OH and β-Ala^1-ACTH(1–10)OH." *Endocrinol. Japon., 19*, 395 (1972).
246. NAKAMURA, M., TANAKA, A., HIROTA, M., INOUE, S., "Structure-melanotropic activity relationship in synthetic polypeptides related to ACTH." *Endocrinol. Japon., 19*, 383 (1972).
247. NEY, R. L., OGATA, E., SHIMIZU, N., NICHOLSON, W. E., LIDDLE, G. W., "Structure–function relationships of ACTH and MSH analogues." *Excerpta Medica Int. Congr. Series* No. *83*, 1184 (1965).
248. NIKOLICS, K., TEPLÁN, I., MEDZIHRADSZKY, K., "Labelled polypeptides. V. Synthesis of α-melanotropin labelled with tritium on the tyrosine residue via the dibromo intermediate." *J. Label. Comp.* In the press.
249. NISHIMURA, O., FUJINO, M., "Synthesis of [4-leucine]- and [4-isoleucine]-α-melanocyte-stimulating hormones. *Takeda Kenkyusho Ho, 31*, 293 (1972); *Chem. Abstr., 77*, 165060 (1972).
250. OELOFSEN, W., LI, C. H., "Adrenocorticotropins. XXXVI. Synthesis of a biologically active prolinol analogue of the nonadecapeptide corresponding to the amino-terminal portion of the ACTH molecule." *J. Am. Chem. Soc., 88*, 4254 (1966).
251. OERTEL, G. W., "Zur Synthese ACTH-verwandter Peptide. I. Darstellung eines Heptapeptid-Derivates." *Angew. Chem., 70*, 51 (1960).
252. OTSUKA, H., INOUYE, K., "Novel tetracosapeptide." Jap. 72, 38966; *Chem. Abstr., 78*, 72598 (1972).
253. OTSUKA, H., INOUYE, K., "The synthesis of an MSH-active tetrapeptide, L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophan." *Bull. Chem. Soc. Japan, 37*, 289 (1964).
254. OTSUKA, H., INOUYE, K., "Syntheses of peptides related to the N-terminal structure of corticotropin. III. The synthesis of L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophan, the smallest peptide exhibiting the melanocyte-stimulating and the lipolytic activities." *Bull. Chem. Soc. Japan, 37*, 1465 (1964).
255. OTSUKA, H., INOUYE, K., JONO, Y., "Syntheses of peptides related to the N-terminal structure of corticotropin. IV. The synthesis of the amino acid sequences Lys-Pro-Val-Gly and Lys-Pro-Val-Gly-Lys." *Bull. Chem. Soc. Japan, 37*, 1471 (1964).
256. OTSUKA, H., INOUYE, K., KANAYAMA, M., SHINOZAKI, F., "The synthesis and biological activities of a hexadecapeptide corresponding to a modified sequence in the corticotropin structure." *Bull. Chem. Soc. Japan, 38*, 679 (1965).
257. OTSUKA, H., INOUYE, K., KANAYAMA, M., SHINOZAKI, F., "The synthesis of a heptadecapeptide amide corresponding to a modified sequence in the corticotropin structure." *Bull. Chem. Soc. Japan, 38*, 1563 (1965).
257. OTSUKA, H., INOUYE, K., KANAYAMA, M., SHINOZAKI, F., "The syntheses of peptides related to the N-terminal structure of corticotropin. VII. The synthesis of peptide derivatives corresponding to positions 11 to 18 in the corticotropin molecule." *Bull. Chem. Soc. Japan, 39*, 882 (1966).

