was dissolved in water and lyophilized to yield 0.21 g. of material, which was then purified by chromatography on carboxymethylcellulose using gradient elution with ammonium acetate. The conditions of chromatography were the same as described previously.[2b,3,5]

Three peaks, A, B, and C (Figure 2A), were isolated. Peak A (0.02 g.) was identified as the acylurea of the N-terminal decapeptide on the basis of electrophoretic mobility (Figure 3) and the amino acid composition of an acid hydrolysate: $Ser_{1.8}Tyr_{1.13}Met_{1.02}Glu_{1.00}His_{0.91}Phe_{1.01}Arg_{0.84}Gly_{0.61}$. Peak B (0.06 g.) was rechromatographed on carboxymethylcellulose to yield 0.045 g. of the hexacosapeptide X (peptide content based on ultraviolet absorption 68%). Peptide X was found to be homogeneous by electrophoresis on paper (mobility relative to lysine 0.67; pH 3.7, 400 volts, 6 hr.). The amino acid composition of X was determined by the chromatographic method[20] after hydrolysis for 20 and 40 hr. and was found to be in good agreement with theoretically expected values (Table I); $[\alpha]^{25}D$ $-126.4°$ (*c* 1, 0.1 *N* acetic acid) based on peptide content determined by ultraviolet absorption. Peak C (0.04 g.) was rechromatographed on carboxymethylcellulose to yield 0.024 g. of an apparently homogeneous peptide with the following amino acid composition: $Ser_{1.88}Tyr_{1.13}Met_{0.91}Glu_{1.00}His_{0.82}Phe_{1.06}Arg_{2.86}Gly_{2.13}Lys_{3.18}Pro_{2.70}Val_{1.30}Asp_{0.07}$.

Acknowledgment. This work was supported in part by United States Public Health Service Research Grant GM-02907, from the Division of General Medicine, National Institute of General Medical Sciences. The authors wish to thank David Chung for many helpful discussions and for invaluable assistance.

Adrenocorticotropins. XXXIV. Aspects of Structure–Activity Relationships of the ACTH Molecule. Synthesis of a Heptadecapeptide Amide, an Octadecapeptide Amide, and a Nonadecapeptide Amide Possessing High Biological Activities

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Contribution from the Hormone Research Laboratory, University of California, Berkeley, California. *Received January 16, 1965*

The synthesis of a heptadecapeptide amide ($\alpha^{1-17}NH_2$-ACTH), an octadecapeptide amide ($\alpha^{1-18}NH_2$-ACTH), and a nonadecapeptide amide ($\alpha^{1-19}NH_2$-ACTH) corresponding to the first 17, 18, and 19 amino acid residues of adrenocorticotropins (ACTH) has been described. By assay procedures both in vivo and in vitro, the adrenal-stimulating activities of the heptadecapeptide amide and the nonadecapeptide amide were found to be considerably higher than those of their respective acid analogs. The adrenocorticotropic potency of the octadecapeptide amide as estimated by bioassay in vivo is almost identical with that of the nonadecapeptide amide. The melanocyte-stimulating activities of the synthetic products are comparable to that of the natural hormone. The relationship of structure to biological activity with respect to the mechanism of action of the hormone is discussed. Comments on the use of sodium in liquid ammonia for the removal of tosyl groups in peptide synthesis are also presented.

During the past 5 years considerable effort has been directed toward the synthesis of various peptides related to pituitary adrenocorticotropins (ACTH, Figure 1) with the aim of delineating the structural features of the molecule responsible for the manifestation of the biological activities associated with the hormone. Thus, a nonadecapeptide,[1] an eicosapeptide amide,[2] a tricosapeptide,[3] and a tetracosapeptide,[4] all related to the NH_2-terminal half of ACTH, were synthesized in three different laboratories.

When a heptadecapeptide[5] corresponding to the first 17 residues of ACTH was synthesized, it was found to be only 15% as potent a steroidogenic agent as the nonadecapeptide.[1] The heptadecapeptide lacks the dipeptide unit arginylproline found at the COOH terminus of the nonadecapeptide and representing positions 18 and 19 of the ACTH molecule. In view of the concentration of basic amino acid residues in the region between 5 and 22 in the ACTH molecule (see Figure 1), it became of interest to determine if the diminished potency of the heptadecapeptide was the result of the loss of positive charge contributed by arginine in position 18. We have now synthesized three new peptides, namely, seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginine amide[6] (X), seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginine amide (XV), and seryltyrosylseryl-

(1) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T-B. Lo, and J. Ramachandran, *J. Am. Chem. Soc.*, **82**, 5760 (1960); **83**, 4449 (1961).

(2) K. Hofmann, H. Yajima, T-Y. Liu, N. Yanaihara, C. Yanaihara, and J. L. Humes, *ibid.*, **84**, 1054 (1962); **84**, 4481 (1962).

(3) K. Hofmann, H. Yajima, T-Y. Liu, and N. Yanaihara, *ibid.*, **83**, 487 (1961); **84**, 4475 (1962).

(4) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1136 (1961); **46**, 1550 (1963).

(5) C. H. Li, J. Ramachandran, D. Chung, and B. Gorup, *J. Am. Chem. Soc.*, **84**, 2460 (1962); **86**, 2703 (1964).

(6) All amino acids occurring in the peptides mentioned in this paper are of the L-configuration with the exception of glycine.

