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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[1-4]

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A synthesis is described of the eicosapeptide amide seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginylprolylvaline amide (18-L), and experimental evidence is presented regarding the stereochemical homogeneity of this peptide. The synthetic preparation exhibited essentially the full adrenocorticotropic activity of pig corticotropin as concerns both adrenal ascorbic acid depletion (111.0 ± 18.0 i.u./mg.) and plasma corticosterone elevation (83 ± 7 i.u./mg.) in the rat when administration was by the intravenous route. The potency ratio subcutaneous/intravenous administration of the synthetic peptide was approximately 0.3 and differed from that of pig corticotropin (approximately 3.0). The eicosapeptide amide possessed *in vitro* melanophoretic activity (1.1×10^8 melanocyte stimulating hormone u./g.) and exhibited marked adrenocorticotropic activity in man. From these and previous results, it was concluded that the smallest N-terminal segment of the corticotropin molecule possessing full adrenocorticotropic activity must be longer than 16 but may be shorter than 20 amino acid residues.

In 1956, Boissonnas, *et al.*,[5] announced a synthesis of the eicosapeptide (I) and reported that their preparation possessed low (2–3 i.u./mg.) *in vitro* adrenocorticotropic activity. Recently, Li, *et al.*,[6] described a preparation of the nonadecapeptide (II) which exhibited *in vivo* adrenal ascorbic acid depleting activity at the level of ~35 i.u./mg. The glutamine analog (III) of the nonadecapeptide according to Schwyzer, *et al.*,[7] appears to possess comparable potency (~30 i.u./mg.).

Since it seemed most unlikely that the shorter chain peptides should be more active than the eicosapeptide (I), we synthesized the eicosapeptide amide (IV) (18-L) and determined its adrenocorticotropic activity, both in the rat and in man. We find our preparation to be essentially as active as pig corticotropin when administration was by the intravenous route.

The synthetic procedures, employed to prepare (IV), are patterned according to a scheme (see flow sheet) which we had developed in connection with the synthesis of a tricosapeptide also possessing essentially the full biological activity of ACTH.[8]

Every intermediate employed in the present synthesis was extensively purified and only fragments of established homogeneity were used for further synthetic work. Purification of final products to "maximal" biological activity, without rigorous analytical scrutiny of intermediates employed in their preparation, does not appear to be the method of choice for synthesizing homogeneous preparations of complex physiologically active polypeptides. Peptides obtained in this manner may consist of a highly complex family of diastereomers with physical properties so similar that separation, even by the most selective of techniques, may become an impossibility. Such mixtures will afford the theoretically expected composition of amino acids on hydrolysis with acid but biological activity may be low. These considerations could offer an explanation for the marked discrepancies between the results reported in this study and those reported by Boissonnas.[5]

Prolylvaline amide (VI) prepared from the carbobenzoxy derivative (V)[9] by hydrogenolysis was treated with a mixed anhydride of carbobenzoxynitroarginine[10] 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.

In the past, and in this study, we have employed a crude preparation of leucine aminopeptidase (LAP) for assessment of stereochemical homogeneity of synthetic peptides and peptide derivatives. Recently, we have conducted similar studies with a commercial preparation of the enzyme and have observed differences in digestibility of certain peptide derivatives, notably those containing the arginyl–proline peptide bond.[11] Pertinent experi-

(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**, 4475 (1962), for paper XXIV in this series.

(4) (a) Some of the results recorded in this paper have been presented at the Laurentian Hormone Conference, September 5, 1961, "Recent Progress in Hormone Research," Vol. XVIII, G. Pincus, editor, Academic Press, Inc., New York, N. Y., 1962 (in press). (b) A preliminary communication has appeared in *J. Am. Chem. Soc.*, **84**, 1054 (1962).

(5) R. A. Boissonnnas, St. Guttmann, J.-P. Waller and P.-A. Jaquenoud, *Experientia*, **12**, 446 (1956).

(6) (a) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T. Lo and J. Ramachandran, *J. Am. Chem. Soc.*, **82**, 5760 (1960). (b) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T. Lo and J. Ramachandran, *ibid.*, **83**, 4449 (1961).

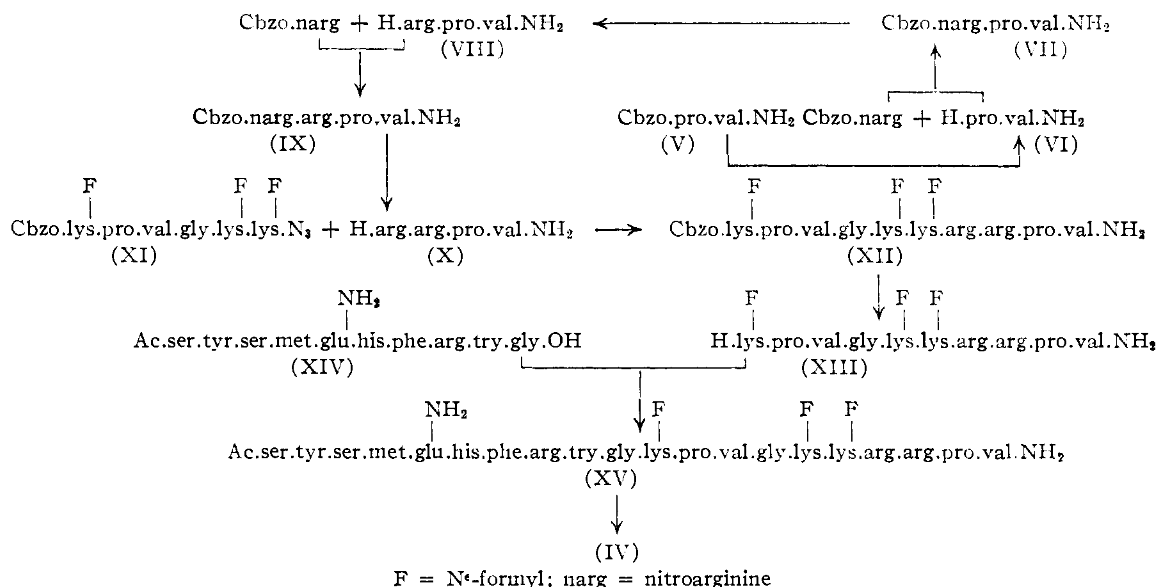
(7) R. Schwyzer, W. Rittel, H. Kappeler and B. Iselin, *Angew.* Chem., **23**, 915 (1960).