259. Otsuka, H., Inouye, K., Shinozaki, F., Kanayama, M., "Synthesis of an octadecapeptide and its 18-amide analogue corresponding to the first eighteen amino acid residues of corticotropin (ACTH), and their biological activities." *J. Biochem. (Tokyo)*, *58*, 512 (1965).
260. Otsuka, H., Inouye, K., Shinozaki, F., Kanayama, M., "Synthesis of peptides related to the N-terminal structure of corticotropin. VI. The synthesis of Ser^1- and Gly^1-decapeptides corresponding to the first ten amino-acid sequence of corticotropin." *Bull. Chem. Soc. Japan*, *39*, 1171 (1966).
261. Otsuka, H., Shin, M., Kinomura, Y., Inouye, K., "Synthesis of corticotropin peptides. IX. Synthesis of [1-glycine]-ACTH-(1-18)-octadecapeptide amide." *Bull. Chem. Soc. Japan*, *43*, 196 (1970).
262. Otsuka, H., Watanabe, K., Inouye, K., "Synthesis of a heptacosapeptide corresponding to the human corticotropin 1–27 sequence." *Bull. Chem. Soc. Japan*, *43*, 2278 (1970).
263. Pickering, B. T., Li, C. H., "On the properties of human β-melanocyte-stimulating hormone." *Biochim. Biophys. Acta*, *74*, 156 (1963).
264. Pickering, B. T., Li, C. H., "Adrenocorticotropins. 29. The action of sodium hydroxide on adrenocorticotropin." *Arch. Biochem. Biophys.*, *104*, 119 (1964).
265. Pless, J., Boissonnas, R. A., Guttmann, St., "Tricosapeptides." French Pat., 1,513.596; *Chem. Abstr.*, *71*, 61764 (1969); Fr. M. 6303; *Chem. Abstr.*, *74*, 88307 (1971).
266. Pless, J., Guttmann, St., Boissonnas, R. A., "Tetracosapeptides." French Pat., 1,515.092; *Chem. Abstr.*, *72*, 44135 (1970).
267. Pless, J., Guttmann, St., Boissonnas, R. A., "Therapeutic pentacosapeptides." Swiss Pat., 494.740; *Chem. Abstr.*, *74*, 54177 (1971).
268. Privat de Garilhe, M., Gros, C., Porath, J., Lindner, E.-B., "Further studies on corticotropin releasing factor (CRF); corticotropin releasing activity of synthetic peptides." *Experientia*, *16*, 414 (1960).
269. Purves, H. D., Sirett, N. E., "Assay of corticotropin in dexamethasone-treated rats." *Endocrinology*, *77*, 366 (1965).
270. Raben, M. S., Landolt, R., Smith, F. A., Hofmann, K., Yajima, H., "Adipokinetic activity of synthetic peptides related to corticotropin." *Nature*, *189*, 681 (1961).
271. Ramachandran, J., Chung, D., Li, C. H., "Adrenocorticotropins. XXXIV. Aspects of structure–activity relationships of the ACTH molecule. Synthesis of a heptadecapeptide amide, an octadecapeptide amide and a nonadecapeptide amide possessing high biological activities." *J. Am. Chem. Soc.*, *87*, 2696 (1965).
272. Ramachandran, J., Lee, V., "Preparation and properties of the *o*-nitrophenyl sulfenyl derivative of ACTH; an inhibitor of the lipolytic action of the hormone." *Biochem. Biophys. Res. Comm.*, *38*, 507 (1970).
273. Ramachandran, J., Lee, V., "Divergent effects of *o*-nitrophenyl sulfenyl ACTH on rat and rabbit fat cell adenyl cyclases." *Biochem. Biophys. Res. Comm.*, *41*, 358 (1970).
274. Ramachandran, J., Li, C. H., The synthesis of L-valyl-L-lysyl-L-valyl-L-tyrosyl-L-prolin. *J. Org. Chem.*, *28*, 173 (1963).
275. Ramachandran, J., Li, C. H., "Adrenocorticotropins. XXXIII. Synthesis of a biologically active hexacosapeptide corresponding to the first 26 residues of bovine ACTH." *J. Am. Chem. Soc.*, *87*, 2691 (1965).
276. Ramachandran, J., Li, C. H., "Structure–activity relationships of the adrenocorticotropins and melanotropins: The synthetic approach." *Advances in Enzymology*, *29*, 391 (1967).
277. Riniker, B., Rittel, W., "Die Synthese des corticotrop hochaktiven [1-D-Serin-17,18-dilysin]-β-corticotropin-(1–18)-octadecapeptidamids." *Helv. Chim. Acta*, *53*, 513 (1970).
278. Riniker, B., Sieber, P., Rittel, W., Zuber, H., "Revised amino acid sequences for porcine and human adrenocorticotrophic hormone." *Nature New Biol.*, *235*, 114 (1972).
279. Rittel, W., "Synthese von Peptid-Zwischenprodukten für den Aufbau eines corticotrop wirksamen Nonadecapeptids. IV. Derivate der Nonapeptidsequenz Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro." *Helv. Chim. Acta*, *45*, 2465 (1962).