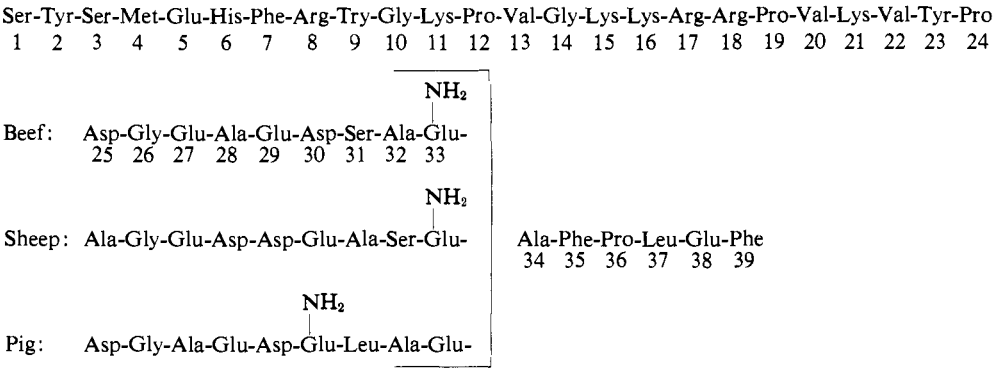

Figure 1. Structure of adrenocorticotropins.

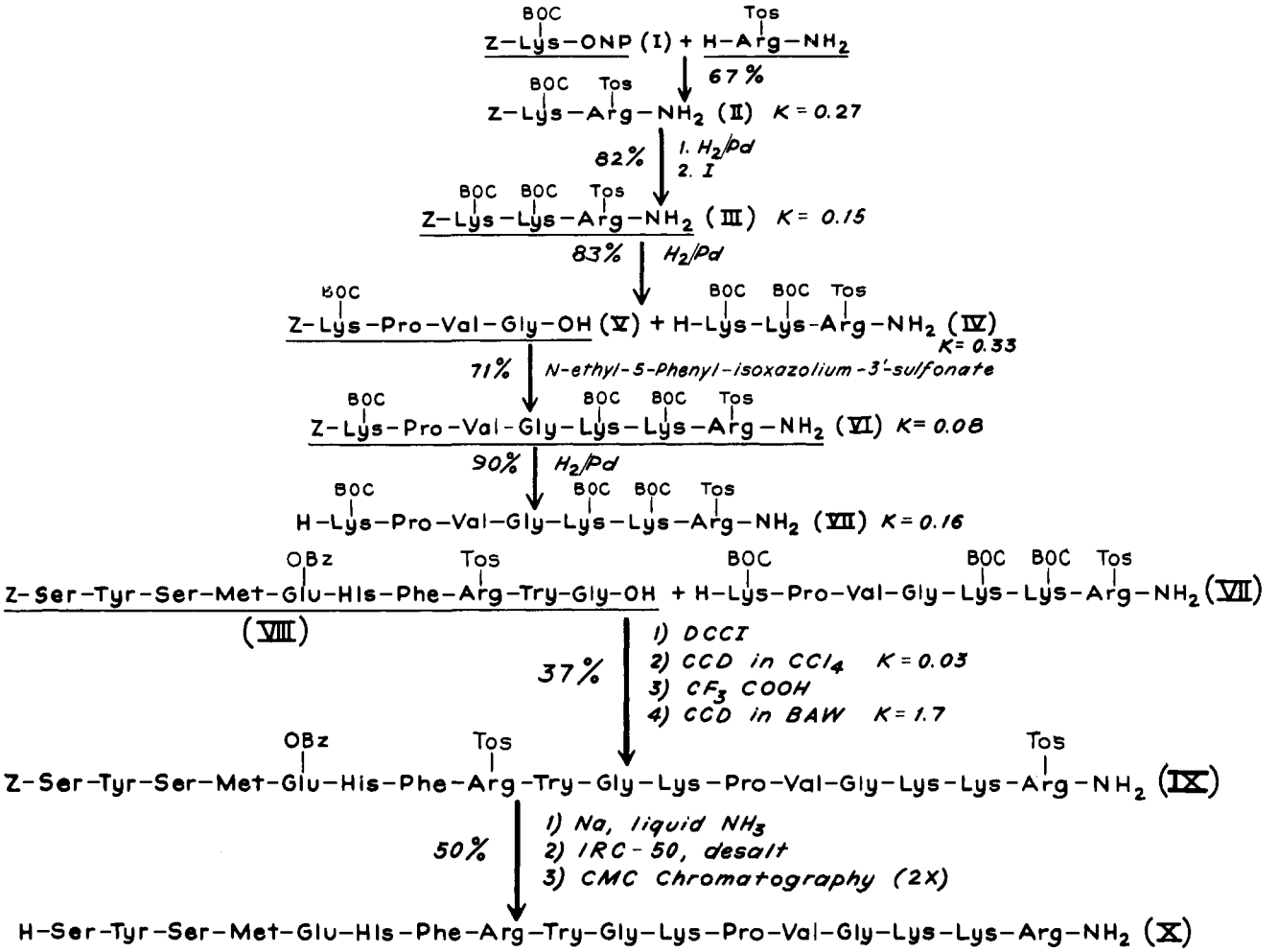

Figure 2. Synthetic scheme for the heptadecapeptide amide (X): Z, carbobenzoxy; Tos, *p*-toluenesulfonyl; BOC, *t*-butyloxycarbonyl; *t*-Bu, *t*-butyl; Bz, benzyl; ONP, *p*-nitrophenoxy.

methionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginylproline amide (XXIII). Of these, peptides X and XXIII are the amide analogs of the heptadecapeptide[5] and the nonadecapeptide[1,7] previously prepared in our laboratory. The biological activities of these synthetic peptides have also been investigated. This paper describes the details of the synthesis and presents a discussion of the relationship of the structure of the ACTH molecule to its biological activities in the light of the results obtained with the various synthetic peptides prepared in our laboratory. A preliminary account of the synthesis of X has appeared.[8]

Synthesis of the Peptides. The synthesis of these three peptides was accomplished by proceeding through routes essentially similar to those employed for the preparation of the heptadecapeptide,[5] pentadecapeptide,[9] and nonadecapeptide.[7] The crystalline protected decapeptide carbobenzoxyseryltyrosylserylmethionyl-γ-benzylglutamylhistidylphenylalanyl-N^G-tos-

(7) C. H. Li, D. Chung, and J. Ramachandran, *J. Am. Chem. Soc.*, **86**, 2715 (1964).

