STRUCTURE–FUNCTION RELATIONS IN THE CORTICOTROPIN SERIES

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INTRODUCTION

The physiological function of the medium-sized polypeptide hormones appears to depend critically on a specific arrangement of the constituent amino-acid residues along their peptide chains. Thus, understanding of the essential structural prerequisites for biological function may be gained from studies relating primary structure to physiological activity.

Recognition that a large spectrum of physiological activities based on the peptide architectural principle is achieved by nature has led to an unprecedented renaissance in peptide chemistry.

Refinement in synthetic methodology, the development of techniques allowing the sharp separation of peptides possessing closely similar properties and the availability of new highly sensitive analytical tools for assessing homogeneity have set the stage for the synthesis of highly complex stereochemically homogeneous peptides possessing a defined arrangement of their amino-acid residues and exhibiting a wide spectrum of physiological activity. As an outgrowth of eight years of systematic studies on the chemistry of peptides related to the melanocyte-expanding hormone α-MSH and to ACTH we have prepared two peptides, one possessing a chain of 20, the other a chain of 23 amino-acid residues. These preparations possess essentially the same adrenocorticotropic activity as a natural ACTH. A derivative of α-MSH possessing a chain of 13 amino-acids was also prepared and possesses the same melanophoretic potency as natural α-MSH. The synthetic preparations have undergone extensive tests in man and were found to be highly active. Some aspects of our work will be presented in this lecture, particularly those relating to peptide structure and physiological function.

At this point I wish to express my most sincere appreciation to my colleagues, Drs Yajima, Yanaihara, Liu, Lande, Mrs Schwartz and Mrs Yanaihara, Miss Woolner and Mr Humes. The investigations which are presented in this lecture could not have been carried out without their untiring and devoted efforts.

STATEMENT OF PROBLEM

In 1954, Bell and collaborators announced the isolation of pure pig corticotropin and its complete amino-acid sequence. Their proposed structure for this polypeptide, which consists of a straight chain of 39 amino-acid residues, is shown in *Figure 1*.

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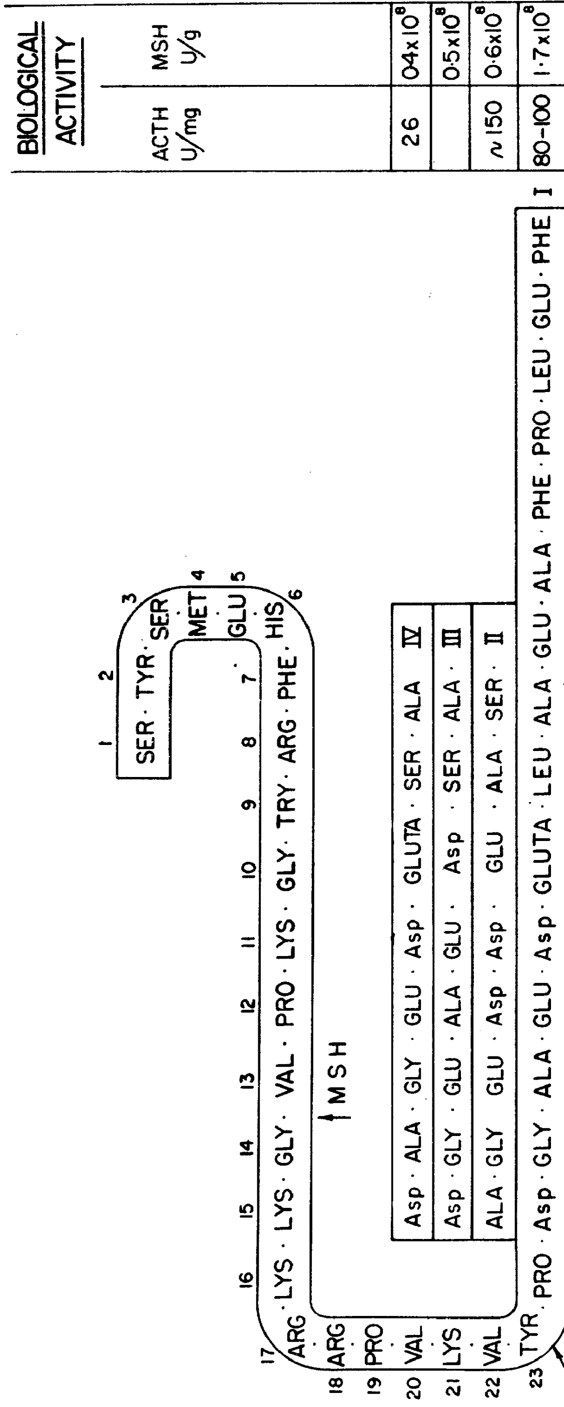

Figure 1. Amino-acid sequence and biological activity of various corticotropins

In their extensive studies of the pig ACTH molecule these investigators made some important observations. First, they found that pure pig corticotropin possessed both adrenocorticotropic and melanocyte-expanding potencies. Secondly, they observed that fragments of the molecule had the ability to bring about adrenal ascorbic acid depletion in the rat. As a matter of fact they deduced from the results on partial hydrolysis that the N-terminal tetracosa-peptide fragment (from the N-terminal serine to position 24) possessed essentially the full biological activity of the complete molecule. The findings of Harris and Lerner, that α-MSH, the most potent melanocyte-stimulating hormone known, exhibits the same arrangement of amino-acids as the N-terminal tridecapeptide of ACTH (namely the portion from the N-terminal serine to the arrow designated MSH) explained the MSH activity of ACTH in concrete structural terms.

ACTH has since been isolated from other species and variations of the peptide sequence have been observed. All of these corticotropins possess the same arrangement of amino-acids as concerns positions 1–24 and 33–39, but the arrangement of amino-acid residues between positions 24 and 33 for sheep (II), beef (III) and human ACTH (IV) is different from that of pig corticotropin. All of these structures must remain hypothetical until synthesis can be achieved and the synthetic product is shown to possess the same physical, chemical and biological properties as the natural hormones.

Our investigations were undertaken with the aim of delineating the minimum structures which will endow a peptide with melanocyte-expanding activity on the one hand, and with adrenocorticotropic potency on the other. Thus, at the present stage of our investigations we are concerned mainly with the relation between the length of the polypeptide chain and its biological function. Future studies will inquire into the essential nature of individual amino-acid residues for biological activity.

GENERAL COMMENTS REGARDING SYNTHESIS OF POLYPEPTIDE HORMONES

Useful procedures for production of polypeptide hormones must allow preparation of these biologically active molecules in quantities permitting detailed evaluation of their physiological spectra on a broad basis.

Selection of suitable subunits from which to construct highly complex peptides without risk of racemization is of critical importance. A minor degree of racemization of every amino-acid residue constituting such a molecule may afford a biologically inactive final product despite the fact that it possesses the correct amino-acid composition and sequence.

Protected peptides terminating in C-terminal glycine or proline residues are desirable subunits from which to assemble large peptides, since absence of an asymmetric centre in glycine and elimination of the possibility for azlactone formation in C-terminal proline preclude racemization during activation of the carboxyl group.

The azide and carbodiimide methods served to combine larger fragments; the mixed anhydride procedure was used *in extenso* to assemble smaller subunits. Only C-terminal glycine residues were activated by the

carbodiimide reagent since racemization has been shown to occur when this reagent activates carboxyl groups in certain acylated peptides C-terminating in an optically active amino-acid.

Low yields are experienced occasionally when attempting to bring about peptide bond formation by the use of *N,N′*-dicyclohexylcarbodiimide between a suitably protected acylpeptide terminating in glycine and a protected peptide ester or amide. Favoured formation of acylurea derivatives seems to provide the explanation for this behaviour. The same coupling reactions may be achieved in excellent yields when Stab's carbonyldiimidazole serves as the carboxyl activator.