280. RITTEL, W., "Techniques for the synthesis of ACTH and MSH peptides and analogues." *Adv. Exp. Med. Biol., 2.* "Pharmacology of Hormonal Polypeptides and Proteins, Proc. Internat. Symp., Milan, 1967," p. 35, Plenum Press, New York, 1968.
281. RITTEL, W., "Structure–activity relationship of synthetic corticotrophin peptides." *Biochem. J., 1971,* 56.
282. RITTEL, W., "β-Ala^1-Lys^{17,18}-β^{1-18}-corticotropin-Lys^{18}-amide." Ger. Offen., 2,124.549; *Chem. Abstr., 76,* 46519 (1972).
283. RITTEL, W., BRUGGER, M., "Peptides." Ger. Offen., 2,228.150; *Chem. Abstr., 78,* 72596 (1973).
284. RITTEL, W., ISELIN, B., KAPPELER, H., RINIKER, B., "Peptides with improved adrenocorticotropic activity." South African Pat., 667.644; *Chem. Abstr., 71,* 113253 (1969).
285. ROSENBERG, L. L., EVANS, E. S., SIMPSON, M. E., "Simple method for the purification of sheep adrenocorticotropin (ACTH)." *Endocrinology, 68,* 1 (1961).
286. RUDMAN, D., MALKIN, M. F., GARCIA, L. A., DI GIROLAMO, M., ABELL, L. L., "Inactivation of the adipokinetic property of adrenocorticotropin, β-melanocyte-stimulating hormone, vasopressin and pituitary fraction H by tissues of the rat and rabbit." *Endocrinology, 75,* 867 (1964).
287. SAFFRAN, M., SCHALLY, A. V., "*In vitro* bioassay of corticotropin: modification and statistical treatment." *Endocrinology, 56,* 523 (1955).
288. SAKAKIBARA, S., SHIMONISHI, Y., "New method for releasing oxytocin from fully-protected nonapeptides using anhydrous hydrogen fluoride." *Bull. Chem. Soc. Japan, 38,* 1412 (1965).
289. SAYERS, M. A., SAYERS, G., WOODBURY, L. A., "The assay of adrenocorticotropic hormone by the adrenal ascorbic acid depletion method." *Endocrinology, 42,* 379 (1948).
290. SCHALLY, A. V., ANDERSEN, R. N., LONG, J. M., GUILLEMIN, R., "Isolation of α- and β-melanocyte stimulating hormones (MSH) on preparative scale." *Proc. Soc. Exptl. Biol. Med., 104,* 290 (1960).
291. SCHENKEL-HULLIGER, L., MAIER, R., BARTHE, P. L., DESAULLES, P. A., JARRET, A., RINIKER, B., RITTEL, W., SIEBER, P., "Biological activity of synthetic human corticotrophin with revised amino acid sequence." *Acta Endocrin., 75.,* 24 (1974).
292. SCHNABEL, E., LI, C. H., "The synthesis of glycyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine and its melanocyte-stimulating activity." *J. Biol. Chem., 235,* 2010 (1960).
293. SCHNABEL, E., LI, C. H., "The synthesis of L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine and its melanocyte-stimulating activity." *J. Am. Chem. Soc., 82,* 4576 (1960).
294. SCHRÖDER, E., GIBIAN, H., "Synthese eines Undekapeptids aus der ACTH-Sequenz." *Ann., 649,* 168 (1961).
295. SCHRÖDER, E., LÜBKE, K., "The Peptides." Vol. II., Academic Press, New York, 1966.
296. SCHWYZER, R., "Molecular mechanism of polypeptide hormone action." in "Peptides. 1972. Proc. 12th European Peptide Symp., Reinhardsbrunn, 1972," (Eds. HANSON, H., JAKUBKE, H.-D.) p. 424, North Holland, Amsterdam, 1973.
297. SCHWYZER, R., COSTOPANAGIOTIS, A., SIEBER, P., "Neue Synthesen auf dem Gebiete der melanophorenstimulierenden und corticotropen Hormone." *Chimia, 16,* 295 (1962).
298. SCHWYZER, R., COSTOPANAGIOTIS, A., SIEBER, P., "Zwei Synthesen des α-Melanotropins (α-MSH) mit Hilfe leicht entfernbarer Schutzgruppen." *Helv. Chim. Acta, 46,* 870 (1963).
299. SCHWYZER, R., ISELIN, B., KAPPELER, H., RINIKER, B., RITTEL, W., ZUBER, H., "Die Synthese des β-Melanotropins (β-MSH) mit der Aminosäuresequenz des bovinen Hormons." *Helv. Chim. Acta, 46,* 1975 (1963).
300. SCHWYZER, R., ISELIN, B., KAPPELER, H., RITTEL, W., RINIKER, B., South African Pat. 61617 (1961).
301. SCHWYZER, R., KAPPELER, H., "Synthese von Zwischenprodukten für den Aufbau corticotrop wirksamer Polypeptide. III. Das Decapeptid H-Ser-Tyr-Ser--Met-Glu-His-Phe-Arg-Trp-Gly-OH und einige seiner Derivate." *Helv. Chim. Acta, 44,* 1991 (1961).