(8) J. Ramachandran, D. Chung, and C. H. Li, *Federation Proc.*, 23, 369 (1964).

(9) C. H. Li, J. Ramachandran, and D. Chung, *J. Am. Chem. Soc.*, 86, 2711 (1964).

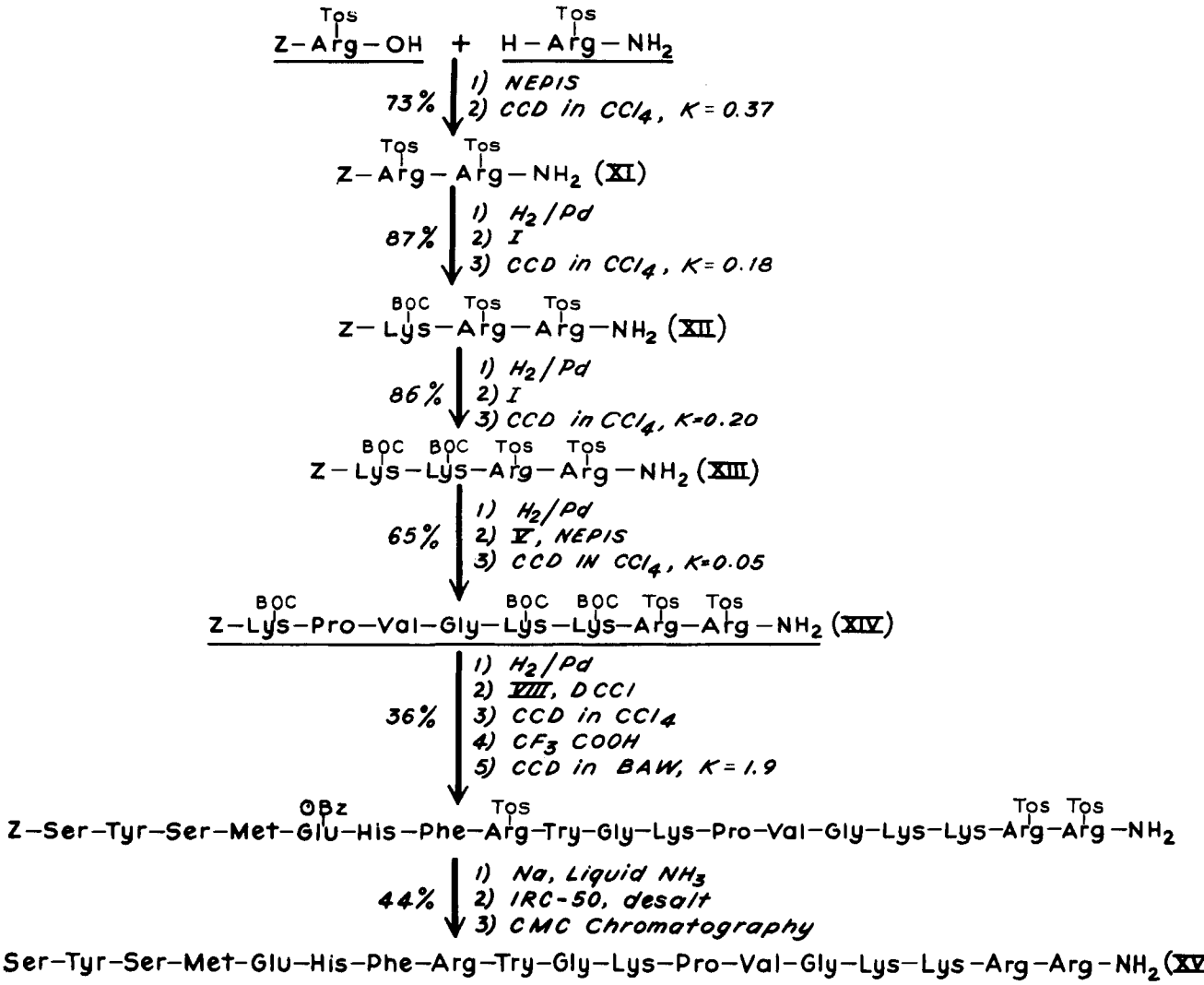

Figure 3. Synthetic scheme for the octadecapeptide amide (XV).

ylarginyltryptophylglycine[5] (VIII) was linked with suitably protected hepta-, octa-, and nonapeptide amides by means of dicyclohexylcarbodiimide[10] (DCCI) in the final step. The complete schemes for the synthesis of X, XV, and XXIII are given in Figures 2, 3, and 4, respectively.

For the synthesis of X and XV, a new N^G-tosyl-protected arginine derivative was prepared, namely, N^G-tosylarginine amide. N^α-Carbobenzoxy-N^G-tosylarginine[11] was converted to the corresponding amide by means of N-ethyl-5-phenylisoxazolium 3′-sulfonate[12] (NEPIS) and ammonia. Although this derivative was obtained only in amorphous form, catalytic hydrogenolysis of this product yielded N^G-tosylarginine amide in crystalline form. This crystalline product was allowed to react with N^α-carbobenzoxy-N^ϵ-*t*-butyloxycarbonyllysine *p*-nitrophenyl ester[13] (I) in a stepwise manner[14] to yield the protected tripeptide III. Peptide III was obtained in crystalline form and in good yield (90%). As in our earlier work[5,7,9] countercurrent distribution was employed routinely for the purification of all intermediates. In view of the higher *K* values exhibited by the amide derivatives compared to the ester analogs[5,7] in the toluene system, the carbon tetrachloride system proved more satisfactory. In addition, these intermediates were characterized by elemental analysis and their homogeneity was checked by means of paper chromatography in two solvent systems.

Peptide III was decarbobenzoxylated and allowed to react with crystalline N^α-carbobenzoxy-N^ϵ-*t*-butyloxycarbonyllysylprolylvalylglycine (V), the synthesis of which has been described.[7] The protected heptapeptide amide VI readily crystallized after purification by means of countercurrent distribution.