We employ the carbobenzoxy group for N-terminal protection of small subunits, and have developed a procedure for producing N^{ϵ}-formyllysine, a protected form of this amino-acid, which we use extensively in our syntheses. The acetyl group serves as the N-terminal protector for large peptide fragments.

PURIFICATION AND ASSESSMENT OF HOMOGENEITY

Purification of intermediates and final products presents the main difficulty in producing complex polypeptides in quantity. In order to facilitate this task it becomes of considerable importance to control the various synthetic steps so as to ensure formation of initial reaction products possessing the highest possible purity. Multi-step syntheses requiring purification of every intermediate by the counter-current procedure are cumbersome. The counter-current procedure, an excellent tool indeed for purification and for assessment of homogeneity of some peptides, becomes impractical when employed for routine purification of gram quantities of these compounds. Also, separation of mixtures composed of basic peptides is frequently difficult to achieve by the counter-current technique. In our work, purification of many intermediates was achieved by carefully selected washing procedures and by chromatography on carboxymethylcellulose. We find chromatography on carboxymethylcellulose of particular value for purification of peptides containing arginine. Ammonium acetate solutions of varying ionic strengths and pH's are employed for development of the columns. Ion-exchange chromatography is readily adaptable to purification of gram lots of peptides.

Our criteria for homogeneity of synthetic polypeptide hormones are based on formation of a single spot on paper chromatography, preferably in more than one solvent system, appearance of a single component on paper electrophoresis at various hydrogen ion concentrations, and finally on formation of a single band on electrophoresis on starch gel. Starch gel electrophoresis appears to represent the most sensitive tool yet devised for detection of minor impurities in polypeptides.

Complete digestibility by leucine aminopeptidase, or by mixtures of this enzyme with prolidase, with formation of the constituent amino-acids in the ratios predicted by theory is accepted as evidence for stereo-chemical homogeneity.

Estimation of the amino-acid composition of acid and enzymatic digests is performed by the automatic procedure of Spackman, Stein and Moore.

It is informative to compute the average recovery of amino-acids in the acid or enzymatic hydrolysates. In conjunction with knowledge of total nitrogen and ammonia nitrogen content, these values provide a sensitive measure for contamination by ammonium salts and non-nitrogenous materials and, in addition, give information regarding the degree of hydration.

The importance for critical evaluation of the homogeneity of intermediates and final products in synthetic work involving production of highly complex biologically active polypeptides cannot be over-emphasized.

SYNTHESIS OF A TRICOSA- AND AN EICOSA-PEPTIDE CORRESPONDING TO N-TERMINUS OF ACTH

The remarkable stability towards acid of adrenocorticotropic activity in crude extracts and of pure corticotropin is well documented. Thus, prolonged heating at 100° in 0·1N hydrochloric acid exerts little, if any, effect on the biological activity of these preparations but short exposure to dilute alkali results in rapid inactivation.

Guided by this information, we synthesized the protected tricosa-peptide amide shown in *Figure 2* which corresponds to the arrangement of the N-terminal 23 amino-acid residues of ACTH, but contains protecting groups attached to certain amino-acid residues. The protecting groups were so selected that they could be removed by exposure to dilute acid.

Two partially protected subunits, a decapeptide terminating with glycine covering positions 1–10 and a tridecapeptide amide corresponding to positions 11–23 were used for construction of the protected tricosa-peptide amide. The subunit covering positions 11–23 shown in *Figure 3* contains four lysine and two arginine units and thus presents a problem of challenging complexity from the point of view of both synthesis and purification. Its synthesis followed the scheme shown in *Figure 4*.

Prolylvalyl-N^{ϵ}-formyllysylvalyltyrosine amide (II) is prepared by stepwise elongation of the peptide chain of valyltyrosine amide with mixed anhydrides of the desired carbobenzoxyamino acids serving as individual building blocks. The intermediate carbobenzoxypeptide amides are deblocked by hydrogenolysis in presence of acetic acid. The first arginine residue is incorporated by our nitroarginine procedure. The ensuing hexapeptide amide (IV) is again acylated with a mixed anhydride of carbobenzoxynitroarginine in aqueous tetrahydrofuran to give (V) in high yields. An excess of the mixed anhydride is employed in this step to ensure complete acylation of the hexapeptide amide since carbobenzoxynitroarginine is removed quantitatively from the reaction product by simple extraction with ethyl acetate. Hydrogenation of (V) affords a crude heptapeptide amide (VI). The azide of carbobenzoxy-N^{ϵ}-formyllysylprolylvalylglycyl-N^{ϵ}-formyllysyl-N^{ϵ}-formyllysine (XI) reacts with the heptapeptide amide (VI) to give the protected tridecapeptide amide (XII) which is decarbobenzoxylated by hydrogenolysis to afford (I).

A paper chromatogram of the analytically pure, partially protected

(I)

DCC

(II)

(III)

Figure 2. Synthesis of protected tricosa-peptide amide

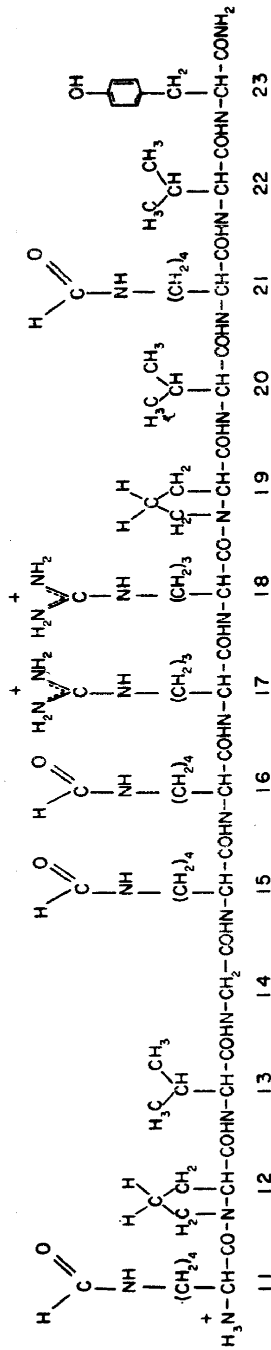

Figure 3. Subunit covering positions 11–23 used in the construction of the protected tricosa-peptide amide

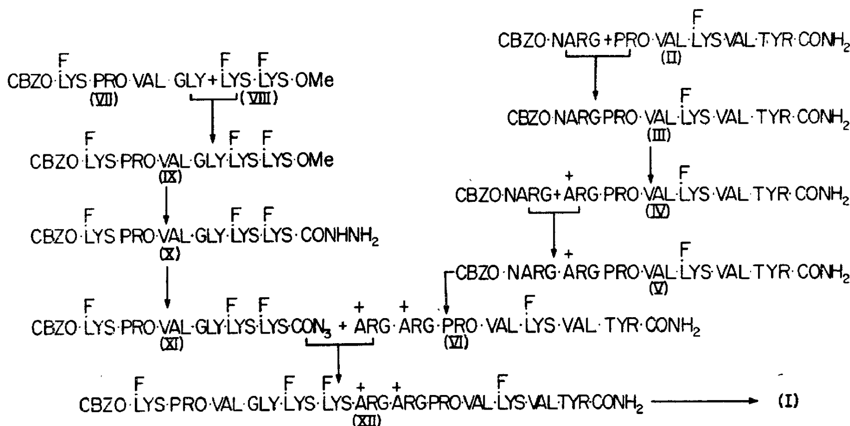

Figure 4. Synthesis of C-terminal partially protected tridecapeptide amide

tridecapeptide amide (11–23), which is completely digestible by LAP, is shown in *Figure 5.*

The characteristic absorption maximum at 275 mμ of the tyrosine residue provides a convenient means for following the various synthetic C-terminal peptides during purification. It is to be noted that both protonization and nitro group-blocking served to protect the arginine residues during the various manipulations involved in construction of this partially protected tridecapeptide amide.