302. SCHWYZER, R., KAPPELER, H., "Synthese eines Tetracosapeptides mit hoher corticotroper Wirksamkeit: β^{1-24}-Corticotropin." *Helv. Chim. Acta, 46,* 1550 (1963).
303. SCHWYZER, R., KAPPELER, H., ISELIN, B., RITTEL, W., ZUBER, H., "Synthese und biologische Aktivität von geschützten Polypeptidsequenzen des β-Melanophoren-stimulierenden Hormons (β-MSH) des Rindes." *Helv. Chim. Acta, 42,* 1702 (1959).
304. SCHWYZER, R., KARLAGANIS, G., "Hormon-Rezeptor Beziehungen: Synthese und Eigenschaften von [Phenylalanin^2,(4,5-dehydro-4,5-ditritio)-norvalin^4]-adrenocorticotropin-(1–24)-tetrakosipeptid." *Ann., 1973,* 1298.
305. SCHWYZER, R., LI., C. H., "A new synthesis of the pentapeptide L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine and its melanocyte-stimulating activity." *Nature, 182,* 1669 (1958).
306. SCHWYZER, R., RITTEL, W., "Synthese von Peptid-Zwischenprodukten für den Aufbau eines corticotrop wirksamen Nonadekapeptids. I. Lys(BOC), Lys(BOC)-Lys(BOC), Lys(BOC)-Pro-Val-Gly und Derivate." *Helv. Chim. Acta, 44,* 159 (1961).
307. SCHWYZER, R., RITTEL, W., COSTOPANAGIOTIS, A., "β^{1-16}-Corticotropin-methylester." *Helv. Chim. Acta, 45,* 2473 (1962).
308. SCHWYZER, R., RITTEL, W., KAPPELER, H., ISELIN, B., "Synthese eines Nonadekapeptides mit hoher corticotroper Wirksamkeit." *Angew. Chem., 72,* 915 (1960).
309. SCHWYZER, R., SCHILLER, P. W., "Hormon-Rezeptor-Beziehungen: Synthese und Eigenschaften von N$^\varepsilon$-Dansyllysin^{21}-adrenocorticotropin-(1–24)-tetrakosipeptid." *Helv. Chim. Acta., 54,* 897 (1971).
310. SCHWYZER, R., SCHILLER, P., SEELIG, S., SAYERS, G., "Isolated adrenal cells: log dose–response curves for steroidogenesis induced by $ACTH_{1-24}$, $ACTH_{1-10}$, $ACTH_{4-10}$ and $ACTH_{5-10}$." *FEBS Letters, 19,* 229 (1971).
311. SCHWYZER, R., SIEBER, P., "Total synthesis of adrenocorticotrophic hormone." *Nature, 199,* 172 (1963).
312. SCHWYZER, R., SIEBER, P., "Die Totalsynthese des β-Corticotropins (adrenocorticotropes Hormon; ACTH)." *Helv. Chim. Acta, 49,* 134 (1966).
313. SCOTT, A. P., LOWRY, P. J., "Adrenocorticotrophic and melanocyte-stimulating peptides in the human pituitary." *Biochem. J., 139,* 593 (1974).