The octapeptide amide XIV required for the synthesis of XV was similarly prepared by coupling V with the product obtained by decarbobenzoxylation of XIII (Figure 3). The latter was prepared from N^G-tosylarginine amide by stepwise synthesis first with N^α-carbobenzoxy-N^G-tosylarginine[11] and subsequently with I. The protected octapeptide amide was obtained in crystalline form.

For the synthesis of the nonapeptide amide intermediate XXI (Figure 4), N^α-carbobenzoxy-N^G-tosylarginyl-N^G-tosylarginylproline (XVI) was converted to the amide by the use of NEPIS and ammonia. Stepwise lengthening of the peptide by reaction with I

(10) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, 77, 1067 (1955).
(11) J. Ramachandran and C. H. Li, *J. Org. Chem.*, 27, 4006 (1962).
(12) R. B. Woodward, R. A. Olofson, and H. Mayer, *J. Am. Chem. Soc.*, 83, 1010 (1961).
(13) R. Schwyzer and W. Rittel, *Helv. Chim. Acta*, 44, 159 (1961).
(14) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, 81, 5688 (1959).

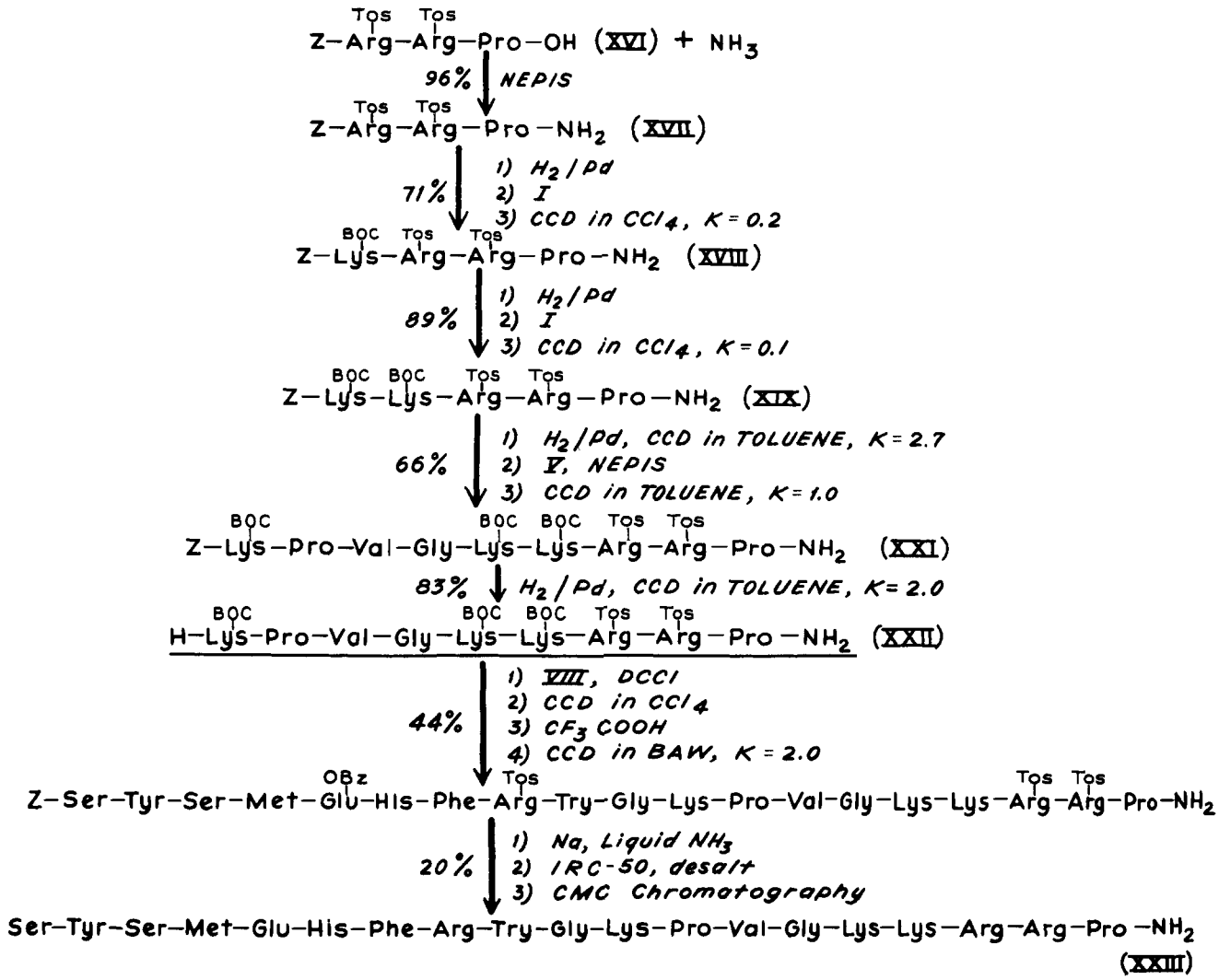

Figure 4. Synthetic scheme for the nonadecapeptide amide (XXIII).

yielded the pentapeptide amide XIX in good yield. XIX was decarbobenzoxylated and allowed to react with V to yield the nonapeptide amide XXI. Although XXI could not be crystallized, the decarbobenzoxylated product XXII readily crystallized after purification by means of countercurrent distribution in the toluene system. It is interesting to note that the nonapeptide[7] and heptapeptide[5] intermediates in which all the basic side chain functions were blocked by the tosyl group could not be induced to crystallize even though their homogeneity was established by the same criteria as those employed in the present work. The *t*-butyloxycarbonyl group seems to be conducive to the formation of crystalline products at least in some of the sequences found in ACTH.