(8) (a) K. Hofmann, H. Yajima, N. Yanaihara, T. Liu and S. Lande, *J. Am. Chem. Soc.*, **83**, 487 (1961); (b) K. Hofmann, H. Yajima, T. Liu and N. Yanaihara, *ibid.*, **84**, 4475 (1962).

(9) K. Hofmann, T. A. Thompson, M. E. Woolner, G. Spühler, H. Yajima, J. D. Cipera and E. T. Schwartz, *ibid.*, **82**, 3721 (1960).

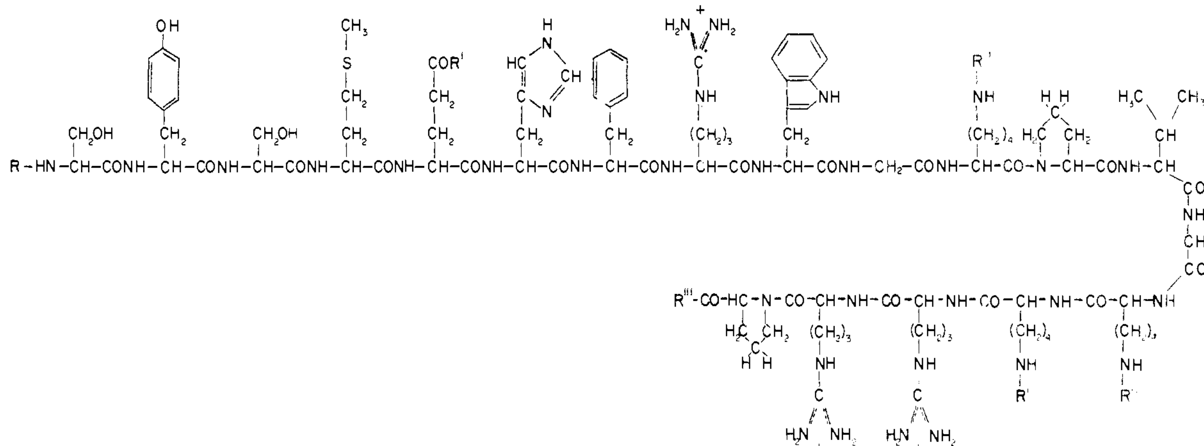
(10) (a) K. Hofmann, A. Rheiner and W. D. Peckham, *ibid.*, **75**, 6083 (1953); (b) K. Hofmann, W. D. Peckham and A. Rheiner, *ibid.*, **78**, 238 (1956).

(11) In preliminary studies we have found that the N^ϵ-formyllysylproline bond of N^ϵ-formyllysylprolylvaline amide, N^ϵ-formyllysylprolylvalylglycyl-N^ϵ-formyllysyl-N^ϵ-formyllysine amide and of N^ϵ-formyllysylprolylvalylglycyl - N^ϵ - formyllysyl - N^ϵ - formyllysylarginylarginylprolylvalyl-N^ϵ-formyllysylvalyltyrosine amide is also resistant to the action of the commercial LAP preparation.



ments are illustrated on Table I. It is apparent that our LAP preparation brought about complete digestion of all three substrates with excellent recovery of the constituent amino acids, but the commercial preparation failed to attack arginylprolylvaline amide and liberated only one of the two arginine residues from the other two substrates. Liberation of other amino acids was low with the commercial preparation and proline could not be detected in the digests. Addition of a preparation rich in prolidase[12] to the commercial enzyme preparation restored its ability to bring about digestion of the three substrates. Thus, the difference between the two LAP preparations appears to be explicable in terms of a difference in the content of prolidase. Our findings are in excellent agreement with those recently reported by Hill and Schmidt.[13]

Interaction between (X) and the azide (XI)[14] resulted in formation of the protected decapeptide amide (XII) which was decarbobenzoxylated to (XIII). N,N'-Dicyclohexylcarbodiimide[15] (DCC) was employed to bring about peptide bond formation between the N-acetyldecapeptide (XIV)[16] and (XIII) to give the protected eicosapeptide amide (XV). In previous studies[8,16] we have resorted to chromatography on carboxymethylcellulose[17] (CMC) as the method of choice for purification of peptides containing arginine and this



Formula 1.

	R	R'	R'	R'''
(I)	H	OH	H	val
(II)	H	OH	H	OH
(III)	H	NH_2	H	OH
(IV)	H	H	H	val amide

(12) We wish to express our sincere appreciation to Dr. R. L. Hill, Biochemistry Department, Duke University, North Carolina, for this preparation.

(13) R. L. Hill and W. R. Schmidt, *J. Biol. Chem.*, **237**, 389 (1962).

(14) K. Hofmann, T. Liu, H. Yajima, N. Yanaihara and S. Lande, *J. Am. Chem. Soc.*, **83**, 2294 (1961).

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

(16) K. Hofmann and H. Yajima, *ibid.*, **83**, 2289 (1961).

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

Anal. Calcd. for $C_{12}H_{23}O_4N_3$: C, 52.7; H, 8.5; N, 15.4. Found: C, 52.6; H, 8.4; N, 15.2.