Figure 6 summarizes the various steps which are used to assemble the

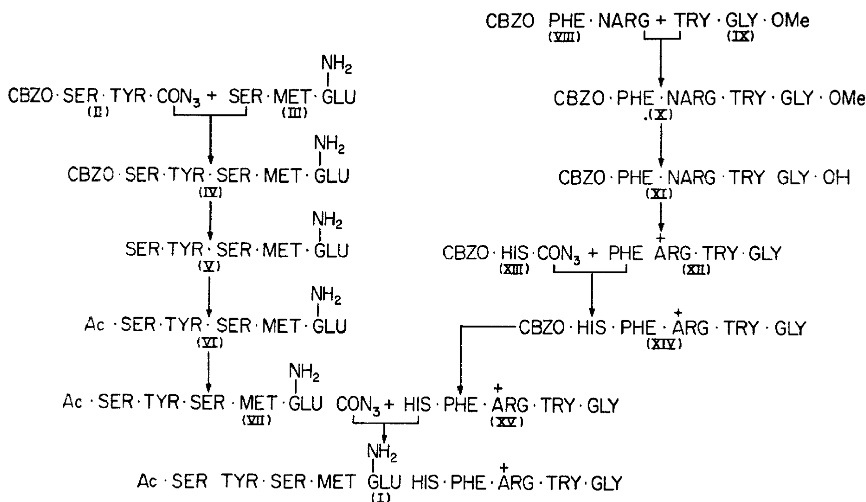

Figure 6. Synthesis of N-terminal partially protected decapeptide

N-terminal protected decapeptide (I). Carbobenzoxyseryltyrosine azide (II) is reacted with the triethylammonium salt of the tripeptide serylmethionylglutamine (III) to give the carbobenzoxy pentapeptide (IV). Exposure to

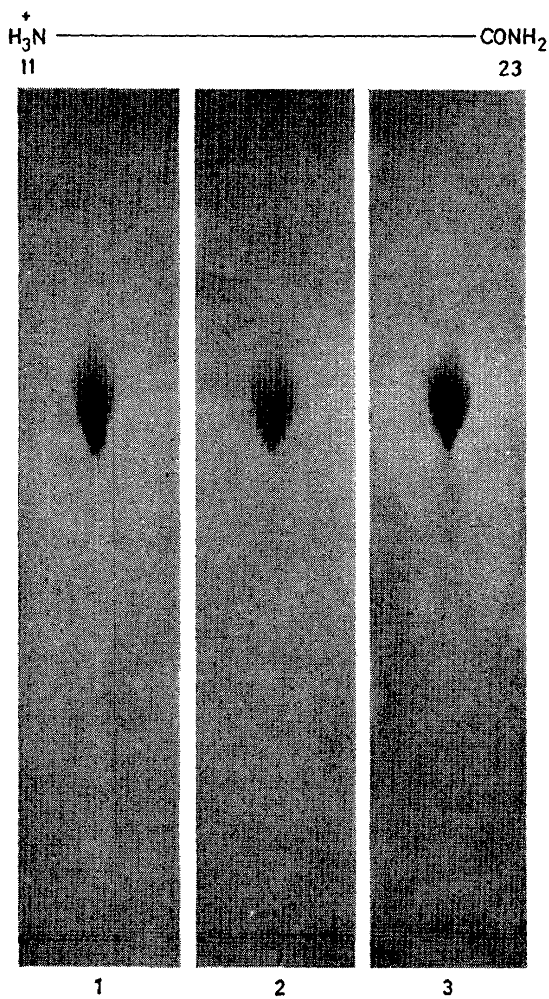

Figure 5. Uni-dimensional paper chromatogram of partially protected tridecapeptide amide

