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Studies on Polypeptides. XXIV. Synthesis and Biological Evaluation of a Tricosapeptide Possessing Essentially the Full Biological Activity of ACTH[1-4]

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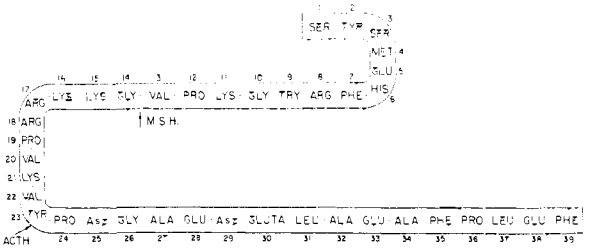
The partially protected tricosapeptide amide N-acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N$^{\epsilon}$-formyllysylprolylvalylglycyl-N$^{\epsilon}$-formyllysyl-N$^{\epsilon}$-formyllysylarginylarginylprolylvalyl-N$^{\epsilon}$-formyllysylvalyltyrosine amide (21-L) was prepared from two subunits of established homogeneity. The peptide amide was found to possess the same level of *in vitro* melanocyte expanding activity as pig corticotropin, but the compound was essentially inactive as concerns adrenocorticotropic potency. The tricosapeptide seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginylprolylvalyllysylvalyltyrosine (21-L) which corresponds to the arrangement of the N-terminal 23 amino acid residues of pig corticotropin was isolated from partial hydrolysates of the protected precursor. The homogeneity of the free peptide was established by paper chromatography and by paper and starch-gel electrophoresis. Acid hydrolysates of the peptide contained the constituent amino acids in the ratios predicted by theory. Microbiological assays of serine, glutamic acid, histidine, phenylalanine, arginine, lysine, proline and valine in the acid hydrolysates demonstrated the presence of the L-forms of these amino acids within the limits of the assay procedures. These data offer convincing evidence in support of the stereochemical homogeneity of the synthetic tricosapeptide. The melanophoretic and adrenocorticotropic activities of the tricosapeptide were of the same order of magnitude as those of pig corticotropin when administration was by the intravenous route. Differences in biological response were noted between the synthetic peptide and pig corticotropin when administration was by the subcutaneous route. Some physiological effects in man of both the partially protected and of the free tricosapeptides are discussed and certain conclusions were drawn relating peptide structure and adrenocorticotropic activity.

Two groups of investigators[5,6] have elucidated the amino acid sequence of pig corticotropin and have arrived at structures for this hormone differing slightly in the arrangement of the amino acid residues in positions 25–30. These differences have been resolved[7] in favor of the sequence proposed by Bell which is illustrated below.

Peptic digestion of pig corticotropin affords, among other products, an N-terminal octacosapeptide fragment (positions 1–28) which is reported to retain essentially the full adrenal ascorbic acid depleting activity of the intact hormone.[8] From studies of partial hydrolysis with acid it was inferred that the four C-terminal amino acid residues may be removed from this octacosapeptide with formation of a tetracosapeptide (positions 1–24) without effecting biological activity, but the tetracosapeptide was neither isolated nor characterized.[9]

Since the corticotropin structure must remain hypothetical until synthesis can be achieved, we undertook the preparation of the tricosapeptide



Formula 1

(IV) which corresponds to positions 1–23 of the molecule.

We find that our synthetic preparation exhibits essentially the full biological activity of the corticotropins thus far isolated from natural materials.

We have observed that a synthetic peptide corresponding to the N-terminal hexadecapeptide sequence (positions 1–16) of pig corticotropin possesses a low but reproducible level of adrenocorticotropic activity.[10] Thus, a structural element in the hormone molecule which is crucial for full biological activity must be located between positions 16 and 23.

Adrenocorticotropic activity, as evaluated by the adrenal ascorbic acid method, both in crude extracts and in pure corticotropin, is remarkably stable to acid. Prolonged heating at 100° in 0.1 *N* hydrochloric acid exerts little if any effect on this biological property.[11]

Our approach to the synthesis of the tricosapeptide takes advantage of this property of the hormone. We assemble the complete amino acid sequence in protected form, using blocking groups removable by exposure to dilute hydrochloric acid. The observation that treatment with hot dilute hydrochloric acid eliminates the formyl group from histidylphenylalanylarginyltryptophyl-

(1) The authors wish to express their appreciation to the U. S. Public Health Service, the National Science Foundation, the American Cancer Society and Armour and Company for generous support of this investigation.

(2) The peptides and peptide derivatives mentioned in this communication (with exception of glycine) are of the L-configuration. In the interest of space conservation the customary L-designation for individual amino acid residues has been omitted.

(3) See *J. Am. Chem. Soc.*, **84**, 4470 (1962), for paper XXIII in accompanying paper series.