314. SEELIG, S., KUMAR, S., SAYERS, G., "Isolated adrenal cells: the partial agonists [Trp(Nps)^9] $ACTH_{1-39}$ and [Trp(Nps)^9] $ACTH_{1-24}$ (Nitrophenyl sulfenyl derivatives of ACTH)." *Proc. Soc. Exp. Biol. Med., 139,* 1217 (1972).
315. SEELIG, S., SAYERS, G., SCHWYZER, R., SCHILLER, P., "Isolated adrenal cells: $ACTH_{11-24}$, a competitive antagonist of $ACTH_{1-39}$ and $ACTH_{1-10}$." *FEBS Letters, 19,* 232 (1971).
316. SHEPHERD, R. G., HOWARD, K. S., BELL, P. H., CACCIOLA, A. R., CHILD, R. G., DAVIES, M. C., ENGLISH, J. P., FINN, B. M., MEISENHELDER, J. H., MOGER, A. W., VAN DER SCHEER, J., "Studies with corticotropin. I. Isolation, purification and properties of β-corticotropin." *J. Am. Chem. Soc., 78,* 5051 (1956).
317. SHEPHERD, R. G., WILLSON, S. D., HOWARD, K. S., BELL, P. H., DAVIES, D. S., DAVIS, S. B., EIGNER, E. A. and SHAKESPEARE, N. E., "Studies with corticotropin. III. Determination of the structure of β-corticotropin and its active degradation products." *J. Am. Chem. Soc., 78,* 5067 (1956).
318. SHIZUME, K., LERNER, A. B., "Determination of melanocyte-stimulating hormone in urine and blood." *J. Clin. Endocrinol: Metabol., 14,* 1491 (1954).
319. SHIZUME, K., LERNER, A. B., FITZPATRICK, T. B., "*In vitro* bioassay for the melanocyte-stimulating hormone." *Endocrinology, 54,* 553 (1954).
320. SIEBER, P., RITTEL, W., RINIKER, B., "Die Synthese von menschlichem adrenocorticotropem Hormon (α_h-ACTH) mit revidierter Aminosäuresequenz." *Helv. Chim. Acta, 55,* 1243 (1972).
321. STARK, E., VARGA, B., MEDZIHRADSZKY, K., BAJUSZ, S., HAJTMAN, B., "Relationships between the structure and the adrenal and extraadrenal effects of ACTH fragments." *Acta Physiol. Acad. Sci. Hung., 38,* 197 (1970).
322. STEELMAN, S. L., GUILLEMIN, R., "Adrenocorticotropic activity of α-melanocyte-stimulating hormone (α-MSH)." *Proc. Soc. Biol. Med., 101,* 600 (1959).
323. STOUFFER, J. E., HSU, J.-S., "Structural relationships in the interaction of adrenocorticotropin with plasma proteins." *Biochemistry, 5,* 1195 (1966).