Carbobenzoxynitroarginylprolylvaline Amide (VII).—A mixed anhydride, prepared in the usual manner from carbobenzoxynitroarginine (14.13 g.) in ice-cold tetrahydrofuran (40 ml.) with tri-*n*-butylamine (9.52 ml.) and ethyl chloroformate (3.8 ml.) was added to a chilled (−5°) DMF (dimethylformamide solution (40 ml.) containing (VI) (6.2 g.) and triethylamine (3.1 ml.), and the mixture was stirred at −5° for 30 minutes and at room temperature for 2 hr. Ether (1000 ml.) was then added, the precipitate collected by centrifugation was dissolved in ethyl acetate (100 ml.). The solution was washed with five 50 ml. portions of 3% ammonium hydroxide, one 50 ml. portion of water, three 50 ml. portions of 1% acetic acid and finally with 50 ml. of water. The aqueous layers were re-extracted with four 50 ml. portions of ethyl acetate in counter-current fashion, and the combined ethyl acetate phases were dried over sodium sulfate and the solvent evaporated. The crystalline product which precipitated during evaporation of the solvent was collected, washed with ethyl acetate and dried; yield 7.95 g. (64%); m.p. 128–130°; $[\alpha]^{30}D$ −30.8° (*c*, 1.73 in DMF).

Anal. Calcd. for $C_{24}H_{36}O_7N_8$: C, 52.5; H, 6.6; N, 20.4. Found: C, 52.3; H, 6.7; N, 20.1.

Arginylprolylvaline Amide Diacetate Dihydrate (VIII).—The carbobenzoxy derivative (VII) (12.6 g.) was hydrogenated over palladium for 12 hr. in 90% aqueous methanol containing 1% v./v. of acetic acid (800 ml.). The catalyst was removed by filtration, the filtrate evaporated to dryness and the oily residue dissolved in methanol (10 ml.). The product precipitated by addition of ether (200 ml.) was collected by centrifugation and dried; yield 11.7 g. (97%); hygroscopic colorless powder. The crude product (1.7 g.) was dissolved in water (500 ml.), and this solution was added to a CMC column (3 × 23 cm.) which was successively eluted with the following ammonium acetate solutions: 0.025 *M*, pH 5.6 (1000 ml.); 0.075 *M*, pH 6.3 (2000 ml.); 0.125 *M*, pH 6.5 (500 ml.). Individual fractions (20 ml. each) were collected at a flow rate of 6 to 8 ml. per minute. Spot tests with the Sakaguchi reagent served to locate the peptides in the various eluates. The 0.075 *M* eluates which contained the desired peptide were pooled, the bulk of the solvent was removed *in vacuo* and the residue was lyophilized. Ammonium acetate was removed by repeated lyophilization to constant weight. The product was dissolved in methanol (10 ml.) precipitated by addition of ether (200 ml.) and dried; yield 1.52 g. (94%); $[\alpha]^{30}D$ −49.0° (c 1.02 in 10% acetic acid); R_f^1 0.39, single spot ninhydrin and Sakaguchi positive; amino acid ratios in acid hydrolysate arg 1.02 pro 0.96 val 1.01 (recovery 96%); amino acid ratios in LAP digest arg 1.00 pro 1.00 val 1.02 (recovery 87%).

Anal. Calcd. for $C_{20}H_{39}O_7N_7.2H_2O$: C, 45.7; H, 8.3; N, 18.6. Found: C, 45.2; H, 8.0; N, 18.7.

Carbobenzoxynitroarginylarginylprolylvaline Amide Acetate Trihydrate (IX).—A mixed anhydride, prepared in the usual manner from carbobenzoxynitroarginine (3.03 g.) in ice-cold tetrahydrofuran (15 ml.) with tri-*n*-butylamine (2.0 ml.) and ethyl chloroformate (0.8 ml.) was added to an ice-cold DMF solution (10 ml.) containing (VIII) (2.05 g.) and triethylamine (0.77 ml.). The mixture was kept at 0° for 60 minutes and at room temperature for 2 hr. Ether (1000 ml.) was added, the precipitate collected by centrifugation was dissolved in 20% acetic acid (100 ml.), and the solution was extracted with five 50 ml. portions of ethyl acetate. The ethyl acetate layers were in turn extracted with four 50 ml. portions of 20% acetic acid and the combined aqueous phases concentrated to a small volume and then lyophilized; yield 3.10 g. (99%). This material (2.02 g.) dissolved in water (250 ml.) was applied to a CMC column (3 × 45 cm.) which was eluted successively with water (1300 ml.) and with pH 6.9, 0.02 *M* ammonium acetate (3000 ml.). Individual fractions (20 ml. each) were collected at a flow rate of 5–6 ml. per minute. The nitroarginine absorption maximum at 270 mμ[14] served to locate the peptides in the various chromatographic fractions. Fractions 96–208, (0.02 *M* eluates) were combined, evaporated to a small volume *in vacuo* and lyophilized. Ammonium acetate was removed from the residue by repeated lyophilization to constant weight; yield 1.16 g. (58%); $[\alpha]^{31}D$ −46.0° (c 0.54 in 10% acetic acid), sharp, single spot, ninhydrin negative, Sakaguchi positive, R_f^1 0.74.

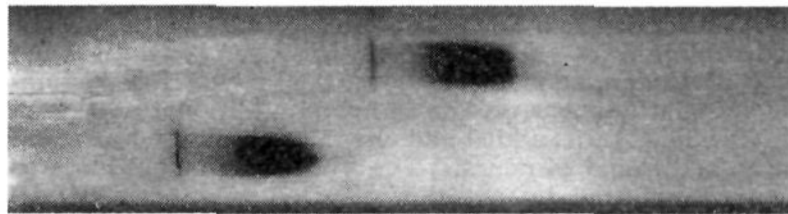


Fig. 3.—Starch-gel electropherogram at pH 8.5 of the eicosapeptide (IV) (Nigrosin stain) (duplicate experiment).