(4) Some of the results recorded in this paper have been presented at: (a) The "First International Symposium on Polyamino Acids," June 19, 1961, "Polyamino Acids, Polypeptides and Proteins" M. Stahmann, editor, University of Wisconsin Press, 1962, p. 21; (b) at the Laurentian Hormone Conference, September 5, 1961, "Recent Progress in Hormone Research," G. Pincus, editor, Academic Press, Inc., New York, N. Y., Vol. XVIII, 1962, p. 41; (c) a preliminary communication has appeared in *J. Am. Chem. Soc.*, **83**, 487 (1961).

(5) P. H. Bell, *ibid.*, **76**, 5565 (1954).

(6) W. F. White and W. A. Landman, *ibid.*, **77**, 1711 (1955).

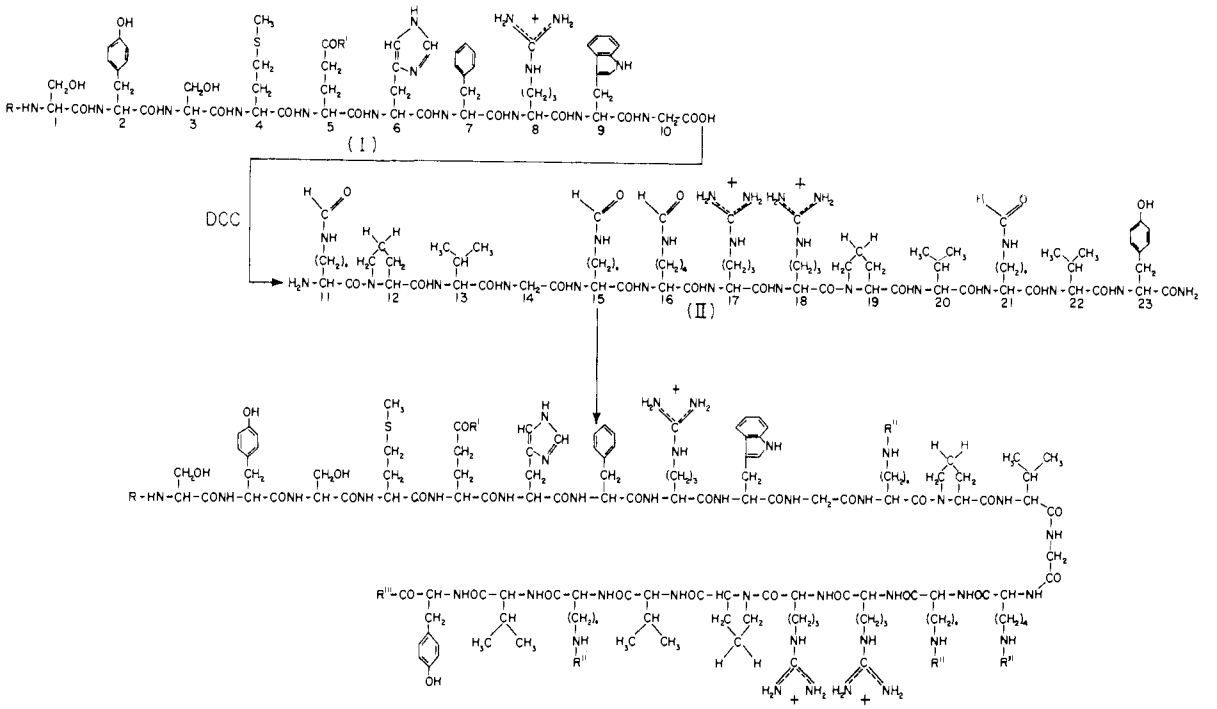
(7) J. I. Harris, *Brit. Med. Bull.*, **16**, 189 (1960).

(8) P. H. Bell, K. S. Howard, R. G. Shepherd, B. M. Finn and J. H. Meisenhelder, *J. Am. Chem. Soc.*, **78**, 5059 (1956).

(9) R. G. Shepherd, S. D. Willson, K. S. Howard, P. H. Bell, D. S. Davies, S. B. Davis, E. A. Eigner and N. E. Shakespeare, *ibid.*, **78**, 5067 (1956).

(10) K. Hofmann, N. Yanaihara, S. Lande and H. Yajima, *ibid.*, **84**, 4470 (1962).

(11) For a review see E. B. Astwood, M. S. Raben and R. W. Payne in "Recent Progress in Hormone Research," Vol. VII, G. Pincus, editor, Academic Press, Inc., New York, N. Y., 1952, p. 1.



Formula 2

	R	R'	R''	R'''
(III)	Acetyl	NH_2	Formyl	NH_2
(IV)	H	OH	H	OH
(V)	H	NH_2	Formyl	NH_2

glycyl-N$^{\epsilon}$-formyllysylprolylvaline amide with formation of the corresponding lysyl peptide and the finding that the same treatment converts N-acetylseryltyrosylserylmethionylglutamine into seryltyrosylserylmethionylglutamic acid causing only minor fragmentation of peptide bonds provided decisive model experiments.[12]

In previous communications[12,13] we have described preparations of the protected decapeptide (I) and of the partially protected tridecapeptide amide (II) and have presented data supporting their homogeneity. The present study records experimental details pertaining to the coupling of these two fragments with formation of the protected tricosapeptide amide (III) and conversion of this compound into the free tricosapeptide (IV). Results of the biological evaluation of (III) and (IV) both in animals and in man also will be discussed.