324. Sturm, K., Geiger, R., Siedel, W., "Synthese eines Tetradecapeptidamid-Derivates mit der Aminosäuresequenz 11–24 des Corticotropins (ACTH)." *Chem. Ber., 96,* 609 (1963).
325. Sturm, K., Geiger, R., Siedel, W., "Synthese von Peptidderivaten mit den Aminosäuresequenzen 11–20, 11–21, 11–22 und 11–23 des Corticotropins (ACTH)." *Chem. Ber., 97,* 1197 (1964).
326. Szporny, L., Hajós, Gy. T., Kárpáti, E., Fekete, Gy., "Investigations of activities of some synthetic ACTH peptides using a new assay method." in "7th Acta Endocrinologica Congress." *Acta Endocr. Suppl., 138,* 21 (1969).
327. Szporny, L., Hajós, Gy. T., Szeberényi, S., Fekete, G., "Pharmacology of synthetic ACTH peptides of different chain length." *Adv. Exp. Med. Biol., 2,* "Pharmacology of Hormonal Polypeptides and Proteins. Proc. Internat. Symp., Milan, 1967." p. 196, Plenum Press, 1968.
328. Szporny, L., Hajós, Gy. T., Szeberényi, S., Fekete, G., "Biological activity of synthetic 1–39 human corticotropin." *Nature, 218,* 1169 (1968).
329. Szporny, L., Hajós, Gy. T., Szeberényi, S., Fekete, G., "Comparative pharmacology of some synthetic human ACTH sequences. Protein and Polypeptide Hormones." *Excerpta Medica Int. Congr. Ser., 161,* p. 492 (1969).
330. Tanaka, A., Pickering, B. T., Li, C. H., "Relationship of chemical structure to *in vitro* lipolytic activity of peptides occurring in adrenocorticotropic and melanocyte-stimulating hormones." *Arch. Biochem., 99,* 294 (1962).
331. Tesser, G. I., Buis, J. T., "Synthesis and biological activity of four analogues of β-corticotropin." *Rec. Trav. Chim., 90,* 444 (1971).
332. Tesser, G. I., Maier, R., Schenkel-Hulliger, L., Barthe, P. L., Kamber, B., Rittel, W., "Biological activity of corticotrophin peptides with homoarginine, lysine or ornithine substituted for arginine in position 8." *Acta Endocrinol., 74,* 56 (1973).
333. Tesser, G. I., Pleumekers, A. W. J., Bassie, W., Balvert-Beers, I. C., "Synthesis of two new corticotrophic peptides containing homoarginine in position 8." *Rec. Trav. Chim., 92,* 1210 (1973).
334. Tesser, G. I., Rittel, W., "Synthese und biologische Eigenschaften der 8-Mono- und 8,17,18-Triornithin-β-corticotropin-(1-24)-tetracosapeptiden." *Rec. Trav. Chim., 88,* 553 (1969).
335. Tesser, G. I., Schwyzer, R., "Synthese des 17,18-Diornithin-β-corticotropin-(1–24)-tetracosapeptides, eines biologisch aktiven Analogons des adrenocorticotropen Hormons." *Helv. Chim. Acta, 49,* 1013 (1966).
336. Vernikos-Danellis, J., Anderson, E., Trigg, L., "Changes in adrenal corticosterone concentration in rats: method of bioassay for ACTH." *Endocrinology, 79,* 624 (1966).
337. Waller, J. P., Dixon, H. B. F., "Selective acetylation of the terminal amino group of corticotropin." *Biochem. J., 75,* 320 (1960).
338. Walser, A., Müller, Th., "Adrenocorticotropic effect in humans of a synthetic β^{1-24}-corticotropin derivative." *Experientia, 24,* 74 (1968).
339. Walser, A., Müller, Th., "The adrenocorticotropic effect in humans of several synthetic peptides related to β^{1-24}-corticotropin. Protein and Polypeptide Hormones." *Excerpta Medica Int. Congr. Ser. 161,* p. 487 (1969).
340. Wang, K.-T., Blake, J., Li, C. H., "The solid-phase synthesis of human and monkey beta-melanotropins." *Int. J. Peptide Protein Res., 5,* 33 (1973).
341. White, J. E., Engel, F. L., "A lipolytic action of epinephrine and norepinephrine on rat adipose tissue *in vitro*." *Proc. Soc. Exp. Biol., 99,* 375 (1958).
342. White, J. E., Engel, F. L., "Lipolytic action of corticotropin on rat adipose tissue *in vitro*." *J. Clin. Invest., 37,* 1556 (1958).
343. White, W. F., "Studies on adrenocorticotropin. V. The isolation of corticotropin-A." *J. Am. Chem. Soc., 75,* 503 (1953).
344. White, W. F., "Studies on adrenocorticotropin. XII. Action of aminopeptidase on corticotropin-A; effect on biological activity." *J. Am. Chem. Soc., 77,* 4691 (1955).
345. White, W. F., Landmann, W. A., "Studies on adrenocorticotropin. XI. A preliminary comparison of corticotropin-A with β-corticotropin." *J. Am. Chem. Soc., 77,* 1711 (1955).