Anal. Calcd. for $C_{32}H_{52}O_{10}N_{12}.3H_2O$: C, 46.9; H, 7.1; N, 20.5. Found: C, 47.2; H, 7.1; N, 20.3.

Arginylarginylprolylvaline Amide Triacetate Tetrahydrate (X).—The carbobenzoxy derivative (IX) (5.9 g.) was hydrogenated for 12 hr. over palladium in 10% aqueous acetic acid (300 ml.). The crude hydrogenation product (5.2 g.) was dissolved in water (400 ml.), and the solution was added to a CMC column (3 × 40 cm.) which was successively eluted with the following pH 6.9 ammonium acetate buffers: 0.01 *M* (500 ml.); 0.025 *M* (500 ml.); 0.05 *M* (500 ml.); 0.075 *M* (500 ml.) and 0.15 *M* (2000 ml.). Individual fractions (20 ml. each) were collected at a flow rate of 6 to 8 ml. per minute. Spot tests with Sakaguchi reagent served to locate the peptides in the various eluates. The 0.15 *M* eluates which contained the desired peptide amide were pooled, concentrated to a small volume and lyophilized. Ammonium acetate was removed by repeated lyophilization to constant weight; yield 4.4 g. (78%); $[\alpha]^{31}D$ −40.2° (*c*, 1.1 in 10% acetic acid); R_f^2 0.39 sharp single ninhydrin and Sakaguchi positive spot; amino acid ratios in acid hydrolysate arg 2.00 pro 0.99 val 1.01 (recovery (89%); amino acid ratios in LAP digest arg 2.01 pro 0.96 val 1.00 (recovery 89%).

Anal. Calcd. for $C_{28}H_{55}O_{10}N_{11}.4H_2O$: C, 43.2; H, 8.2; N, 19.8. Found: C, 43.7; H, 8.2; N, 19.8.

Nα-Carbobenzoxy-Nε-formyllysylprolylvalylglycyl-Nε-formyllysyl - Nε - formyllysylarginylarginylprolylvaline Amide Diacetate Hexahydrate (XII).—A tetrahydrofuran solution of the azide (XI), prepared from 1.32 g. of the hydrazide in a manner previously described,[14] was added to a DMF solution (4 ml.) containing (X) (0.525 g.) plus 10% triethylamine in DMF (3.0 ml.), and the mixture remained at 5° for 24 hr. when it was dissolved in water (250 ml.). This prepared solution was added to a CMC column (3 × 25 cm.) which was eluted successively with the following pH 6.9 ammonium acetate buffers: 0.001 *M* (250 ml.); 0.025 *M* (500 ml.); 0.05 *M* (1500 ml.). Individual fractions (15 ml. each) were collected at a flow rate of 4 to 5 ml. per minute. Spot tests with the Sakaguchi reagent served to locate the peptides in the various eluates. The 0.05 *M* eluates which contained the desired compound were pooled, concentrated to a small volume *in vacuo* and the residue was lyophilized. Ammonium acetate was removed by repeated lyophilization to constant weight; yield 0.892 g. (80%); $[\alpha]^{30}D$ −79.4° (*c*, 0.34 in 10% acetic acid); R_f^1 0.52, sharp single spot, ninhydrin negative, Sakaguchi positive; amino acid ratios in acid hydrolysate lys 3.01 pro 2.03 val 2.13 gly 1.08 arg 1.77 (recovery 96%).

Anal. Calcd. for $C_{67}H_{112}O_{19}N_{20}.6H_2O$: C, 50.0; H, 7.8; N, 17.4. Found: C, 49.6; H, 7.9; N, 17.4.

Nε-Formyllysylprolylvalylglycyl-Nε-formyllysyl-Nε-formyllysylarginylarginylprolylvaline Amide Triacetate Octahydrate (XIII).—The carbobenzoxy derivative (XII) (245 mg.) was hydrogenated for 3 hr. over palladium in 2% aqueous acetic acid (30 ml.). The catalyst was removed by filtration and the filtrate lyophilized; yield 233 mg. (100%); $[\alpha]^{30}D$ −73.5° (c. 0.25 in 10% acetic acid); R_f^2 0.60, sharp single spot, ninhydrin and Sakaguchi positive; amino acid ratios in acid hydrolysate lys 3.02 pro 2.02 val 2.04 gly 1.07 arg 1.80 (recovery (97%); amino acid ratios in LAP digest formlys 3.03 pro 2.07 val 2.03 gly 0.97 arg 1.93 (recovery 85%).

Anal. Calcd. for $C_{61}H_{110}O_{19}N_{20}\cdot 8H_2O$: C, 46.6; H, 8.1; N, 17.8. Found: C, 46.5; H, 8.1; N, 18.2.

N-Acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl - Nε - formyllysyl-Nε - formyllysylarginylarginylpropylvaline Amide Triacetate Decahydrate (XV).—N,N′-Dicyclohexylcarbodiimide (150

TABLE I

DIGESTIBILITY OF THREE SYNTHETIC PEPTIDES BY CERTAIN ENZYME PREPARATIONS[a]

Peptide	Amino acids in digest % of theory					Enzyme preparation
	tyr	F lys	arg	pro	val	
			87	87	89	LAP
H.arg.pro.val.NH_2			0.8	0	6	LAP (G)
			88	87	87	LAP (G) + P
			90	88	90	LAP
H.arg.arg.pro.val.NH_2			46	0	8	LAP (G)
			91	88	88	LAP (G) + P
	98	92	98	102	101	LAP
H.arg.arg.pro.val.lys.val.tyr.NH_2	17	17	36	0	17	LAP (G)
	102	109	103	107	107	LAP (G) + P

[a] LAP = crude leucine aminopeptidase prepared in our Laboratory[16]; LAP (G) = leucine aminopeptidase (General Biochemicals, Lot 1267H); P = prolidase preparation[12]; F = N^ϵ-formyl. See text for experimental details.

technique was used again extensively in the present investigation with excellent results.