Paper chromatograms of the acetate salts of (I) and (II), the form in which they were isolated originally, have been reproduced.[4a] In order to eliminate competition with acetate ions during the final coupling step, these sub-units were converted into the corresponding hydrochlorides. The crude, protected tricosapeptide amide (III) which ensued when the hydrochlorides of (I) and (II) were coupled by the use of N,N'-dicyclohexylcarbodiimide (DCC)[14] was subjected to chromatography on carboxymethylcellulose (CMC)[15] and apparently homogeneous samples of (III) were obtained.

The highly purified protected tricosapeptide amide (III) isolated as the triacetate octahydrate, behaved as a single component on paper chromatography and on paper electrophoresis at various pH values and its acid hydrolysate contained the constituent amino acids (minus tryptophan) in the ratios predicted by theory. The average recovery of amino acids from the hydrolysate was 94%.[16]

Microbiological determination of certain amino acids in acid hydrolysates of (III) (Table I) showed them to be of the L-variety within the limits of error of the assay procedure.

The protected tricosapeptide amide (III) possessed marked *in vitro* melanocyte expanding activity (2.0×10^8 MSH units/g.[17]) and on administration to a light colored negro (14 mg. per day for seven days) brought about marked darkening of the skin as observed by both visual observation and by skin surface reflectance measurements.[18] The compound exhibited a low level of *in vivo* adrenal cortical stimulation (0.05 to 0.1 i.u./mg.) as determined by rat adrenal ascorbic

(12) K. Hofmann and H. Yajima, *J. Am. Chem. Soc.*, **83**, 2289 (1961).

(13) K. Hofmann, T. Liu, H. Yajima, N. Yanaihara and S. Lande, *ibid.*, **83**, 2294 (1961).

(14) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1955).

(15) E. A. Peterson and H. A. Sober, *ibid.*, **78**, 751 (1956).

(16) Several grams of the protected tricosapeptide amide (III) were prepared with yields ranging from 40–53%. A paper chromatogram of the highly purified compound has been reproduced.[4b]

(17) We wish to express our thanks to Drs. A. B. Lerner and J. S. McGuire of the Department of Medicine, Yale University School of Medicine, for the MSH assays which were performed according to the method of K. Shizume, A. B. Lerner and T. B. Fitzpatrick, *Endocrinol.*, **54**, 553 (1954).

(18) J. S. McGuire and A. B. Lerner, Fifth International Pigment Cell Conference, Annals of the New York Academy of Sciences (in press).

TABLE I

AMINO ACID COMPOSITION OF ACID HYDROLYSATES OF THE PROTECTED AND FREE SYNTHETIC TRICOSAPEPTIDES 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 (III)													
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 (IV)													
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	

acid assay[19] but the stimulation resembled the non-dose related all-or-none type of response. A dose of 0.05 mg. administered by the subcutaneous route failed to produce a significant depression of the adrenal ascorbic acid level in 24 hr. hypophysectomized rats, but a significant rise in plasma corticosterone (from 4 to 6 μg. per 100 ml. to 10.3 to 15.0 μg. per 100 ml.) ensued and continued to be maintained for 3 hr. Administration of the material to four healthy males (6.7 mg. per day for seven days) showed the preparation to be essentially ineffective in bringing about adrenal cortical stimulation.[20] Thus, the protected tricosapeptide amide (III) is practically devoid of adrenal cortex stimulating potency but exhibits potent melanocyte expanding activity both *in vitro* in frogs and *in vivo* in man.

Samples of the protected tricosapeptide amide were exposed to 0.5 *N* hydrochloric acid at the temperature of a boiling water bath for various time intervals and following exchange of the chloride by acetate ions the ensuing hydrolysates were lyophilized. Assays of the residues by the rat adrenal ascorbic method gave the results shown on Table II. It is apparent that partial hydrolysis converts the essentially inactive starting compound into materials possessing significant adrenocorticotropic potency. 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 protecting groups, *i.e.*, the N-terminal acetyl, the glutamine amide and the N^{ϵ}-formyl groups from the lysines in positions 11, 15, 16 and 21. The slow decline in biological activity on prolonged exposure to acid appears to be the result of gradual peptide backbone fragmentation. A hydrolysis time of 80 minutes was selected for isolation of the biologically active compound on a preparative scale since it was assumed that the protecting groups had been removed quantitatively at this point and that the hydrolysate contained a mixture composed of completely deblocked peptide (IV) plus smaller peptide fragments. Such a mixture, it was reasoned, would be easier to separate than a conglomeration of closely similar partially protected tricosapeptides as appears to be present after shorter exposure to the acid.