For the synthesis of the heptadecapeptide amide, VIII was allowed to react with VII with the aid of DCCI. The product was purified by means of countercurrent distribution. The protecting groups derived from *t*-butyl alcohol were removed by treatment with trifluoroacetic acid. Further purification was achieved at this stage by means of countercurrent distribution in the BAW system. This step serves to separate the partially deblocked heptapeptide amide (K = 1.7) from the acylurea[15] of the decapeptide[16] (K = 7) as well as unreacted decapeptide VIII. Any unreacted heptapeptide amide VII which has not already been removed by countercurrent distribution in the carbon tetrachloride system is partially deblocked by the trifluoroacetic acid and has a low K value (0.5) in the BAW system, and is also conveniently removed.

The partially protected heptadecapeptide amide was then completely deblocked by treating with sodium in liquid ammonia.[17] The product was desalted on IRC-50 resin[18] and purified by chromatography on carboxymethylcellulose[19] using gradient elution with ammonium acetate (Figure 5). The main peak was isolated and rechromatographed on carboxymethylcellulose to yield the heptadecapeptide amide X. The octadecapeptide amide XV and the nonadecapeptide amide were prepared similarly and purified on carboxymethylcellulose as described above. All the three peptides were found to be homogeneous by electrophoresis on paper as well as polyacrylamide gel. The electrophoretic mobilities of the heptadecapeptide amide X and the nonadecapeptide amide XXIII are compared with the heptadecapeptide acid[5] and the nonadecapep-

(15) H. G. Khorana, *Chem. Ind.* (London), 1087 (1955).
(16) See preceeding paper.
(17) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, 117, 27 (1937).
(18) H. B. F. Dixon and M. B. Stack-Dunne, *Biochem. J.*, 61, 483 (1955).
(19) E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.*, 78, 751 (1956).

Table I. Amino Acid Composition of the Heptadeca-, Octadeca-, and Nonadecapeptide Amides

Amino acid	Heptadecapeptide amide (X)		Octadecapeptide amide (XV)		Nonadecapeptide amide (XXIII)	
	Theor.	Found[a]	Theor.	Found[a]	Theor.	Found[a]
Serine	2	1.85	2	1.74	2	1.75
Tyrosine	1	1.04	1	1.08	1	0.91
Methionine	1	0.90	1	0.91	1	1.02
Glutamic acid	1	1.04	1	1.00	1	1.00
Histidine	1	1.02	1	0.86	1	0.95
Phenylalanine	1	1.06	1	1.04	1	0.91
Arginine	2	2.06	3	2.83	3	3.00
Glycine	2	2.08	2	2.01	2	2.08
Lysine	3	3.12	3	3.04	3	2.93
Proline	1	0.91	1	0.97	2	2.06
Valine	1	1.02	1	1.05	1	1.02
Tryptophan[b]	1	0.82	1	0.92	1	0.85
Amide	1	1.00[c]	1	..	1	..

[a] Chromatography. [b] Determined spectrophotometrically. [c] Determined by the micro-Conway method. We wish to thank Mr. W. P. Reinhardt for this determination.

tide acid[7] in Figure 6. It is evident that X and XXIII move faster toward the cathode at pH 3.7 because of their lack of a free carboxyl terminal.

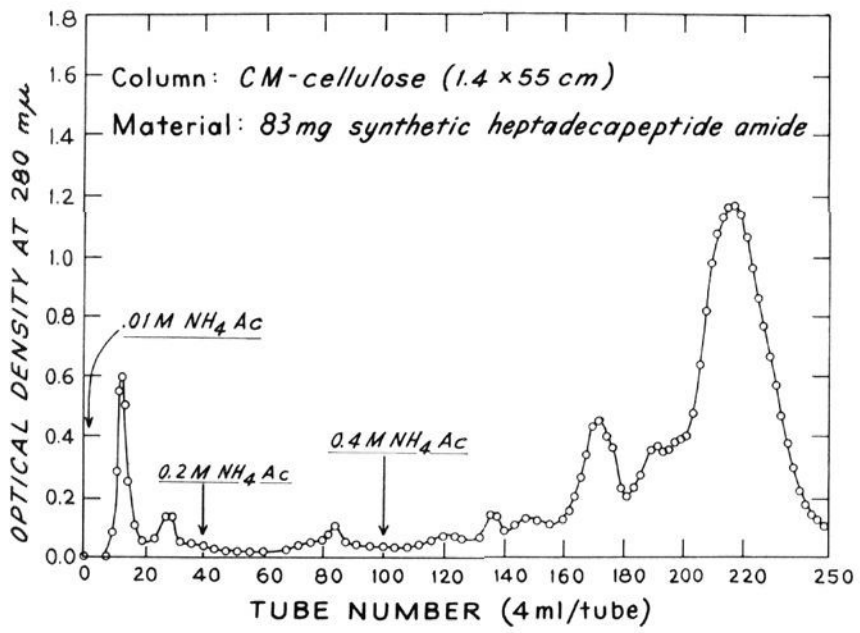

Figure 5. CM cellulose chromatography of the heptadecapeptide amide (X). The initial buffer was 0.01 *M* ammonium acetate, pH 4.5. After 3–4 hold-up volumes (4 cc./tube) had been collected, a gradient with respect to pH and concentration of salt was started by introducing 0.2 *M* ammonium acetate buffer of pH 6.7 through a 500-cc. mixing flask containing the starting buffer. Later, the gradient was increased by substituting 0.4 *M* ammonium acetate of pH 6.7 as the solution flowing into the mixing flask.

The amino acid composition of acid hydrolysates of the three peptides was determined by the chromatographic method[20] and is given in Table I. The values obtained experimentally are seen to be in good agreement with the theoretical values.

Since all the peptides contain a proline residue in position 12, total enzymatic digestion with leucine aminopeptidase[5] was not attempted. The decapeptide intermediate employed is the same one used in previous work[5] and has been well characterized. Since the same precautions were taken to avoid the danger of racemization as in earlier work[5,7,9] and since a crystalline peptide was obtained in each case prior to linking with the glycine residue of the decapeptide, the resulting peptides (X, XV, XXIII) may be considered to possess a high degree of optical purity.