The highly purified protected eicosapeptide amide (XV) isolated as the triacetate decahydrate behaved as a single component on paper chromatography and afforded paper electropherograms at various *p*H values exhibiting a single Pauly positive spot (Fig. 1). 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%. These findings in conjunction with the fact that the protected eicosapeptide amide was synthesized from intermediates of proven homogeneity appears to justify the conclusion that the compound is of a high level of purity. The peptide derivative possessed marked *in vitro* melanocyte expanding activity (4.2 × 10^8 MSH u./g.[18]) but was essentially inert as concerns adrenocorticotropic potency.

Exposure of (XV) to 0.5 *N* hydrochloric acid for 80 minutes at the temperature of a boiling water-bath[8] followed by exchange of chloride ions by acetate ions and lyophilization gave a material which possessed *in vivo* adrenocorticotropic activity (30–35 i.u./mg. in various experiments).[19] This product was subjected to chromatography on CMC to give a material containing a major component of $R_f{}^2$ 1.35 × his and two minor components with $R_f{}^2$ values of 0.55 and 0.84 × his, respectively (adrenal ascorbic acid depleting activity of 94.8 ± 31.2 i.u./mg.). Apparently homogeneous samples of the eicosapeptide amide were isolated from the CMC purified product by cellulose-block electrophoresis at pH 7.0 followed by a second chromatographic step on CMC. Since the possibility existed that the evaporation of large volumes of solvents during these various fractionations could have brought about some oxidation of the methionine sulfur, the material from the electrophoretic purification was incubated with thioglycolic acid prior to the second chromatographic purification. The final product was homogeneous as judged by paper chromatograms. It produced a single Pauly positive spot on paper electropherograms at various pH values (Fig. 2), exhibited a single spot on starch-gel electrophoresis at pH 8.5 (Fig. 3), and its acid hydrolysate contained the constituent amino acids (minus tryptophan) in the ratios predicted by theory. The eicosapeptide amide was digestible 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.

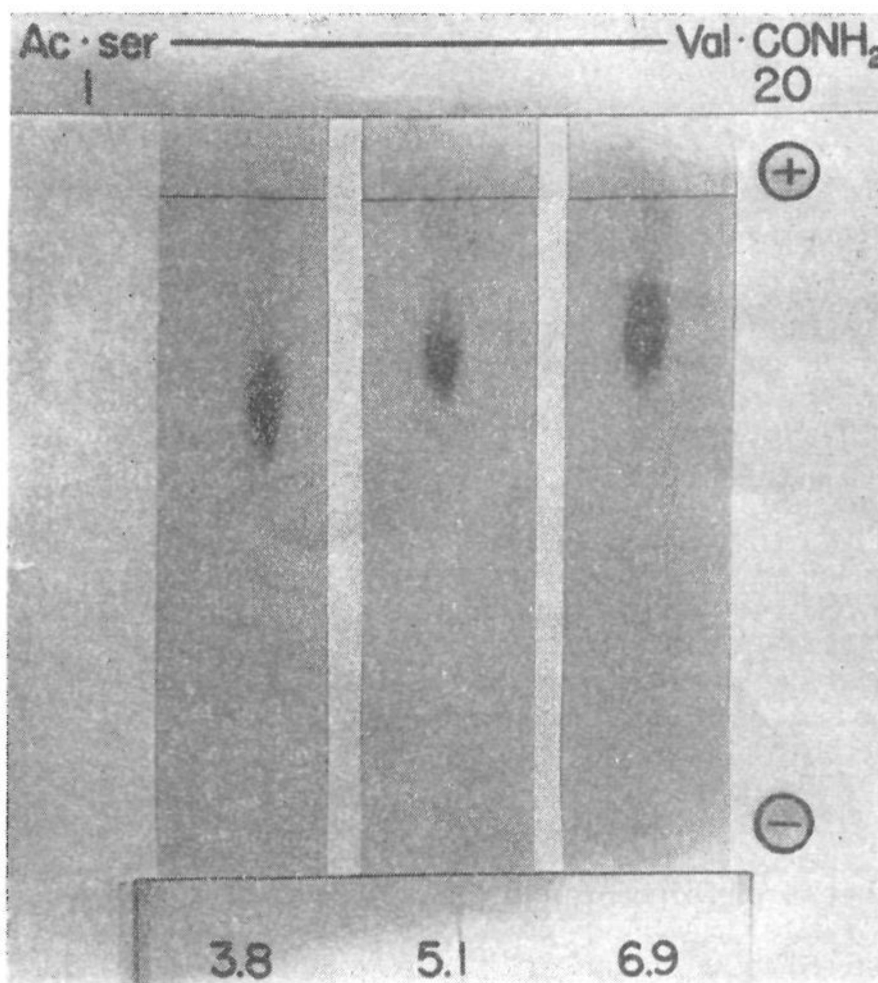


Fig. 1.—Paper electropherograms of the protected eicosapeptide amide (XV) (Pauly stain).

(18) 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).

(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, *ibid.*, **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.