TABLE II

ADRENAL ASCORBIC ACID DEPLETING ACTIVITY OF PARTIAL HYDROLYSATES OF (III) AT VARIOUS INTERVALS OF TIME (SEE TEXT FOR CONDITIONS)

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

Chloride ions were exchanged for acetate ions in an 80 minute hydrolysate and the ensuing mixture of peptides (biological activity 30–40 i.u./mg.) was subjected to CMC chromatography. A typical chromatogram is illustrated on Figure 1. Of the various chromatographic fractions only those derived from peaks 5, 6 and 7 showed a significant *in vivo* adrenocorticotropic activity. Fractions corresponding to peak 6 which exhibited the highest specific potency were pooled and evaporated to give samples of (IV) which were contaminated by two minor impurities. Preparations of the tricosapeptide homogeneous on paper chromatograms, on paper electropherograms at various hydrogen ion concentrations and which produced a single spot on starch-gel electrophoresis at pH 8.5 were isolated from the CMC purified material by cellulose-block electrophoresis at pH 7.0 followed by a second chromatographic purification on CMC using ammonium formate buffers for column development.[21] Acid hydrolysates of this material gave the amino acid ratios (minus tryptophan) predicted by theory and microbiological assays of a number of amino acids in the hydrolysate (Table I) showed them to be of the L-variety within the limits of assay errors.

In a preliminary communication[4c] we have formulated the tricosapeptide (IV) as terminating with an amide group. The observation that carboxypeptidase A releases 86% of the C-terminal

(19) Ascorbic acid depleting activity was determined in 24 hr. hypophysectomized rats according to the method of "U. S. Pharmacopeia," Vol. XV against the U.S.P. reference standard. The plasma corticosterone levels were determined 15 minutes following administration (R. Guillemin, G. W. Clayton, J. D. Smith and H. S. Lipscomb, *Endocrinol.*, **63**, 349 (1958)). The free steroid was separated chromatographically and assayed by a modification of the method of H. Kalant, *Biochem J.*, **69**, 93 (1958). We are much indebted to Dr. Joseph D. Fisher of Armour Pharmaceutical Company, Kankakee, Illinois, for the biological assays.

(20) T. S. Danowski, K. Hofmann, H. Yajima and C. Moses, *Proc. Soc. Expl. Biol. Med.*, **108**, 559 (1961).

(21) See ref. 4b for reproductions of a paper chromatogram and of electropherograms of highly purified (IV).

tyrosine and 93% of the valine in position 22 demonstrates the presence of a free C-terminal carboxyl group.

The constituent amino acids were liberated from the peptide by leucine aminopeptidase, but recovery of amino acids located in the N-terminal portion of the molecule was higher than that from the C-terminal end. Pig corticotropin behaves similarly.[22] Liberation of glutamic acid and not glutamine on enzymic digestion of the tricosapeptide corroborated the presence of the glutamic acid residue in position 5. We have previously shown[12] that our LAP (leucine aminopeptidase) preparation does not remove the amide group from glutamine or from peptides containing this amino acid residue.

The experimental evidence cited appears to justify the conclusion that the synthetic tricosapeptide possesses a high degree of homogeneity.[23]

Realization that the entire molecule of certain pituitary hormones is not required for biological activity is of rather recent origin. The observation that peptic digests and partial acid hydrolysates of highly purified pig corticotropin possess potent adrenal ascorbic acid depleting activity provided the first indirect evidence.[11] Our demonstration that a chemically defined octapeptide, corresponding to a section of the α-MSH molecule, exhibits significant melanocyte expanding activity,[24] afforded the first direct evidence, in the anterior pituitary hormone series.

Since biological evaluation of peptide hormone fragments or derivatives 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 passage through 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 identical 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.

The tricosapeptide (IV) exhibits marked *in vitro* melanocyte expanding activity (2.0×10^8 MSH units/g.), and in this respect it is essentially as potent as the protected precursor (III) and pig corticotropin. Biological evaluation by the rat adrenal ascorbic assay of various batches of material from the first CMC chromatogram gave a weighted mean potency of 103 ± 10.4 i.u./mg. (p 95% confidence limits 84.8–126 i.u./mg.). Our most highly purified samples of the tricosapeptide (CMC chromatography, electrophoresis, CMC chromatography) possessed comparable biological potency (116 ± 21 i.u./mg.). Pig corticotropin by this assay is reported to exhibit an activity of 80–100 i.u./mg.[25] When assayed for its ability to bring about elevation in plasma corticosterone levels in the rat, an activity of 91 ± 12.6 i.u./mg. was found for the synthetic hormone.[19] Pig corticotropin (Bell) is reported to possess a potency of 94.5 ± 10.6 i.u./mg. in this assay.[26] Intravenous infusion of the tricosapeptide at dose levels above 5 i.u. brings about marked elevation of the plasma 17-hydroxysteroids in man.[27]

The results of this extensive comparison of certain biological properties of the synthetic preparation with the International Standard appears to justify the conclusion that the tricosapeptide (IV) possesses essentially the same biological activity as natural ACTH both in animals and in man when comparison is made on the basis of intravenous administration.