Removal of Tosyl Groups by the Use of Sodium in Liquid Ammonia. In all our work the guanidino group of arginine residues, and in some cases the ε-amino group of lysine residues as well, have been protected by blocking with the *p*-toluenesulfonyl (tosyl) group.[21] The tosyl group as well as the carbobenzoxy

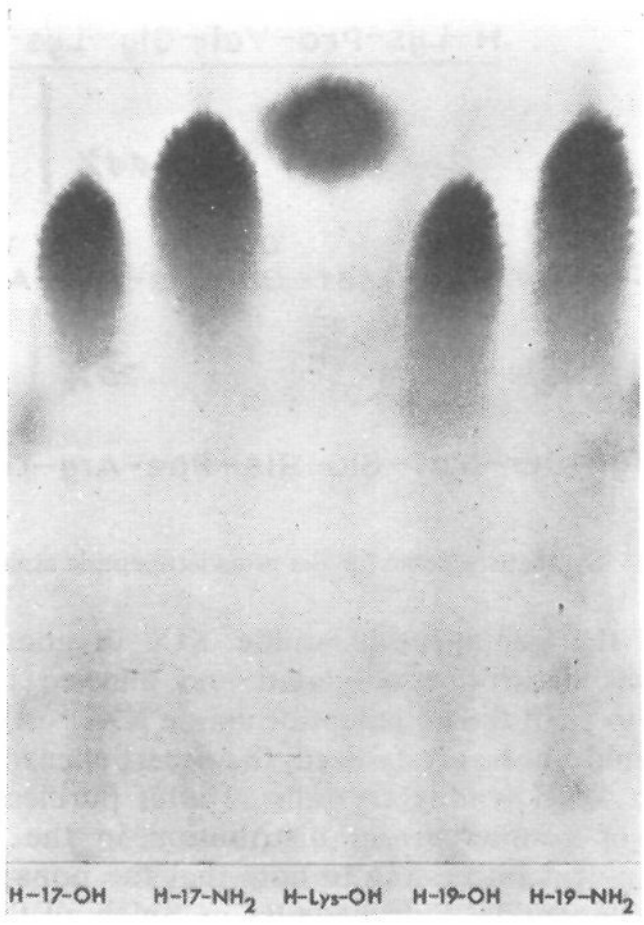

Figure 6. Electrophoresis on Whatman No. 3 filter paper in pyridine acetate buffer, pH 3.7; 6 hr., 400 volts; stained with ninhydrin. The peptides migrated toward the cathode. H-17-OH, heptadecapeptide; H-17-NH_2, heptadecapeptide amide; H-Lys-OH, lysine; H-19-OH, nonadecapeptide; H-19-NH_2, nonadecapeptide amide.

group and the benzyl moiety have all been removed in the final stages of the synthesis by reductive cleavage by the use of sodium in liquid ammonia. Recently, the report[22] of cleavage of the peptide bond Lys-Pro during this deblocking step in the case of an analog of α-melanocyte-stimulating hormone has caused concern about the usefulness of the tosyl group as a protecting group.

It has been our experience that optically and chemically homogeneous peptides related to ACTH can be prepared in reasonable yields using the tosyl group for

(20) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(21) J. Ramachandran, D. Chung, and C. H. Li, *Metabolism*, **13**, 1043 (1964).

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

Table II. Removal of Protecting Groups from Synthetic Peptides by Treatment with Sodium in Liquid Ammonia

Starting material	Peptide isolated	Protecting groups removed, no.	Peptide isolated, %[a]
Protected pentapeptide	α^{20-24}-ACTH	1	70
Protected hexapeptide	α^{5-10}-ACTH	2	67
Protected decapeptide	α^{1-10}-ACTH	3	65
Protected pentadecapeptide	$\alpha^{(1-10)+(15-19)}$-ACTH	5	50
Protected heptadecapeptide	α^{1-17}-ACTH	8	54
Protected heptadecapeptide amide	α^{1-17NH_2}-ACTH	4	50
Protected octadecapeptide amide	α^{1-18NH_2}-ACTH	5	44
Protected nonadecapeptide	α^{1-19}-ACTH	5	43
Protected nonadecapeptide amide	α^{1-19NH_2}-ACTH	5	20
Protected hexacosapeptide	$\alpha_b{}^{1-26}$-ACTH	9	20

[a] Homogeneous free peptide isolated after chromatography on carboxymethylcellulose.

protecting basic side chain functions. Table II lists the peptides prepared in our laboratory and characterized as mentioned above, along with the yields obtained with the use of sodium and liquid ammonia for the removal of the protecting groups. It can be noted that the yields drop with increase in the number of protecting groups and compare quite favorably with those obtained in the removal of other protecting groups such as the formyl group.[2,3] Of course the *t*-butyloxycarbonyl group[4,13,23] has proved to be excellent for the protection of the ϵ-amino groups of lysine, and we have employed it for this purpose. The use of the nitro group for protecting the guanidine function of arginine residues precludes the use of the carbobenzoxy group for α-amino protection. Besides, the nitro group does not efficiently suppress the reactivity of the highly basic guanidino group.[24] The use of tosyl protection ensures smooth synthesis of all intermediates in high yields in our experience, and this outweighs the minor disadvantages associated with its removal at the completion of the synthesis.

Recent work[25,26] with model peptides has clarified some of the controversial observations reported earlier[22] concerning the use of sodium in liquid ammonia for the removal of tosyl groups in peptide synthesis. Guttmann[25] studied the effect of sodium in liquid ammonia on peptides containing the sequence glycyllysylproline under various conditions and presented evidence to show that cleavage of the lysylproline bond is the result of the presence of water in the medium. This cleavage was found to be negligible if the reductive removal of protecting groups is carried out under anhydrous conditions. Similar results were obtained by Bajusz and Medzihradszky[26] who observed quantitative deblocking of the peptide carbobenzoxyglycyl-N$^{\epsilon}$-tosyllysylproline amide without any damage to the peptide backbone. Guttmann[27] further stated that quantitative cleavage of the lysylproline bond in the sequence X-Lys-Pro-Y is achieved in the presence of 5 to 20 equiv. of ammonium chloride. These results clearly imply that the cleavage of peptide bonds involving proline is due to the presence of water or other proton donors in the medium.