346. YAJIMA, H., "Synthetic approach to studies on the structure–function of melanocyte-stimulating hormone." *Gunma Symp. on Endocrinol., 5*, 73 (1968).
347. YAJIMA, H., KAWASAKI, K., "Studies on peptides. XVII. Synthesis of N-(N^{α}-acetylseryltyrosylserylmethionylglutamylhistidylphenylalanylarginyl)-5-methoxytryptamine and its physiological properties on frog melanocyte *in vitor*." *Chem. Pharm. Bull., 16*, 1379 (1968).
348. YAJIMA, H., KAWASAKI, K., "Studies on peptides. XIX. Synthesis of a stereoisomer of α-melanocyte-stimulating hormone." *Chem. Pharm. Bull., 16*, 1387 (1968).
349. YAJIMA, H., KAWASAKI, K., MINAMI, H., KAWATANI, H., MIZOKAMI, N., KISO, Y., TAMURA, F., "Studies on peptides. XXVIII. Synthesis of the docosapeptide which embodies the entire amino acid sequence of β-melanocyte-stimulating hormone from human pituitary gland." *Chem. Pharm. Bull., 18*, 1394 (1970).
350. YAJIMA, H., KAWASAKI, K., MINAMI, H., KAWATANI, H., MIZOKAMI, N., OKADA, Y., "Studies on peptides. XXI. Synthesis of the docosapeptide corresponding to the entire amino acid sequence of human β-melanocyte-stimulating hormone." *Biochim. Biophys. Acta, 175*, 228 (1969).
351. YAJIMA, H., KAWASAKI, K., OKADA, Y., MINAMI, H., KUBO, K., YAMASHITA, I., "Studies on peptides. XVI. Regeneration of lysine from N^{ε}-formyllysine by aqueous hydrazine or hydroxylamine and their application to the synthesis of α-melanocyte-stimulating hormone." *Chem. Pharm. Bull., 16*, 919 (1968).
352. YAJIMA, H., KAWATANI, H., WATANABE, H., "Studies on peptides. XXVII. Synthesis of the decapeptide, ACTH (5–14) as an example of peptide-fragment condensation on the polymer support." *Chem. Pharm. Bull., 18*, 1333 (1970).
353. YAJIMA, H., KINOMURA, Y., OSHIMA, T., OKADA, Y., "Studies on peptides. XV. Synthesis of Pro-Tyr-Arg-Met the tetrapeptide situated within the amino acid sequence of monkey β-MSH." *Chem. Pharm. Bull., 15*, 1922 (1967).
354. YAJIMA, H., KUBO, K., "The synthesis of D-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine and L-histidyl-L-phenylalanyl-L-arginyl-D-tryptophylglycine and their physiological properties in frog melanocyte." *Biochim. Biophys. Acta, 97*, 596 (1965).
355. YAJIMA, H., KUBO, K., "Studies on peptides. II. Synthesis and physiological properties of D-histidyl-D-phenylalanyl-D-arginyl-D-tryptophylglycine, an optical antipode of an active fragment of α-melanocyte-stimulating hormone." *J. Am. Chem. Soc., 87*, 2039 (1965).
356. YAJIMA, H., KUBO, K., "Studies on peptides. IV. The synthesis of D-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine and L-histidyl-L-phenylalanyl-L-arginyl-D-tryptophylglycine and their physiological properties in frog melanocyte *in vitro*." *Chem. Pharm. Bull., 13*, 759 (1965).
357. YAJIMA, H., KUBO, K., KINOMURA, Y., "Studies on peptides. XIII. Contribution of the arginine residue in L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine to its melanotropic activity." *Chem. Pharm. Bull., 15*, 504 (1967).
358. YAJIMA, H., KUBO, K., KINOMURA, Y., LANDE, S., "Studies on peptides. XI. The effect on melanotropic activity of altering the arginyl residue in L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine." *Biochim. Biophys. Acta, 127*, 545 (1966).
359. YAJIMA, H., KUBO, K., OKADA, Y., "Studies on peptides. VI. Synthesis of three stereoisomeric pentapeptides of histidylphenylalanylarginyltryptophylglycine possessing D-phenylalanyl-D-arginyl moiety within the sequence and their melanocyte-stimulating activities *in vitro*." *Chem. Pharm. Bull., 13*, 1326 (1965).
360. YAJIMA, H., KUBO, K., OSHIMA, T., HANO, K., KOIDA, M., "Studies on peptides. VII. Synthesis of three stereoisomeric pentapeptides of histidylphenylalanylarginyltryptophylglycine and their melanocyte-stimulating activities *in vitro*." *Chem. Pharm. Bull., 14*, 775 (1966).
361. YAJIMA, H., OKADA, Y., KAWATANI, H., MIZOKAMI, N., "Studies on peptides. XXII. Synthesis of the partially protected pentadecapeptide related to monkey and human β-melanocyte-stimulating hormones." *Chem. Pharm. Bull., 17*, 1229 (1969).