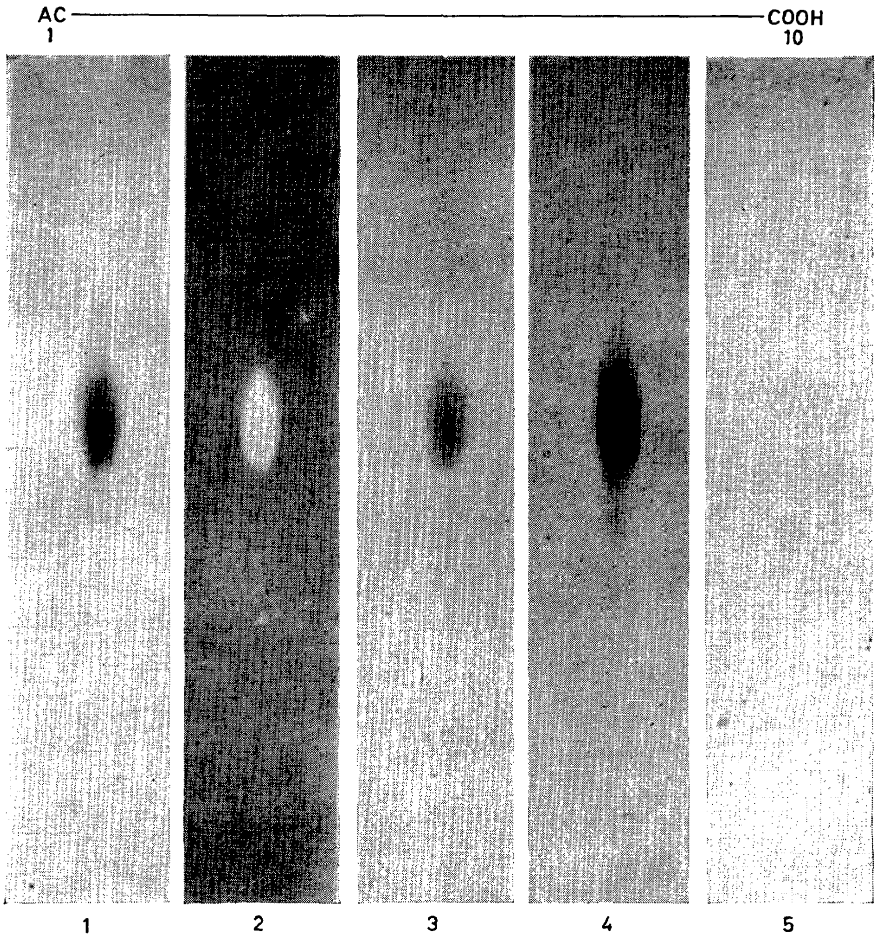

Figure 7. Uni-dimensional paper chromatogram of acetyldecapeptide

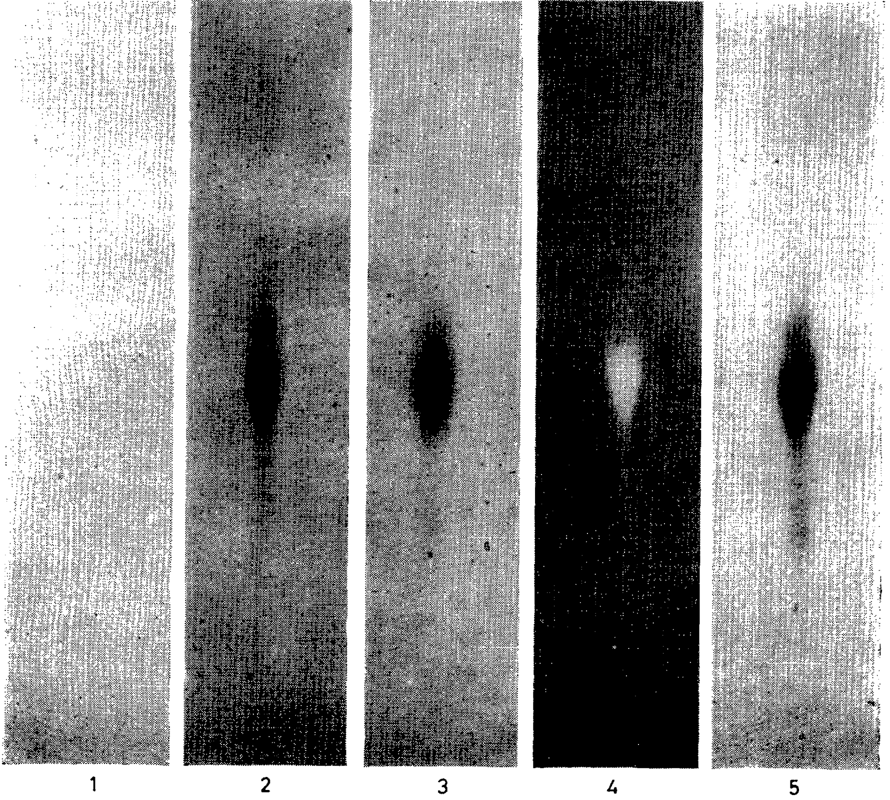

Figure 8. Uni-dimensional paper chromatogram of partially protected *N*-acetyl tricosapeptide

Figure 9. Starch gel electrophoresis of tricosapeptide at pH 8·5

sodium in liquid ammonia converts (IV) into the free pentapeptide (V). Acetylation of (V) in aqueous solution with acetic anhydride in presence of sodium bicarbonate affords (VI) which is converted *via* the methyl ester and hydrazide into the solid azide (VII).

Carbobenzoxyphenylalanylnitroarginine (VIII) is coupled with the methyl ester of tryptophylglycine (IX) to give the carbobenzoxypentapeptide methyl ester (X). The methyl ester is converted to (XI) by exposure to sodium hydroxide. Exhaustive hydrogenation in acetic acid of (XI) results in simultaneous removal of both the protecting nitro-, and carbobenzoxy functions with formation of the tetrapeptide salt (XII) which is coupled with the azide of carbobenzoxyhistidine (XIII) to give (XIV). Interaction of the triethylammonium salt of (XV), obtained from (XIV) by hydrogenolysis, with the azide (VII) affords the acetate salt of the acetyldecapeptide (I). A paper chromatogram of the homogeneous compound is shown in *Figure 7.*

Peptide bond formation between the hydrochlorides of the two aforementioned fragments was achieved by the use of *N,N′*-dicyclohexylcarbodiimide, and the ensuing protected tricosa-peptide amide was isolated from the reaction products by chromatography on carboxymethyl callulose.

As shown in *Figure 8* the purified compound was homogeneous as judged by paper chromatography and on hydrolysis with acid liberated the constituent amino-acids in the ratios predicted by theory.

The protected tricosa-peptide amide is essentially inert as concerns adrenocorticotropic potency, but as shown in *Table 1,* activity appears when the compound is exposed to 0·5N hydrochloric acid at thet emperature of a boiling water-bath.

Table 1. Adrenal ascorbic acid depleting activity of partial hydrolysates of (III, *Figure 2*) at various intervals of time

Hydrolysis time (min)	*Biological activity* (I.U./mg)
0	~0·05
20	7·5 ± 1·2
40	40·8 ± 6·4
60	29·8 ± 6·0
80	28·5 ± 5·7
100	10·7 ± 3·0
120	9·1 ± 3·3
140	6·2 ± 1·5

Biological activity reached a maximum following exposure to the acid for 40 minutes, then declined slowly, but significant activity was still present in samples which were hydrolyzed for as long as 140 minutes. It appears reasonable to assume that the initial rise in biological activity reflects elimination of the various blocking groups such as the N-terminal acetyl, the glutamine amide and the formyl groups. The slow decline in biological potency on prolonged exposure to acid is probably the result of gradual backbone fragmentation. The free tricosa-peptide was isolated from 80 minute hydrolysates

by chromatography on carboxymethylcellulose followed by electrophoresis on cellulose powder and a second purification on carboxymethylcellulose.

The extensively purified free tricosa-peptide was homogeneous with reference to paper chromatography and paper electrophoresis at various pH values and, as shown in *Figure 9*, produced a single spot on starch gel electrophoresis at pH 8.5.

Complete digestibility by leucine aminopeptidase and the fact that eight of the elevent constituent optically active amino-acids were shown to be of the L-variety by microbiological assay leaves little doubt regarding the high degree of stereochemical purity of the tricosa-peptide. Data pertaining to the ratios and stereochemical nature of the amino-acids in hydrolysates of the protected and of the free tricosa-peptide are shown in *Table 2*. These analytical data, and the fact that the tricosa-peptide was obtained by the linking of two subunits of established homogeneity by a procedure which eliminates the possibility for racemization appear to justify the conclusion that both the protected precursor and the free tricosa-peptide are essentially pure compounds. The observation that carboxypeptidase liberated tyrosine and valine from the peptide establishes the presence of a free C-terminal carboxylic acid function.

Table 2. Amino-acid composition of acid hydrolysates of the protected and free synthetic tricosa-peptide determined by the Stein–Moore procedure and by microbiological assay

	Ser	Tyr	Met	Glu	His	Phe	Arg	Try	Gly	Lys	Pro	Val	Recovery (%)
Theory	2	2	1	1	1	1	3	1	2	4	2	3	
Protected peptide													
Stein–Moore	2·00	2·00	0·97	1·03	0·97	1·00	2·77	—	2·07	3·93	2·00	3·03	94
Microbiological	2·08	—	—	0·98	0·92	1·02	2·70	—	—	3·60	1·98	2·91	
Free peptide													
Stein–Moore	1·96	1·86	0·84	1·04	1·00	1·00	3·00	—	2·04	3·96	1·96	3·08	95
Microbiological	2·06	—	—	1·15	0·93	1·13	2·94	—	—	3·96	2·02	3·15	

Our synthetic route to the eicosa-peptide amide, which is patterned along the lines used in the preparation of the tricosa-peptide is illustrated in *Figure 10*.

Prolylvaline amide (VI), prepared from the carbobenzoxy derivative (V) by hydrogenolysis, was reacted with a mixed anhydride of carbobenzoxynitroarginine to give (VII) which was transformed into (VIII) by hydrogenolysis. Acylation of (VIII) with carbobenzoxynitroarginine *via* a mixed anhydride afforded (IX) and this intermediate was converted into (X) by catalytic hydrogenation.

Interaction between (X) and the azide (XI) resulted in formation of the protected decapeptide amide (XII) which was decarbobenzoxylated to (XIII). Sheehan's *N,N'*-dicyclohexylcarbodiimide reagent was employed to bring about peptide bond formation between the *N*-acetyldecapeptide (XIV) and (XIII) to give the protected eicosa-peptide amide (XV). As