In our work, we have maintained anhydrous conditions and no source of protons such as ammonium chloride or acetate was added to discharge the blue color. Rather, the blue color could be maintained for long periods with a very minute excess of sodium (0.1 equiv.). The liquid ammonia was freshly distilled from sodium immediately before use. The results in Table II suggest that the limiting features in the use of sodium in liquid ammonia may be the number of protecting groups to be removed and the solubility of the peptide, rather than any side reactions. The danger of peptide bond cleavage is not peculiar to this treatment alone but is rather general and is dependent on other factors such as the sequence of amino acids. Thus, intramolecular catalysis through the side chain functions of neighboring amino acids can cause peptide bond cleavage under as mild conditions as 50% acetic acid at room temperature.[28a] From our experience, as well as from others,[28b] it can be said that the tosyl group is a very useful and efficient protecting group, especially for the highly reactive guanidino moiety of arginine residues, and can be removed easily from peptides of medium chain length if anhydrous conditions are employed during the treatment with sodium in liquid ammonia.

Structure–Activity Relationships. The apparent similarities in the biological activities of ACTH derived from bovine, ovine, and porcine pituitaries (Figure 1) suggested that the portion of the peptide chain near the carboxyl terminal (wherein lie the structural differences) may not be essential for the manifestation of the biological activities associated with this hormone. Chemical and enzymatic degradative studies[29,30] performed in the early 1950's confirmed this suspicion. Limited digestion of both the ovine and bovine hormones with pepsin resulted in the release of 11 amino acids from the carboxyl end. The biological activity of the peptide, however, was not significantly impaired. From studies of partial acid hydrolysates of the peptic core, it was further inferred that four more residues could be removed without loss of activity. On the other hand, alteration of the NH_2-terminal serine by

(23) L. A. Carpino, *J. Am. Chem. Soc.*, **79**, 98, 4427 (1957); **82**, 2725 (1960); F. C. McKay and N. F. Albertson, *ibid.*, **79**, 4686 (1957); G. W. Anderson and A. C. McGregor, *ibid.*, **79**, 6180 (1957); R. Schwyzer, 7th Giornate Biochimiche Latine, St. Margherita (Ligure), May 25,1963.

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(25) St. Guttmann in "Peptides," G. T. Young, Ed., The Macmillan Co., New York, N. Y., 1963, pp. 41–47.

(26) S. Bajusz and K. Medzihradszky, pp. 49–52 of ref. 25.

(27) St. Guttmann, ref. 25, p. 54.

(28) (a) R. H. Mazur and J. M. Schlatter, *J. Org. Chem.*, 28, 1025 (1963); (b) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon, *J. Am. Chem. Soc.*, **75**, 4879 (1953); V. du Vigneaud, M. D. Bartlett, and A. Jöhl, *ibid.*, **79**, 3355 (1957).

(29) C. H. Li, *Recent Progr. Hormone Res.*, **18**, 1 (1962).

(30) C. H. Li, *Advn. Protein Chem.*, **11**, 101 (1956).

Table III. Biological Activities of Synthetic Peptides

Peptide	Partial amino acid sequence	Steroidogenic potency *in vivo*, U.S.P.U./μmole	Steroidogenic potency *in vitro*, I.U./μmole	Melanocyte-stimulating activity *in vivo*, μmole	Melanocyte-stimulating activity *in vitro* u./μmole
α_s-ACTH	Ser-Tyr....Lys-Lys-Arg-Arg-Pro....Phe 1 2 15 16 17 18 19 39	481 (318–722)[a]	617 (917–418)[a]	4.4×10^{-5}[b]	2.9×10^{5}
$\alpha_b{}^{1-26}$-ACTH (XXIV)	Ser-Tyr....Lys-Lys-Arg-Arg-Pro....Gly 1 2 15 16 17 18 19 26	390 (237–636)	279 (369–210)	6.6×10^{-5}	4.8×10^{5}
$\alpha^{1-19}NH_2$-ACTH (XXIII)	Ser-Tyr....Lys-Lys-Arg-Arg-Pro-NH_2 1 2 15 16 17 18 19	345 (270–441)	122 (178–84)	8.7×10^{-5}	4.2×10^{5}
α^{1-19}-ACTH (XXV)	Ser-Tyr....Lys-Lys-Arg-Arg-Pro-OH 1 2 15 15 17 18 19	110 (68–180)	82 (129–54)	8.7×10^{-5}	3.3×10^{5}
$\alpha^{1-18}NH_2$-ACTH (XV)	Ser-Tyr....Lys-Lys-Arg-Arg-NH_2 1 2 15 16 17 18	299 (214–419)	74 (103–54)	8.9×10^{-5}	4.5×10^{5}
$\alpha^{1-17}NH_2$-ACTH (X)	Ser-Tyr....Lys-Lys-Arg-NH_2 1 2 15 16 17	69 (54–88)	88 (123–63)	9.6×10^{-5}	4.2×10^{5}
α^{1-17}-ACTH (XXVI)	Ser-Tyr....Lys-Lys-Arg-OH 1 2 15 16 17	11.3 (7.7–16.5)	10.9 (17.1–6.9)	9.6×10^{-5}	2.1×10^{5}
$\alpha^{(1-10)+(15-19)}$-ACTH (XXVII)	Ser-Tyr....Gly-Lys-Lys-Arg-Arg-Pro-OH 1 2 10 15 16 17 18 19	0.0118 (0.0078–0.0137)	0.8 (1.2–0.6)	1×10^{-2}	3.7×10^{3}

[a] Mean of at least three assays; 95% confidence limits are given in parentheses. [b] The dose produces a change in melanophore index in hypophysectomized *Rana pipiens* from 1+ to 3+ within 1 hr.

selective acetylation of the amino group[31] or by periodate oxidation[32] which converts the amino terminus to an aldehyde resulted in almost complete loss of adrenal-stimulating potency. Hence, the idea that the full biological activity of the molecule resides in a sequence smaller than the tetracosapeptide containing the first 24 residues with its amino terminus intact gained ground. After the synthesis of the eicosapeptide amide,[2] it was claimed that this molecule represented the smallest peptide related to ACTH which exhibits the full activity found to be associated with the natural hormone.