The biological properties of the tricosapeptide differ somewhat from those of highly purified pig corticotropin and of the biological standards when comparison is based on subcutaneous administration. The subcutaneous (s.c.) potency of two preparations of (IV) was 38.5 ± 4.0 and 43.6 ± 5.0 i.u./mg., respectively. By definition the potency ratio subcutaneous to intravenous administration (s.c./i.v.) of the U.S.P. or International Standards of ACTH is unity. Pig corticotropin exhibits an s.c./i.v. potency ratio of approximately 3.[28] The s.c./i.v. potency ratio of the synthetic tricosapeptide is approximately 0.3.

Recently we have completed[29] a synthesis of an eicosapeptide amide corresponding to positions 1–20 of the corticotropin molecule and find that this compound which exhibits a high level of adrenal ascorbic acid depleting potency (111.0 ± 18.0 i.u./mg.) also possesses an s.c./i.v. ratio of approximately 0.3. Thus, our studies suggest that low s.c./i.v. ratios are a characteristic property of adrenocorticotropically active chemically defined subunits of the ACTH molecule. These findings are in marked disagreement with observations by Li, *et al.*,[30] who report an s.c./i.v. ratio of approximately 2 for a nonadecapeptide which corresponds to positions 1–19 of the ACTH molecule. It should be noted that the i.v. potency of the Li preparation is at best 30% that of our eicosa- and tricosapeptides.

(22) W. F. White, *J. Am. Chem. Soc.*, **77**, 4691 (1955).

(23) The preparation of CMC purified (IV) acetate by partial hydrolysis with acid of (III) has been repeated many times with yields ranging from 25–35%.

(24) K. Hofmann, T. A. Thompson and E. T. Schwartz, *J. Am. Chem. Soc.*, **79**, 6087 (1957).

(25) R. G. Shepherd, K S. Howard, P. H. Bell, A. R. Cacciola, R. G. Child, M. C. Davies, J. P. English, B. M. Finn, J. H. Meisenhelder, A. W. Moyer and J. van der Scheer, *ibid.*, **78**, 5051 (1956).

(26) R. Guillemin, *Endocrinol.*, **66**, 819 (1960).

(27) These results were obtained by Dr. F. C. Engel, Department of Medicine, Duke Medical School and by Dr. H. S Lipscomb, Department of Physiology, Baylor University Medical School, and will be presented in separate publications. We wish to thank these colleagues for their cooperation.

(28) For a review see E. E. Hays and W. F. White in "Recent Progress in Hormone Research," Vol. X, G. Pincus, editor, Academic Press, Inc., New York, N. Y., 1954, p. 265.

(29) K. Hofmann, T. Liu, H. Yajima, N. Yanaihara, C. Yanaihara and J. Humes, *J. Am. Chem. Soc.*, **84**, 4481 (1962).

(30) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T. Lo and J. Ramachandran, *ibid.*, **83**, 4419 (1961).

When administered intramuscularly to five human subjects (160 i.u. per patient per day) the tricosapeptide elicited a number of responses qualitatively similar to those seen following administration of 200 i.u. per patient per day of commercial ACTH.[31]

The observation that a synthetic peptide of established structure which corresponds to the arrangement proposed for the N-terminal 23 amino acid residues of pig corticotropin possesses essentially the full adrenocorticotropic potency of the natural hormone appears to corroborate the proposed structure of this section of the molecule. However, comparison of biological activity alone does not provide unequivocal proof of structure until it can be demonstrated that full hormonal function is not only critically dependent on the position but also on the specific nature of each of the constituent amino acid residues.

It is significant that protection of the N-terminus by an acetyl group and formylation of the lysine ϵ-amino groups brings about a marked change in the biological properties of the tricosapeptide. This modification eliminates almost completely the adrenocorticotropic activity but does not measurably alter the melanophoretic properties of the molecule. Waller and Dixon[32] have selectively acetylated the N-terminus of pig corticotropin and have found that this modification markedly diminishes the adrenocorticotropic potency of the hormone. We have prepared the partially protected tricosapeptide amide (V) and find that it possesses only a low level of adrenal ascorbic acid depleting activity (0.5 to 0.7 i.u./mg.).[33]

This observation demonstrates that in addition to an unprotected N-terminus, all or some of the lysine ϵ-amino groups in positions 11, 15 and 16 are essential for high level adrenal cortical stimulation. The high biological activity of the eicosapeptide amide (1–20)[28] eliminates the lysine in position 21 as a crucial structural element for ACTH activity.

Experimental[34]

Acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine Dihydrochloride Octahydrate (I).—Acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine (I)[12] (600 mg.) was dissolved in 0.2 *N* hydrochloric acid (10 ml.) with short warming at 50°, and the solution was lyophilized; yield essentially quantitative.

Anal. Calcd. for $C_{61}H_{81}O_{16}N_{17}S\cdot 2HCl\cdot 8H_2O$: C, 47.0; H, 6.4; N, 15.3. Found: C, 47.5; H, 6.5; N, 14.9.