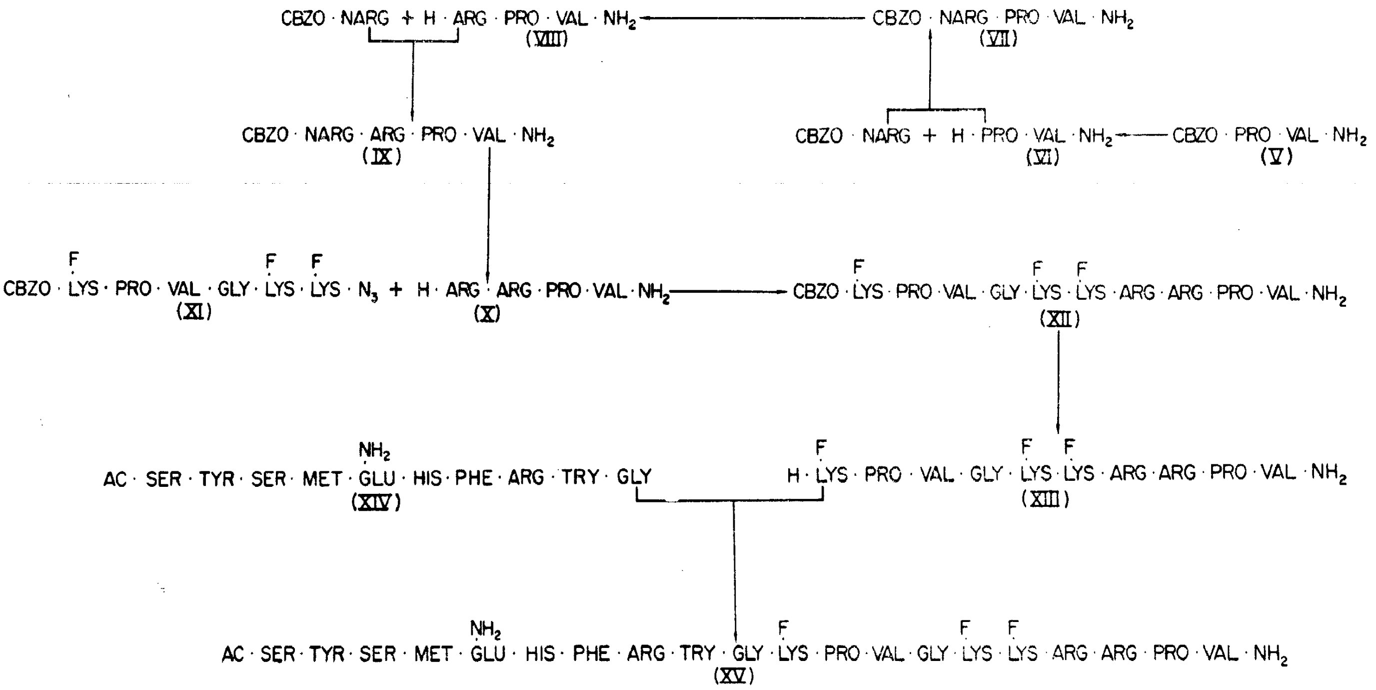

Figure 10. Synthesis of eicosa-peptide

illustrated in *Figure 11*, the protected eicosa-peptide amide behaved as a single component on paper chromatography and afforded paper electropherograms at pH 3·8, 5·1 and 6·9 exhibiting a single Pauly positive spot. Acid hydrolysates of the peptide derivative contained the constituent amino-acids in the ratios predicted by theory. The average recovery of amino-acids from the hydrolysate was 93 per cent. These findings in conjunction with the fact that the protected eicosa-peptide amide was synthesized from intermediates of proven homogeneity appears to justify the conclusion that the compound is of a high level of purity. The compound is inactive as concerns adrenocorticotropic activity, but on exposure to 0·5N hydrochloric acid for 80 minutes at the temperature of a boiling water-bath was converted to a material which possessed *in vivo* adrenocorticotropic activity (30–35 I.U./mg in various experiments). Apparently homogeneous samples of the eicosapeptide amide were isolated from a carboxymethylcellulose purified concentrate by cellulose-block electrophoresis at pH 7·0 followed by a second chromatographic step on carboxymethylcellulose. The final product was homogeneous as judged by paper chromatograms. As may be seen in *Figure 12* the peptide amide produced a single Pauly positive spot on paper electropherograms at various hydrogen ion concentrations, and most important, behaved as a single component on starch gel electrophoresis at pH 8·5 (*Figure 13*).

Acid hydrolysates of the compound contained the constituent amino-acids (minus tryptophan) in the ratios predicted by theory. The average recovery of amino-acids was 80 per cent. The eicosa-peptide amide was completely digestible by leucine aminopeptidase.

This rather extensive analytical evaluation appears to justify the conclusion that our eicosapeptide amide possesses a high degree of homogeneity. The observation that the peptide resisted the action of carboxypeptidase A established the presence of a C-terminal carboxamide function.

BIOLOGICAL EVALUATION OF SYNTHETIC PITUITARY HORMONES

The concept that the entire peptide sequence is not required for biological activity of certain pituitary hormones is a rather recent one. Since biological evaluation of peptide hormone fragments involves comparison with the genuine hormone (standard) in assays using intact animals, numerous factors must be considered which may influence the apparent biological potency. These include relative affinities of the hormone subunits for plasma constituents and their rate of release at the receptor sites, the rate of transport from the locus of administration to the receptors, the rate of transport across cell membranes, the rate of breakdown, the time required for onset of maximal response and possibly many other as yet unrecognized factors. In assigning definite potencies to such preparations it is tacitly assumed that standard and unknown are effected in a quantitatively indentical manner by all these variables. Since the validity of such an assumption remains to be demonstrated, assignment of potencies to fragments of peptide hormones relative to a standard must be regarded as approximations. The following conclusions should be interpreted with this in mind.

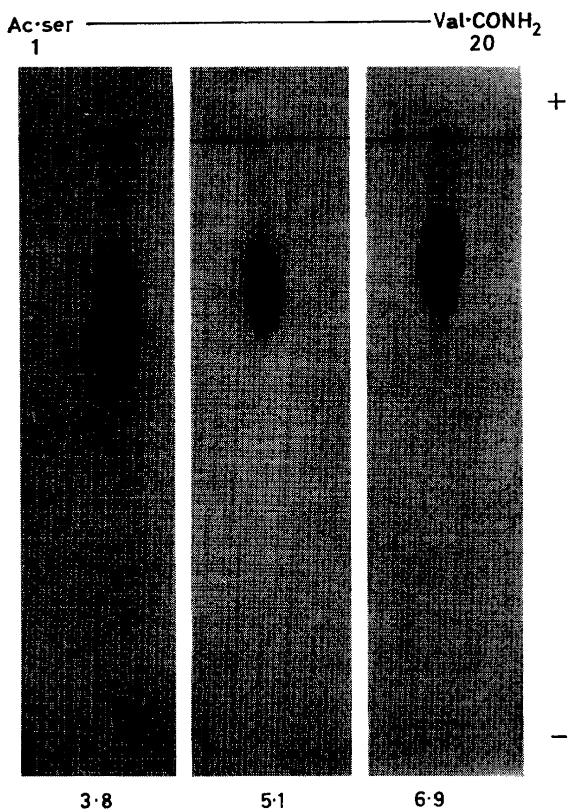

Figure 11. Paper electrophoretic behaviour of protected eicosapeptide amide at various pH values

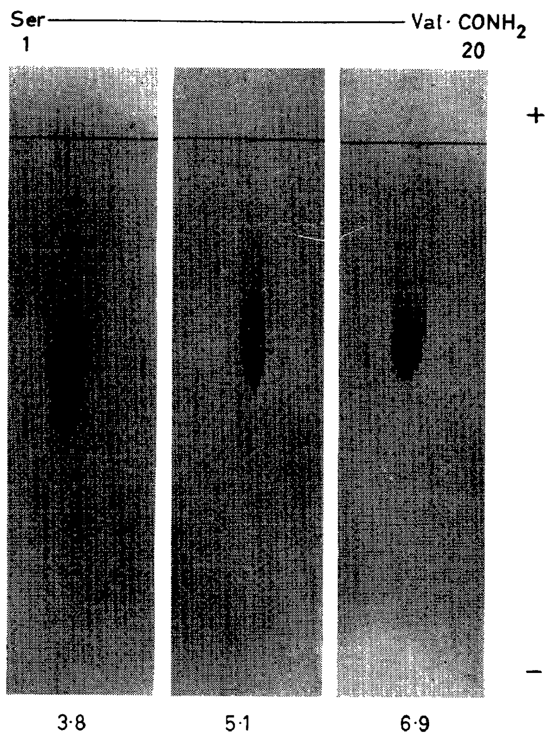

Figure 12. Paper electrophoretic behaviour of eicosapeptide amide at various pH values