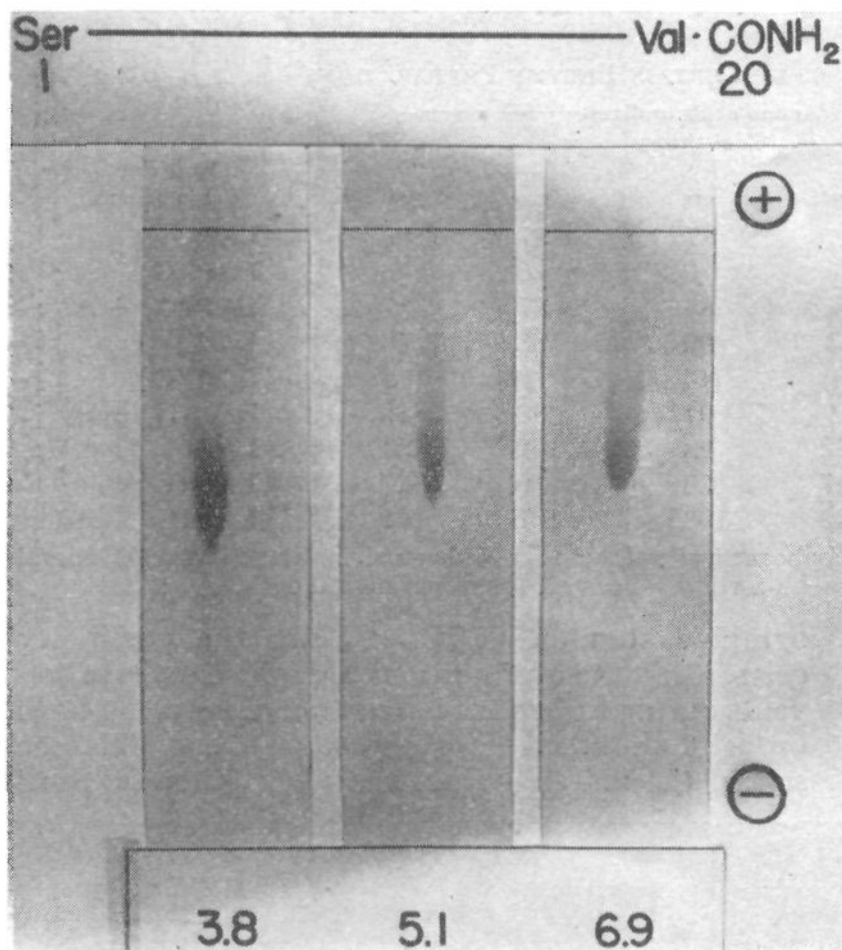


Fig. 2.—Paper electropherograms of the eicosapeptide amide (IV) (Pauly stain).

This rather extensive analytical evaluation appears to justify the conclusion that our preparation of the eicosapeptide amide exhibits a high degree of homogeneity.

The observation that the peptide amide resisted the action of carboxypeptidase A established the presence of a C-terminal carboxamide function. The eicosapeptide amide (IV) possessed *in vitro* melanocyte expanding activity (1.1×10^8 MSH u./g.) and adrenal ascorbic acid depleting potency (111.0 ± 18.0 i.u./mg.). The adrenal ascorbic acid depleting activity of pig corticotropin is reported as 80–100 i.u./mg.[20]; its *in vitro* MSH activity appears to be of the order of 1.7×10^8 MSH u./g.[21]

The plasma corticosterone elevating potency of the eicosapeptide (83 ± 7 i.u./mg.) approaches that of pig corticotropin (94.5 ± 10.6 i.u./mg.).[22] To date the eicosapeptide amide has been administered to 12 humans by intravenous infusion over a 15 minute period, and at all levels above 5 i.u. there was a significant rise in plasma 17-hydroxysteroids which occurred maximally at 30 minutes but persisted into the 60 minute period.[23] These biological results support the conclusion, previously stated, that the eicosapeptide amide possesses essentially the same biological potency as pig corticotropin when administration is by the intravenous route.

(20) 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, *J. Am. Chem. Soc.*, **78**, 5051 (1956).

(21) C. H. Li, *Laboratory Investigation*, **8**, 574 (1959).

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

(23) We are much indebted to Dr. H. S. Lipscomb, Department of Physiology, Baylor University Medical School, for these results which will be published in a separate communication.

The s.c./i.v. (subcutaneous/intravenous) potency ratio observed with the synthetic eicosa- and tricosapeptides[8] (approximately 0.3) as contrasted by that of pig corticotropin (approximately 3.0) differentiates these preparations somewhat from the natural hormone.[24]

The synthetic approach, applied systematically in our Laboratory to the study of the relation between polypeptide chain-length and adrenocorticotropic activity, has provided significant results. We have demonstrated that elongation of the chain of a peptide which corresponds to the arrangement of the N-terminal 16 amino acid residues of ACTH to a chain corresponding to the arrangement of the N-terminal 20 amino acid residues brings about a more than thousand-fold increase in adrenocorticotropic potency. Thus, the sequence arginylarginylprolylvaline which occupies positions 17–20 in the corticotropin molecule (or a portion thereof) must be essential for high level activity. Elongation of the chain from 20 to 23 amino acid residues does not appear to effect adrenocorticotropic or melanophoretic activity significantly.

Accepting the chemical and biological data presented by Li, *et al.*,[6] it would appear that adrenocorticotropic potency decreases markedly (from ~100 to ~30 i.u./mg.) when the N-terminal portion of the peptide chain of corticotropin is shortened from 20 to 19 amino acid residues. Careful scrutiny of the data presented by these authors, pertaining to the stereochemical homogeneity of their final product and of many intermediates employed in its preparation, suggests caution in accepting these data as final. The possibility still exists that *pure* peptides longer than 16 but shorter than 20 amino acid residues corresponding to the N-terminus of pig corticotropin may possess the full adrenocorticotropic potency of that hormone. It has become apparent also that structural elements located between positions 23 and 39 exert a significant effect on the s.c./i.v. potency ratios as concerns adrenal ascorbic acid depleting activity. Further study will be required to clarify these points.