N$^{\epsilon}$-Formyllysylprolylvalylglycyl-N$^{\epsilon}$-formyllysyl-N$^{\epsilon}$-formyllysylarginylarginylprolylvalyl - N$^{\epsilon}$ - formyllysylvalyltyrosine Amide Trihydrochloride Trihydrate (II).—N$^{\epsilon}$-Formyllysyprolylvalylglycyl - N$^{\epsilon}$ - formyllysyl - N$^{\epsilon}$-formyllysylarginylarginylprolylvalyl - N$^{\epsilon}$ - formyllysylvalyltyrosine amide triacetate (II)[18] (553 mg.), carefully dried *in vacuo*, was dissolved in ice water (4 ml.). Under ice cooling, 0.9 ml. of 1 *N* hydrochloric acid was added and the solution was lyophilized; yield essentially quantitative.

Anal. Calcd. for $C_{76}H_{125}O_{18}N_{24}\cdot 3HCl\cdot 3H_2O$: C, 49.9; H, 7.5; N, 18.4. Found: C, 49.9; H, 8.1; N, 18.3.

N-Acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl - N$^{\epsilon}$ - formyllysylprolylvalylglycyl - N$^{\epsilon}$ - formyllysyl - N$^{\epsilon}$ - formyllysylarginylarginylprolylvalyl - N$^{\epsilon}$ - formyllysylvalyltyrosine Amide Triacetate Octahydrate (III).—To a DMF (dimethyl formamide) solution (10 ml.) containing (I) (551 mg.) and (II) (522 mg.) 0.95 ml. of a 10% solution of triethylamine in DMF was added followed by DCC (dicyclohexylcarbodiimide) (180 mg.), and the mixture was kept at room temperature with stirring for 24 hr. An additional quantity of DCC (100 mg.) was then added and stirring continued for an additional 24 hr. The N,N'-dicyclohexylurea which had precipitated was removed by filtration, and the peptide material was precipitated from the filtrate by addition of 250 ml. of ethyl acetate. The material collected by centrifugation was resuspended in ethyl acetate (100 ml.), recentrifuged and dried *in vacuo* over phosphorus pentoxide; yield 1.08 g. Paper chromatography revealed the presence of four components with R_f^1 values of 0.40, 0.44, 0.66 and 0.68, respectively. This product was dissolved in water (250 ml.) and the solution applied to a column of carboxymethylcellulose (3 × 21 cm.) which was successively eluted with the following pH 6.8 ammonium acetate buffers: 0.075 *M* (1500 ml.), 0.09 *M* (300 ml.), 0.15 *M* (1500 ml.) and finally 0.25 *M* (500 ml.). Individual fractions of 10 ml. each were collected at a flow rate of 5 to 6 ml. per minute. The chromatogram showed the presence of four peaks. The contents of tubes corresponding to each of these were pooled, the bulk of the solvent was removed *in vacuo* and the residue was lyophilized. Ammonium acetate was removed by repeated lyophilization of the residues from small volumes of water to constant weight. The material corresponding to peak 1 (91 mg.) (0.075 *M* eluates, tubes 53–62); R_f^1 0.68; represented unreacted acetyldecapeptide (I); the material corresponding to peak 2 (0.075 *M* eluates, tubes 65–120) (130 mg.); R_f^1 0.66; appears to represent the acylurea derivative of (I); the material corresponding to peak 3 (0.15 *M* eluates, tubes 220–290) (463 mg.; 49%); R_f^1 0.44; represented the desired peptide (III); the material corresponding to peak 4 (0.25 *M* eluates, tubes 353–382) (138 mg.); R_f^1 0.40; represented unreacted partially protected tridecapeptide amide (II). The triacetate octahydrate of (III) was obtained in the form of a colorless fluffy powder forming a single ninhydrin negative, Pauly, Sakaguchi, methionine and Ehrlich positive single spot on paper chromatograms; a single Pauly positive component was present in paper electropherograms in pyridinium acetate buffers at pH 3.8, 5.1, 6.0 and 6.5 and in collidinium acetate buffer at pH 7.0; see Table I for amino acid ratios in acid hydrolysate; average recovery of amino acids in hydrolysate 94%; $[\alpha]^{25}D$ −71.3° (c, 0.29 in 10% acetic acid); λ_{max} 275 mμ, log ϵ 3.913 (in 10% acetic acid); see text for biological activity.

Anal. Calcd. for $C_{143}H_{219}O_{39}N_{41}S\cdot 8H_2O$: C, 51.8; H, 7.2; N, 17.3. Found: C, 51.6; H, 7.3; N, 17.5.

Seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginylprolylvalyllysylvalyltyrosine (IV). a. Pilot Studies.—Seven test tubes, each charged with (III) (6 mg.) and 0.5 *N* hydrochloric acid containing 0.2% v./v. of thioglycolic acid (0.5 ml.) were immersed in a boiling water bath, and individual tubes were removed at 20, 40, 60, 80, 100, 120 and 140 minutes respectively and cooled immediately in an ice bath. Water, (approximately 2 ml.) was added to each tube followed by Amberlite IRA-400 (acetate cycle) (approximately 300 mg.), and the suspensions were kept at room temperature with frequent shaking for 20 minutes. The Amberlite was removed by filtration, was washed repeatedly with water and the combined fil-

(31) T. S. Danowski, K. Hofmann, H. Yajima and C. Moses, *Metabolism*, **10**, 835 (1961).