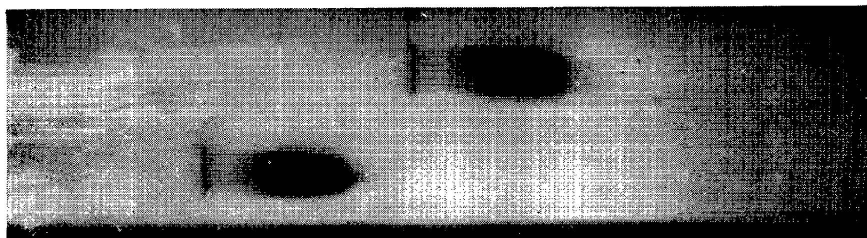

Figure 13. Starch gel electrophoresis of eicosapeptide amide at pH 8·5; duplicate experiment

The melanocyte-expanding activity of the various peptides was determined by Lerner and his staff at Yale Medical School, using the method of Shizume, Lerner and Fitzpatrick. Adrenocorticotropic activity was determined both by the adrenal ascorbic acid depleting method and by measuring the increase in plasma corticosterone 15 minutes after administration of the material to 24-hour hypophysectomized rats. We wish to express our sincere thanks to Dr Fisher of Armour Pharmaceutical Company for these determinations.

RELATION BETWEEN STRUCTURE AND ADRENOCORTICOTROPIC ACTIVITY IN THE RAT *IN VIVO*

Our procedures for producing homogeneous peptides possessing amino-acid sequences corresponding to that of the N-terminal section of the corticotropins provided us with the tools to investigate in a systematic manner the relation between chain length and adrenocorticotropic activity. In particular, we are interested in identifying the smallest fragment which is endowed with this important physiological property. Pertinent results are summarized in *Table 3*.

Steelman and Guillemin evaluated the adrenocorticotropic activity of natural α-MSH (I) and reported the activities shown. We have evaluated the adrenocorticotropic activity of the partially protected tridecapeptide amide (II) which differs from natural α-MSH by replacement of glutamic acid by glutamine and by a formyl group which is attached to the lysine N^{ϵ}-amino group. The compound is devoid of measurable activity but the deblocked tridecapeptide amide (III) shows definite and reproducible activity at a level of less than 100 milliunits per milligram. Not only does this substance bring about depletion of adrenal ascorbic acid in the hypophysectomized rat but it also stimulates steroidogenesis. This compound, then, is the first synthetic peptide reported in the literature possessing this type of physiological activity.

The observation that this tridecapeptide amide exhibits low but reproducibly significant *in vivo* adrenocorticotropic activity prompted further extension of its peptide chain in order to explore the importance in biological activity of the two lysine residues in positions 15 and 16. To this end, we prepared the partially protected hexadecapeptide amide (IV) and removed the protecting groups by treatment with 0·5N hydrochloric acid to obtain the unprotected hexadecapeptide (V).

Both the partially protected and the unprotected compounds were evaluated for ability to stimulate the adrenal cortex. The protected hexadecapeptide amide (IV) is inactive; the adrenocorticotropic potency of the unprotected peptide (V) is of the same order of magnitude as that of the tridecapeptide amide (III). Thus, one may conclude that a peptide corresponding to the N-terminal-16-amino-acid residues of the corticotropin sequence is capable of bringing about stimulation of the adrenal cortex of the hypophysectomized rat but that this stimulation is of a low order of magnitude compared to that elicited by the natural hormone.

The corticotropins whose structures have been elucidated to date contain a sequence composed of four basic amino-acid residues which occupies

Table 3. Relation between structure and physiological activity of peptide sequences related to corticotropin

	Compound (1 5 11 13 14 15 16 17 18 19 20 21 22 23 ... 39)	MSH in vitro U/g	ACTH adrenal ascorbic U/mg	ACTH plasma B U/mg
I	Ac · SER · · · GLU · · · LYS · · · VAL · $CONH_2$	2×10^{10}	0·22	0·12
	(NH_2 under GLU; F under LYS)			
II	Ac SER GLU LYS · VAL · $CONH_2$	$2{\cdot}2 \times 10^{10}$		
III	H_3^+N SER · GLU LYS · VAL · $CONH_2$	$1{\cdot}9 \times 10^{9}$	< 0·1	< 0·1
	(NH_2 under GLU; F under LYS 11, LYS 15, LYS 16)			
IV	Ac · SER · · · GLU · · · LYS · · VAL · GLY · LYS · LYS · $CONH_2$	$2{\cdot}0 \times 10^{9}$	INACTIVE	INACTIVE
V	H_3^+N SER · · GLU LYS · VAL · GLY · LYS · LYS · COO^-	$3{\cdot}7 \times 10^{8}$	< 0·1	< 0·1
VI	H_3^+N SER GLU LYS VAL · GLY LYS LYS · ARG · COO^-		∽ 6	
	(NH_2 under GLU)			
VII	H_3^+N · SER GLU LYS VAL GLY LYS LYS · ARG · ARG · PRO · COO^-		20 – 30	
VIII	H_3^+N · SER · · · GLU · · LYS · · · VAL · GLY · LYS · LYS ARG · ARG · PRO · COO^-	$1{\cdot}4 \times 10^{7}$	35	
	(NH_2 under GLU; F under LYS 11, LYS 15, LYS 16)			
IX	Ac · SER · · · GLU · · LYS · · · · VAL · GLY · LYS · LYS · ARG · ARG · PRO · VAL · $CONH_2$	$4{\cdot}2 \times 10^{8}$		
X	H_3^+N · SER · · GLU · LYS VAL · GLY · LYS · LYS · ARG · ARG · PRO · VAL · $CONH_2$	$1{\cdot}1 \times 10^{8}$	111·0 ± 18·0	83 ± 7
	(NH_2 under GLU; F under LYS 11, LYS 15, LYS 16, LYS 21)			
XI	Ac SER · · GLU · · LYS · · VAL · GLY · LYS · LYS ARG · ARG PRO VAL LYS VAL · TYR · $CONH_2$	$2{\cdot}0 \times 10^{8}$	INACTIVE	INACTIVE
XII	H_3^+N SER · · GLU · · · LYS VAL · GLY LYS · LYS ARG ARG · PRO VAL LYS · VAL TYR · COO^-	$2{\cdot}0 \times 10^{8}$	103 ± 10·4	91 ± 12·6
XIII	SER · · · · · · · · · · CORTICOTROPIN · · · · · · · · · · PHE	$1{\cdot}7 \times 10^{8}$	80 – 100	94·5 ± 10·6

positions 15 to 18 in the polypeptide chain. The observation that the hexadecapeptide (V) possesses only a low level of adrenocorticotropic activity points to this basic tetrapeptide moiety as essential for high level potency.

Li *et al.* find that the nonadecapeptide (VIII) has the ability to simulate the adrenal cortex *in vivo*. Their compound exhibits an activity of approximately 30 I.U. per mg. Essentially the same biological activity was reported by Schwyzer *et al.* for the glutamine analogue of this peptide (VII). Thus, there remains little doubt that elongation of the peptide chain from 16 to 19 amino-acid residues is accompanied by a striking increase in biological activity, pointing to the importance of the lysyllysylarginylarginyl sequence for high level potency. In 1956, Boissonnas *et al.* reported a synthesis of the eicosa-peptide which corresponds in its arrangement of amino-acids to the N-terminus of the corticotropin molecule. This product (carboxylic acid analogue of X) according to them, possessed only the very limited *in vitro* adrenocorticotropic activity, of 2 to 3 units per mg.