Experimental[25]

Prolylvalyl Amide Acetate (VI).—Carbobenzoxyprolylvaline amide (V)[9] (1 g.) was hydrogenated over palladium in methanol containing 1% of acetic acid v./v. (100 ml.). The dipeptide amide salt isolated in the usual manner was recrystallized from a mixture of ethanol and ether; yield 0.640 g. (85%); m.p. 138–140°; $[\alpha]^{29}D$ −54.6° (*c*, 1.02 in 10% acetic acid); $R_f{}^1$ 0.52.

(24) See *J. Am. Chem. Soc.*, **84**, 4475 (1962), for a discussion of this point and for a critical delineation of various factors which may limit the accuracy of the assay results.

(25) The organic solvents were freshly distilled. Doubly distilled water from which a sizable forerun was removed was employed. The melting points are uncorrected. Rotations were determined with a Rudolph Precision Polarimeter, Model 80 with a model 200 photoelectric attachment. The amino acid composition of the acid and enzymatic hydrolysates was determined with a Beckman-Spinco Amino Acid Analyzer, Model 120, according to the method of S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958). $R_f{}'$ values refer to the Partridge system (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). The carboxymethylcellulose (Cellex-CM) was obtained from the Bio·Rad Corporation, Richmond, California. Unless stated otherwise, solvents were evaporated at a bath temperature of 40–50° in a rotatory evaporator.

mg.) was added to a DMF solution (2.0 ml.) containing the hydrochlorides of (XIII) (270 mg.), (XIV) (353) mg.) and 10% triethylamine in DMF v./v. (0.60 ml.). After standing at room temperature for 24 hr. an additional portion of DCC (80 mg.) was added and the mixture allowed to stand for an additional 24 hours. The crude reaction product was then precipitated by addition of ethyl acetate (200 ml.) and the precipitate was dried, dissolved in water (150 ml.) and the solution added to a CMC column (3 × 15 cm.) which was eluted successively with the following pH 6.9 ammonium acetate buffers: 0.075 *M* (1000 ml.); 0.10 *M* (250 ml.) and 0.15 *M* (750 ml.). Individual fractions (10 ml. each) were collected at a flow rate of 3 and 4 ml. per minute. Spot tests with the Sakaguchi reagent and absorbancy at 280 mμ served to locate the peptides in the various eluates. The 0.15 *M* eluates which contained the desired material were pooled, the bulk of the solvent was removed *in vacuo* and the residue was lyophilized. Ammonium acetate was removed by repeated lyophilization to constant weight; yield 293 mg. (59%); $[\alpha]^{25}D$ −56.0° (*c*, 0.28 in 10% acetic acid); R_f^1 0.34 single spot ninhydrin negative, Sakaguchi, Pauly, Ehrlich and methionine positive; amino acid ratios in acid hydrolysate ser 2.08 tyr 0.97 met 0.93 glu 1.04 his 1.00 phe 1.00 arg 2.85 gly 1.96 lys 2.92 pro 2.04 val 1.96 (recovery 93%).

Anal. Calcd. for $C_{122}H_{189}O_{34}N_{37}S \cdot 10H_2O$: C, 50.0; H, 7.2; N, 17.7. Found: C, 50.1; H, 7.0; N, 17.1.

Seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginylprolylvaline Amide Formate (IV).—A solution of the protected eicosapeptide amide (XV) (350 mg.) in 0.5 *N* hydrochloric acid containing 0.2% of thioglycolic acid (21 ml.) was heated in a boiling water bath for 80 minutes (gas-phase nitrogen) and then quickly cooled in an ice-bath. The hydrolysate was diluted with water (20 ml.) and Amberlite IRA-400 (acetate cycle) was added with shaking until the supernatant was free of chloride ions (approximately 35 g. required). The resin, removed by filtration, was washed with four 50 ml. portions of water and the combined filtrate and washings were evaporated to a small volume *in vacuo* and then lyophilized; yield 347 mg.; ascorbic acid depleting activity of various batches varied from 30–35 i.u./mg. An aqueous solution of this material (150 ml.) was added to a CMC column (3 × 10 cm.) which was eluted with the following ammonium acetate buffers: 0.075 *M*, pH 6.6 (600 ml.); 0.225 *M*, pH 6.6 (250 ml.); 0.25 *M*, pH 6.6 (650 ml.); 0.25 *M*, pH 9.0 (500 ml.). Individual fractions (10 ml. each) were collected at a flow rate of 3 ml. per minute. Absorbancy measurements at 280 mμ revealed the presence of at least 8 components in the chromatogram. The contents of the tubes containing the pH 9.0, 0.25 *M* eluates which corresponded to a major peak on the chromatogram were pooled and the water and ammonium acetate removed in the usual manner. The residue (101 mg.; 29%) exhibited adrenal ascorbic acid depleting activity (94.8 ± 31.2 i.u./mg.). Material corresponding to a peak in the pH 6.6, 0.25 *M* eluates (63 mg.) was less active (~ 20 i.u./mg.). Paper chromatograms of the highly active material showed the presence of one major (R_f^2 1.35 × his) and at least two minor components (R_f^2 0.55 and 0.84 × his respectively); amino acid ratios in acid hydrolysate ser 1.8 tyr 0.8 met 0.9 glu 1.0 his 1.0 phe 1.0 arg 3.4 gly 2.4 lys 3.8 pro 2.4 val 2.4. This material (70 mg.), dissolved in a collidinium acetate buffer pH 7.0 (2 ml.) (20 ml. of collidine, 93 ml. of 1 N acetic acid to 2000 ml. with water) was applied to the anode side of a cellulose powder block (38.5 × 7.7 × 0.9 cm.), prepared from Whatman cellulose powder with the same buffer, and electrophoresis was conducted for 15 hr. at 4° (800 volts, 37 milliamps.). A contact print from the surface of the block (ninhydrin) revealed the presence of two bands located 13–18 and 20.5–24 cm., respectively, from the origin. The section of the block correponding to the slower moving zone (ninhydrin and Pauly positive) was eluted with four 70 ml. portions of water which were combined, concentrated to a small volume *in vacuo* and lyophilized. Paper chromatographic evaluation of the residue revealed one major component (R_f^2 1.35 × his) and two minor impurities with R_f^2 values of 0.55 and 0.84 × his, respectively. For further purification the material from two identical electrophoretic experiments (104 mg.) was dissolved in 3 ml. of 2 *N* acetic acid containing 1% of thioglycolic acid, and the solution was kept at 75° for 7 hr. and at room temperature for an additional 12 hr., when it was lyophilized. The residue, dissolved in water (100 ml.) was added to a CMC column (1 × 25 cm.) which was eluted, successively, with the folloing pH 6.0 ammonium formate buffers: 0.1 *M* (150 ml.); 0.225 *M* (150 ml.); 0.25 *M* (200 ml.). At this point pH gradient elution was employed by adding 0.25 *M* pH 8.5 buffer (500 ml.) through a mixing flask containing 0.25 *M* pH 6.0 buffer (150 ml.). Fractions (5 ml. each) were collected at a flow rate of 3 ml. per minute. Absorbancy measurements at 280 mμ revealed the presence of a single major peak in the fractions derived from gradient elution. Tubes corresponding to this peak were combined and the solvent and ammonium formate removed in the usual manner; yield 77 mg.; single ninhydrin, Pauly, Sakaguchi, methionine and Ehrlich positive component with R_f^2 1.35 × his; homogeneous on paper electrophoresis at pH 3.8, 5.1 and 6.9 (Fig. 2); and on starch-gel electrophoresis at pH 8.5 (Fig. 3); $[\alpha]^{26}D$ −56.8° (*c*, 0.15 in 10% acetic acid); amino acid ratios in acid hydrolysate ser 1.81 tyr 0.76 met 0.95 glu 1.00 his 1.00 phe 1.00 arg 3.00 gly 2.10 lys 3.10 pro 2.05 val 2.10 (average recovery 80%); amino acid ratios in LAP digest ser 1.5 tyr 0.87 met 1.04 glu 1.10 his 1.04 phe 0.93 arg 1.45 try 0.99 gly 1.45 (lys + orn) 3.43 pro 1.45 val 1.40; MSH activity *in vitro* 1.1 × 10^8 MSH u./g.; adrenal ascorbic acid depleting activity i.v., 111.0 ± 18 i.u./mg.; s.c., 33.3 ± 3.9 i.u./mg.

Enzymatic Experiments.—Experiments with crude leucine aminopeptidase prepared in our Laboratory were conducted essentially as previously described.[16] Leucine aminopeptidase (General Biochemicals, Lot. 1267H) (0.25 mg.) in 0.025 *M* "tris" buffer, pH 8.5 (1 ml.), 0.005 *M* with respect to magnesium chloride was incubated for 3 hr. at 40°. Digests were prepared by incubating for 24 hr. at 40° solutions containing the peptides (2–4 mg.) in 0.025 *M*, pH 8.5 "tris" buffer (0.60 ml.), 0.005 *M* with respect to magnesium chloride plus activated enzyme solution (0.40 ml.). Digestion was stopped by heating the digest in a boiling water bath for 2 minutes. The ensuing coagulate was removed by centrifugation and the supernatant evaporated to dryness *in vacuo* over P_2O_5 and KOH. The residue was dissolved in either water or 0.2 *N* sodium citrate, pH 2.2 buffer for paper or Dowex-50 chromatographic amino acid analyses. Carboxypeptidase A (Worthington Lot 6002) (0.20 mg.) was added to a solution of the highly purified eicosapeptide amide formate (IV) (1.87 mg.) in 0.1 *M* ammonium acetate pH 7.5 (0.5 ml.), and the solution was incubated for 24 hr. at 37°. The digest, acidified with 2 *N* acetic acid, was evporated to dryness *in vacuo* over P_2O_5 and KOH and the residue analyzed by paper chromatography. The chromatogram revealed the absence of valine and proline. Experiments using combinations of LAP and prolidase were carried out as follows: A prolidase preparation (8 mg. of protein/ml.; C_1 = 2)[12] was dialyzed against 0.005 *M* "tris" buffer, pH 8.0 for 48 hr. at 5°. The dialyzed solution (0.16 ml.) and leucine aminopeptidase (General Biochemicals Co., Lot 1267H) (0.25 mg.) were added to 0.005 *M* "tris" buffer, pH 8.5 0.005 *M* with respect to magnesium chloride (1.0 ml.), and the solution was incubated for 3 hr. at 40°. The peptides to be digested (approximately 2 mg.) were dissolved in pH 8.5, 0.005 *M* "tris" buffer (0.6 ml.), 0.005 *M* with respect to magnesium chloride and activated enzyme solution (0.40 ml.) was added. The mixtures were incubated for 24 hr. at 40°, when the protein was precipitated by 2 minutes heating in a boiling water-bath. The coagulum was removed by centrifugation, was washed with 0.5 ml. of water and the combined supernatant and washing were evaporated to dryness over P_2O_5 and KOH. The residue was dissolved in either water or 0.2 *N* sodium citrate, pH 2.2 buffer for paper and Dowex-50 chromatographic analyses.

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