(32) J.-P. Waller and H. B. F. Dixon, *Biochem. J.*, **75**, 320 (1960).

(33) K. Hofmann and N. Yanaihara, unpublished.

(34) The organic solvents were freshly distilled. Doubly distilled water from which a sizeable forerun was removed was employed. Optical rotations were determined in a Rudolph Precision Polarimeter, Model 80 with model 200 photoelectric attachment. The enzymatic techniques (LAP) were essentially those previously described.[12] The amino acid composition of acid and enzymatic hydrolysates was determined with a Beckman-Spinco Amino Acid Analyzer, Model 120 according to the method of D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1185 (1958). R_f^1 values refer to the Partridge systems, (S. M. Partridge, *Biochem. J.*, **42**, 238 (1948); R_f^2 values refer to the system 1-butanol, pyridine, acetic acid, water 30:20:6:24 (S. G. Waley and G. Watson, *ibid.*, **55**, 328 (1953)) and are expressed as a multiple of the distance traveled by a histidine marker. The carboxymethylcellulose (Cellex-CM) was obtained from the Bio-Rad Corporation, Richmond, California. Unless stated otherwise, solvents were evaporated in a rotatory evaporator at a bath temperature of 40–50°. Absorbancy at 275 mμ served to locate peptides in the various chromatographic fractions.

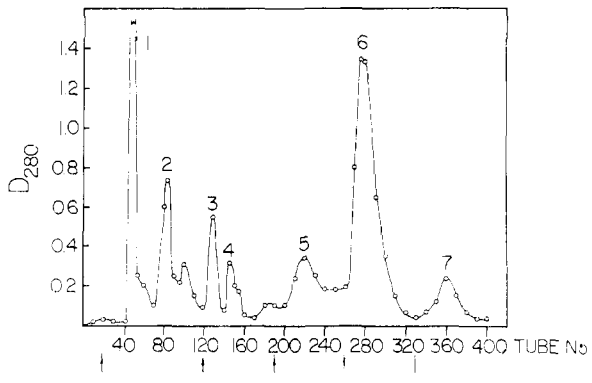


Fig. 1.—Chromatogram of partial hydrolysate of (III) on carboxymethylcellulose. Buffer changes are indicated by arrows. See text for composition and volumes of buffers used.

trates and washings from each tube were lyophilized and the residues assayed for adrenocorticotropic activity by the ascorbic acid depleting method.[19] The results are shown on Table II.

b. Preparative Scale, CMC Purified Acetate.—Each of five test tubes was charged with the protected tricosapeptide amide (III) (120 mg.), 0.5 *N* hydrochloric acid containing 0.2% v./v. of thioglycolic acid (8.5 ml.) and the tubes were immersed in a boiling water bath for 80 minutes (gas-phase nitrogen). The tubes were cooled at room temperature, their contents pooled, diluted with water (20 ml.) and Amberlite IRA-400 (acetate cycle) was added with stirring until the solution was free of chloride ions (approximately 40 g. required). The resin was removed by filtration, was washed with four 30 ml. portions of water and the combined filtrate and washings were lyophilized to give a colorless fluffy powder (600 mg.) which possessed adrenocorticotropic activity (rat ascorbic acid depleting assay 30–40 i.u./mg. in various experiments).

This material was dissolved in water (200 ml.), and the solution was applied to a CMC column (3 × 15 cm.) which was then eluted successively with the following ammonium acetate buffers: 0.075 *M*, pH 6.0 (1000 ml.), 0.225 *M*, pH 6.7 (750 ml.), 0.25 *M*, pH 6.8 (700 ml.), 0.25 *M*, pH 8.7 (800 ml.), and finally 0.3 M, pH 8.9 (500 ml.). Individual fractions (10 ml. each) were collected at a flow rate of 5 to 6 ml. per minute. Appropriate fractions were pooled, evaporated to a small volume *in vacuo* and the residues were lyophilized from small volumes of water to constant weight. A typical elution pattern is shown on Fig. 1. Certain properties of the materials corresponding to individual peaks were as follows: peak 1 (0.075 *M* eluates, tubes 40–50) (120 mg.), R_f^2 1.97 × his; amino acid ratios in acid hydrolysate ser 2.06 tyr 1.06 met 1.06 glu 1.14 his 1.12 arg 0.96 gly 0.88 lys 0.44 pro 0.34 val 0.52. The low lys, pro and val content points to the presence of impure decapeptide 1–10 arising from cleavage of the gly-lys bond between positions 10 and 11; peak 2 (0.075 *M* eluates, tubes 73–89) (32 mg.), R_f^2 1.67 × his; amino acid ratios in acid hydrolysate ser 2.0 tyr 1.02 met 0.93 glu 1.00 his 0.97 phe 1.00 arg 1.21 gly 1.82 lys 1.03 pro 1.08 val 1.29; appears to represent impure tetradecapeptide 1–14 arising from cleavage of the gly-lys bond in position 14–15; peaks 3 and 4 combined (0.225 *M* eluates, tubes 125–151) (39 mg.); adrenal ascorbic acid depleting activity ~6 i.u./mg.; peak 5 (pH 6.8, 0.25 *M* eluates, tubes 207–235) (57 mg.); adrenal ascorbic acid depleting activity ~28 i.u./mg.; peak 7 (0.30 *M* eluates, tubes 350–375) (40 mg.); adrenal ascorbic acid depleting activity ~37 i.u./mg. The tricosapeptide was isolated from the material corresponding to peak 6 (pH 6.8, 0.25 *M* eluates, tubes 262–315); yield 208 mg.; ninhydrin, Pauly, Sakaguchi, methionine and Ehrlich positive spot; R_f^2 1.18 × his with minor impurities of R_f^2 0.47 and 0.78 × his respectively; amino acid ratios in acid hydrolysate ser 1.7 tyr 1.8 met 0.8 glu 0.9 his 0.8 phe 0.9 arg 3.1 gly 2.0 lys 4.3 pro 2.3 val 3.4 (average recovery 95%); see text for biological activity.