We have developed a synthesis, previously discussed in this lecture, of the eicosapeptide amide (X) which contains the same amino-acid sequence as the compound prepared by Boissonnas. We find that this substance possesses essentially the full adrenocorticotropic activity of natural ACTH namely $111{\cdot}0 \pm 18{\cdot}0$ I.U./mg. The protected precursor in the synthesis of the eicosa-peptide amide (IX) is practically devoid of adrenocorticotropic activity. Extension of the chain of the eicosapeptide to the tricosapeptide (XII) does not appear to effect significantly the adrenocorticotropic potency. The biological activity of the tricosa-peptide of $103 \pm 10{\cdot}3$ I.U./mg is essentially the same as that of the shorter unit. Here again the protected precursor (XI) is essentially inert.

Summarizing these findings it is apparent that the unit of the ACTH molecule which is endowed with full ACTH activity must be longer than 16 but may be shorter than 20 amino-acid residues. A structural element located in the region between position 16 and position 20, most likely the segment lysyllysylarginylarginine, appears to play an important rôle in connection with adrenocorticotropic potency.

We have noted that the protected precursors (IX) and (XI) of the highly active eicosa-, and tricosa-peptides (X) and (XII) exhibit practically no adrenocorticotropic activity. A simplified version of the structure of the protected tricosapeptide amide is illustrated in *Figure 14*.

It is of some interest that the various blocking groups, namely the N-terminal acetyl, the glutamine amide, and the formyl group are among a class of substances which may be designated as "physiological". This implies that these groups are normally present in cells and that there are metabolic pathways available for their attachment to, or elimination from, other molecules. It is thus rather surprising that both the rat and, as we shall see later, the human seem to possess at best only a very limited ability to convert the biologically inert protected material into the biologically active species.

From the point of view of the mode of action of the corticotropins, it is of considerable importance to ascertain whether all of the functional groups which are covered in the tricosa-peptide amide are essential for activity or whether only one or more key functional groups are involved. There is

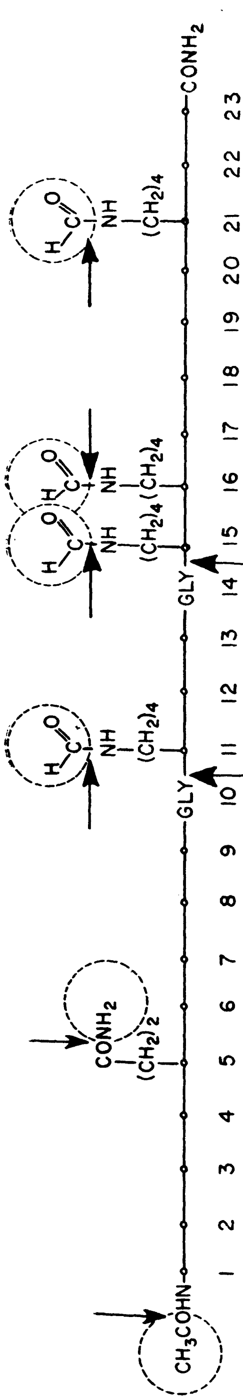

Figure 14. Location of bonds labile to dilute hydrochloric acid of an adrenocorticotropically inactive, partially protected tricosa-peptide amide

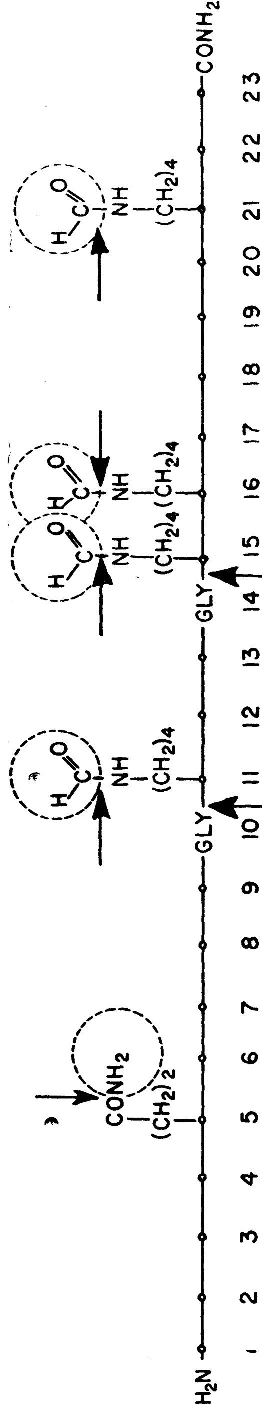

Figure 15. Partially protected tricosa-peptide amide

experimental evidence to show that the free N-terminus is necessary for high level adrenocorticotropic activity. For example, Waller and Dixon have shown that selective acetylation of the N-terminal serine in corticotropin from natural sources brings about a marked drop in biological activity. Utilizing the differential stability towards trifluoroacetic acid between the t-butyloxycarbonyl and the formyl group, we have been able to secure the partially protected tricosa-peptide amide shown in *Figure 15.*

This compound exhibits adrenocorticotropic activity at a level of 0·5–0·7 units per mg. This finding attests to the importance of the free N-terminus for biological activity and illustrates convincingly that all or some of the lysine ε-amino groups in positions 11, 15 and 16 must be present in unprotected form for high level activity. The high degree of biological activity of the eicosa-peptide amide eliminates the lysine residue in position 21 as a significant structural element for adrenocorticotropic activity.

Returning for a moment to *Table 3*, it should be noted that all of the compounds listed possess a significant level of melanocyte-expanding activity. Differences between the protected or free peptides as concerns this physiological property are not impressive. Earlier in this presentation I mentioned the fact that ACTH possesses a dual specificity since it possesses both melanocyte-expanding and adrenocorticotropic activity. It has now become possible to dissociate these activities through chemical manipulation in some of the synthetic peptides. For example, the eicosa-peptide amide (X) possesses both activities but the protected derivative (IX) is devoid of adrenal stimulating activity; it retains the ability to expand melanocytes. The same relation holds as concerns compounds (XI) and (XII).

PHYSIOLOGICAL EFFECTS OF SYNTHETIC PITUITARY HORMONES IN MAN

Lerner and McGuire injected our synthetic α-MSH derivative (4 to 8 mg daily for 14 days) into two negroes and observed marked darkening of the skin. This experiment demonstrated for the first time that a pure synthetic peptide closely related to natural α-MSH exerts striking effects on human melanocytes. Results of blood studies before and after administration of the synthetic preparation showed essentially no change and there were no alterations in blood pressure and pulse rate. Danowski *et al.* administered the same compound (8 mg daily for 7 days) to a negro patient with vitiligo and observed darkening of the normal skin, but the diseased areas remained unchanged. There were no demonstrable electrolyte, steroid, carbohydrate or lipid effects in this patient.

These findings with human material are in full agreement with previous observations which have indicated that this synthetic α-MSH derivative, although exhibiting a high level of melanocyte-expanding activity *in vitro*, is without measurable effect on the adrenal cortex of the rat *in vivo*.

The ACTH-like activity in the human of the adrenocorticotropically active synthetic tricosa-peptide was also assessed by Danowski *et al.* The polypeptide was incorporated into the gelatin base employed for production of commercial ACTH and amounts of this material equivalent to 160 units were administered once daily for seven days into the deltoid muscle of four

healthy adult male prisoners and into a 53-year-old female with disseminated sclerosis (scleroderma).

Observations on the effects of 200 units of Acthar gel administered once daily for seven days to a total of 21 healthy adult male prisoners and 5 laboratory workers (2 males, 3 females) served as controls.