362. YAJIMA, H., OKADA, Y., KINOMURA, Y., MINAMI, H., "Studies on peptides. XX. Synthesis of the octadecapeptide corresponding to the entire amino acid sequence of monkey β-melanocyte-stimulating hormone." *J. Am. Chem. Soc.*, *90*, 527 (1968).
363. YAJIMA, H., OKADA, Y., KINOMURA, Y., MIZOKAMI, N., KAWATANI, H., "Studies on peptides. XXIII. Total synthesis of monkey β-melanocyte-stimulating hormone." *Chem. Pharm. Bull.*, *17*, 1237 (1969).
364. YAJIMA, H., OKADA, Y., KINOMURA, Y., SETO, E., "Studies on peptides. XII. Synthesis of the decapeptide corresponding to the C-terminal portion of β-melanocyte-stimulating hormone." *Chem. Pharm. Bull.*, *15*, 270 (1967).
365. YAMASHIRO, D., LI, C. H., "Adrenocorticotropins. 44. Total synthesis of the human hormone by the solid phase method." *J. Am. Chem. Soc.*, *95*, 1310 (1973).
366. YOUNG, G. T. (sen. reporter), "Amino Acids, Peptides and Proteins: a Specialist Periodical Report, Vol. 1–4, 1969–1972." The Chemical Society, Burlington House, London.
367. ZONDEK, B., KROHN, H., "Hormon des Zwischenlappens der Hypophyse (Intermedin)." *Klin. Wochenschr.*, *11*, 405 (1932).

SUBJECT INDEX

RECENT DEVELOPMENTS
IN THE CHEMISTRY
OF NATURAL CARBON
COMPOUNDS

Volume 6

The sixth volume of the series contains three independent reviews written by distinguished authors who are themselves active workers in the fields concerned.

APPROACHES TO CAMPTOTHECIN

by *E. Winterfeldt*
Institute of Organic Chemistry
Technical University,
Hannover, F. R. G.

THE PYRROLIZIDINE ALKALOIDS

by *A. Klásek*
and *O. Weinbergová*
Institute of Chemistry,
Medical Faculty Palacký University,
Olomouc, Czechoslovakia

ADVANCES IN THE CHEMISTRY OF GLYCOSAMINIDES

by *A. Ya. Khorlin*
and *S. E. Zurabyan*
Shemyakin Institute
for Chemistry of Natural Products
Academy of Sciences
of the USSR, Moscow

Distributors
KULTURA
H-1389 Budapest, P.O.B. 149

Series ISBN 963 05 0702 1
Vol. 7. ISBN 963 05 0784 6