c. Highly Purified Formate.—The CMC purified material (65 mg.) was dissolved in collidinium acetate buffer pH 7.0 (1.2 ml.) (collidine 20 ml., *N* acetic acid 93 ml. with water to 2000 ml.) and the solution was applied to the anode side of a Whatman cellulose powder block (1.1 × 7.7 × 38.5 cm.) previously equilibrated with the same buffer, and electrophoresis was conducted at a temperature of 4° for 15 hr. (800 volts, 41 milliamp.). A contact print from the surface of the block (ninhydrin) revealed the presence of two components which occupied zones 12–18 cm. and 19.5–24 cm. respectively from the origin. The peptide material was eluted from each of these zones and the eluates were evaporated to dryness. Comparable fractions from two experiments were combined to give 30 mg. of faster and 107 mg. of slower moving component. The former was not investigated; paper chromatography of the latter showed the presence of mainly (IV) slightly contaminated by impurities with R_f^2 values of 0.47 and 0.78 × his respectively. This material was dissolved in 3 ml. of 2 *N* acetic acid, containing 1% of thioglycolic acid, and the solution was kept at 75° for 8 hr. and at room temperature for 12 hr. The solution was lyophilized, the residue was dissolved in water (100 ml.) and the solution was applied to a CMC column (1.5 × 17 cm.) which was eluted with the following pH 5.6 ammonium formate buffers: 0.1 *M* (100 ml.); 0.225 *M* (100 ml.); 0.25 *M* (350 ml.). At this point gradient elution was employed. Gradient established by adding 0.25 *M*, pH 7.0 ammonium formate buffer (800 ml.) through a mixing flask containing 150 ml. of pH 5.6, 0.25 *M* ammonium formate. Individual fractions (5 ml. each) were collected at a flow rate of 2–3 ml. per minute. Tubes 142–185 from the gradient elution were pooled, the bulk of the solvent was removed *in vacuo* and the residue was lyophilized to constant weight; yield 84 mg.; $[\alpha]^{29}D$ −73.2 (*c*, 0.20 in 5% acetic acid); single ninhydrin, Pauly, Sakaguchi, methionine and Ehrlich positive spot with R_f^2 1.18 × his; single Pauly positive component on paper electrophoresis at pH 3.8, 5.1 and 6.5 in pyridinium acetate and in pH 6.9 collidinium acetate; single component on starch-gel electrophoresis at pH 8.5[4b]; see Table I for amino acid ratios in acid hydrolysate by Stein–Moore and microbiological assays; amino acid ratios in LAP digest ser 2.28 tyr 1.83 met 1.00 glu 1.00 his 0.80 phe 0.66 arg 1.10 try 0.66 gly 1.00 (lys + orn) 2.50 pro 1.06 val 1.60.

Enzymatic Studies and Microbiological Assays.—Carboxypeptidase A (Worthington Lot. 6002) (0.25 mg.) was added to a solution containing the tricosapeptide formate (IV) (0.28 μmoles) in 0.1 *N* ammonium acetate buffer pH 7.5 (0.5 ml.), and the mixture was incubated for 24 hr. at 37°. The digest was acidified with 2 *N* acetic acid and the solution evaporated to dryness *in vacuo* at room temperature over KOH and P_2O_5. The residue was analyzed with an automatic amino acid analyzer; recovery of tyr 0.24 μmoles (86%); recovery of valine 0.26 μmoles (93%).

Arginine, lysine, valine and histidine were determined with *Streptococcus faecalis* 9790; *Leuconostoc mesenteroides* D-60 was used to determine phenylalanine, proline, serine and glutamic acid. The methods employed were essentially those described in ref. 35.

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