None of the subjects experienced local reactions to the peptide or the Acthar gel. Increases in body weight in the two groups were comparable and there was no discernible trend in the blood pressure fluctuations recorded prior to and during the period of therapy. The clinical status of the patient with scleroderma definitely improved during treatment with the synthetic polypeptide. None of the subjects receiving the synthetic polypeptide manifested a discernible Cushing's syndrome facies. As illustrated in *Figure 16* the synthetic peptide produced in all five subjects a several-fold increase in urinary 11-oxysteroids as reflected in Porter-Silber chromogens. The magnitude of this rise compares favourably with that seen with 200 units of Armour's ACTH daily in the group of control subjects as a whole. The tricosa-peptide raised the 24-hour output of urinary 17-ketosteroids in three

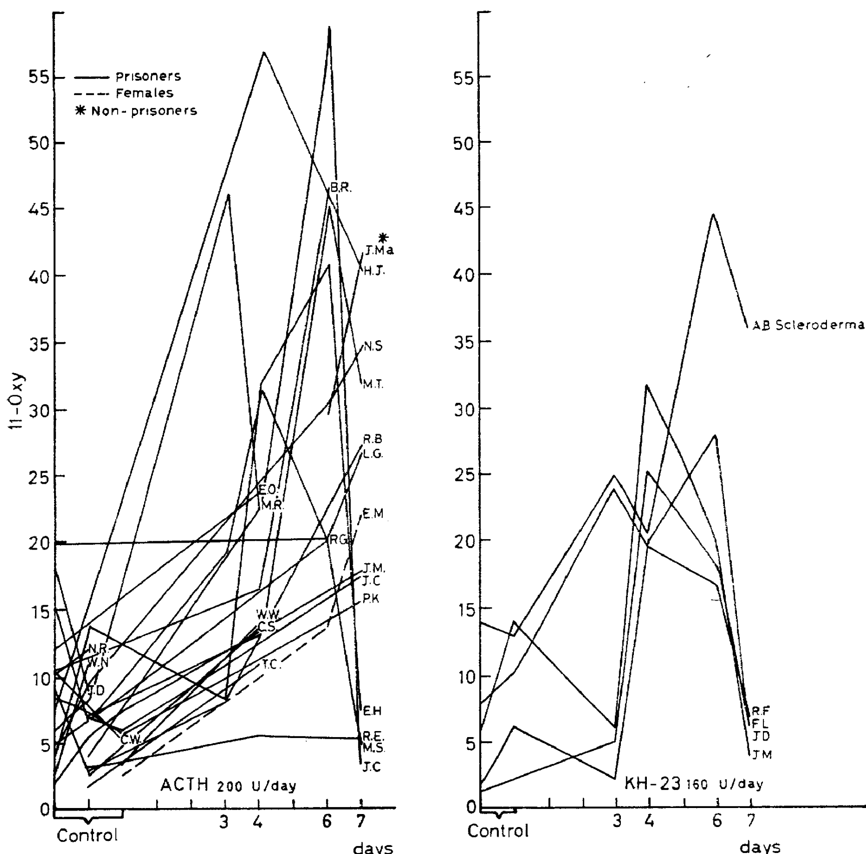

Figure 16. Increases in daily urinary excretion of 11-oxysteroids (measured as milligrams of Porter-Silber chromogens per 24 hours) during administration of commercial ACTH (200 units per day) and of synthetic tricosa-peptide (160 units per day)

of the four men and increased the urinary excretion of 11-desoxysteroids. The synthetic material did lower the total serum protein, serum albumin and serum calcium values. Similar quantities of commercial ACTH usually raise the serum sodium and CO_2 and lower the serum potassium, total protein, albumin, globulin and calcium.

These and other results indicate that the synthetic tricosa-peptide elicits a number of responses qualitatively similar to those seen following administration of commercial ACTH of animal origin. Additional studies are necessary for evaluation of the significance of certain quantitative differences in the urinary and serum responses to the peptide and to commercial ACTH but the somewhat lower dosages of tricosa-peptide which were employed may provide the answer. In assessing these differences, it must be realized that comparison is made between a crude mixture of compounds as it occurs in commercial ACTH with a compound that is well defined chemically. Some of the physiological effects of the commercial preparation may not be those of corticotropin but may reflect contamination by other biologically active materials.

An identical study using the protected tricosa-peptide amide was conducted with four healthy male prisoners. The peptide derivative, in gelatin, was injected intramuscularly at a dose level of 6·7 mg per day for seven days. This dose corresponds closely to that employed with the free peptide. There were no increases in blood pressure or body weight nor did any of the subjects develop Cushingoid manifestations. In contrast to the free tricosa-peptide which increases the urinary 11-oxysteroids and to a lesser degree the 17-ketosteroids the protected molecule fails to produce these changes. With exception of a decrease in serum potassium, the protected compound failed to elicit significant alterations in serum electrolytes.

Earlier, we have observed little if any difference between a synthetic peptide corresponding to the first 20, or one corresponding to the first 23 amino-acid residues of the corticotropin molecule as concerns adrenocorticotropic activity in the rat. The results shown in *Table 4* demonstrate essentially

Table 4. Effect of intravenously administered peptides on plasma 17-hydroxy-corticoid levels in the human (mean values in μg/100 ml plasma)

Compound	*Dose* (units)	*Time* (min) 0	15	30	60	90	120	240
Eicosa-peptide amide (12)	2–8*	14·2 ± 1·4	17·5 ± 1·0	22·8 ± 3·0	21·1 ± 1·4			
Tricosapeptide (14)	2–8*	16·4 ± 2·2	18·0 ± 1·6	21·3 ± 1·8	24 ± 2·1			
Tricosapeptide (1)	30†	5·7	13·7	19·0	28·3	32·2	30·9	12·9
ACTH (1)	24†	5·7	12·5	17·7	19·5	25·9	30·4	23·4

Numbers in parentheses refer to the number of subjects used.
* Courtesy of H. S. Lipscomb.
† Courtesy of F. Engel and H. Lebowitz, endogenous ACTH blocked by administration of Dexamethasone (3·5 mg every eighth hour).

identical potencies of these preparations as concerns elevation of plasma 17-hydroxysteroids in man. When administered at dose levels of 2 to 8 units per patient, both synthetic preparations bring about essentially the same

significant increases in the plasma steroid levels. The data pertaining to the eicosa-peptide amide are mean values derived from the study of 12 patients; those pertaining to the tricosa-peptide derive from studies on 14 human subjects. The response to a dose of 300 μg of the tricosa-peptide administered to a patient whose endogenous ACTH level was blocked by administration of dexamethasone appears to be roughly equivalent to a dose of 24 units of a commercial ACTH preparation.

These results with human material correspond to findings based on studies in the rat. They support the conclusion that our synthetic ACTH preparations exhibit essentially the full potency of the natural hormone.

In a previous section of this lecture we have shown that it is possible to dissociate the melanophoretic and adrenocorticotropic activities of certain peptide hormones through selective chemical manipulation of structure. We have pointed out that simple blocking of certain functional groups of the adrenocorticotropically highly active tricosa-peptide affords a material which retains practically the full melanophoretic potency of the starting compound but which had lost almost completely the adrenal stimulating properties. These results, based on studies in the rat, have been fully substantiated in the human. McGuire and Lerner injected the protected tricosa-peptide amide into a light coloured negro volunteer at a dose of 14 mg per day for seven days and observed the striking darkening of the skin. Careful blood and urinary studies on this subject revealed none of the characteristic manifestations of adrenal cortical stimulation.

SUMMARY AND OUTLOOK

In this paper I have reviewed some of the results of our investigations in the pituitary hormone field. I have sketched some of the chemical developments, have stressed the importance of careful evaluation of homogeneity of synthetic peptides and have given you a survey of some of the biological findings both in animals and in man. I believe that these results are but the beginning of remarkable advances in the understanding of the physiology and chemistry of those hormones which are based on the polypeptide architectural principle. Let us never forget that these advances, whatever they may be, will stem from a solid chemical foundation.

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