In all the studies mentioned above the adrenal-stimulating activity was measured, usually by the adrenal ascorbic acid depletion method[33] or by the adrenal weight assay.[34] With the development of more specific assays measuring steroidogenesis in the adrenals *in vitro*[35] and *in vivo*,[36] many of the synthetic peptides have been assayed by these latter procedures. Some confusion has arisen owing to the fact that the adrenal-stimulating potencies of some peptides determined by one method have been compared with the activities of the natural hormones assayed by a different procedure. Although the adrenal ascorbic acid depletion assay provided considerable stimulus to the investigations on ACTH during the late 1940's and early 1950's, the relationship of adrenal ascorbic acid depletion to adrenal steroidogenesis has not been satisfactorily established. Furthermore, some of the synthetic peptides have been compared with different standard preparations and the potencies have been expressed in terms of percentage of activity of the standard rather than in international units. Lebovitz and Engel[37] studied the synthetic eicosapeptide amide in detail and found it to possess the following potencies expressed as percentage of activity of pig ACTH (corticotrophin A_1) by weight: steroidogenesis measured *in vivo*, 176%; steroidogenesis measured *in vitro*, 77%. Purified pig ACTH (corticotrophin A_1) has been shown[38] to possess 65–70 units/mg. when assayed *in vivo* and 49 units/mg. when assayed *in vitro*. Thus, in terms of units the eicosapeptide amide has a potency of 114–120 units/mg. *in vivo* and 38 units/mg. *in vitro*. In order to obtain a meaningful picture of the relationship between structure and biological activity, it is imperative that all the synthetic peptides be compared with the highly purified hormone under identical conditions. With this aim we have synthesized several peptides related to ACTH. The syntheses have been described above and in previous publications.[1,5,7,9] In Table III biological activities of highly purified sheep ACTH (α_s-ACTH) are compared with those of a hexacosapeptide (XXIV), a nonadecapeptide (XXV), a nonadecapeptide amide (XXIII), an octadecapeptide amide (XV), a heptadecapeptide (XXVI), a heptadecapeptide amide (X), and a pentadecapeptide with an altered sequence (XXVII).

It is apparent that the results obtained *in vitro* do not parallel those obtained *in vivo*.[39] The reason for this is not clear, but the explanation may be a more rapid destruction of the smaller peptides *in vitro* by the enzymes released during the quartering of the adrenals. As the structure of the native hormone is approached, the activities measured *in vitro* and in *in vivo* approach the same value. From the *in vitro* measurements it is clear that the intact natural product is the only fully active product. Even *in vivo* the intact hormone appears to be somewhat more potent than the synthetic peptides.[40]

Although the native hormone appears to be the most active molecule, the results in Table III show that the structural features essential for eliciting steroidogenesis probably reside in the amino terminal half of the molecule. The *in vivo* activities of the octadecapeptide amide, nonadecapeptide amide, and hexa-

(31) J. P. Waller and H. B. F. Dixon, *Biochem., J.*, 75, 320 (1960).
(32) H. B. F. Dixon, *ibid.*, 62, 25p (1956).
(33) M. A. Sayers, G. Sayers, and L. A. Woodbury, *Endocrinology*, 42, 379 (1948).
(34) J. B. Collip, E. M. Anderson, and D. L. Thomson, *Lancet*, 2, 347 (1933).
(35) M. A. Saffran and A. V. Schally, *Endocrinology*, 56, 523 (1955).
(36) H. S. Lipscomb and D. H. Nelson, *ibid.*, 71, 13 (1962).
(37) H. E. Lebovitz and F. L. Engel, Abstracts of the 45th Meeting of the Endocrine Society, Atlantic City, N. Y., June 1963.
(38) H. A. Boright, F. L. Engel, H. E. Lebovitz, J. L. Kostyo, and J. E. White, Jr., *Biochem., J.*, 83, 95 (1962).
(39) These assays were kindly performed by Dr. G. W. Liddle and associates at Vanderbilt University.
(40) (a) This conclusion is in agreement with the findings of Schwyzer[40b] that synthetic β^{1-39}-corticotropin[40c] (porcine ACTH in Figure 1) is almost twice as active (140 I.U./mg., 736 I.U./μmole) as β^{1-24}-corticotropin[4] (106 I.U./mg., 376 I.U./μmole) when compared on the basis of molecular size in the adrenal ascorbic acid depletion assay.[33] (b) R. Schwyzer, *Ann. Rev. Biochem.*, 33, 276 (1964). (c) R. Schwyzer and P. Sieber, *Nature*, 190, 172 (1963).

cosapeptide are comparable although the hexacosapeptide appears to be more potent *in vitro*.

An examination of the structure of ACTH (Figure 1) shows that all the basic amino acid residues are found in the region between positions 5 and 22 and that a core of basic amino acids occurs in positions 15–18. In addition, the first acidic amino acid beyond position 5 is found in position 25. Thus it appears that the positive charge contributed by the basic amino acids may play an important role in the action of this hormone. Evidence in support of this is the significant loss of activity found when the biological potencies of the heptadecapeptide and the nonadecapeptide are compared. That this decrease in potency as the peptide chain length decreases from the nonadecapeptide to the heptadecapeptide is due to the loss of the positive charge contributed by the arginine residue in position 18 is easily seen when the activities of the heptadecapeptide are compared with that of the corresponding amide (see Table III). There is a nearly eightfold increase in potency when the free carboxyl group is blocked, thereby increasing the net positive charge. This hypothesis was confirmed by the synthesis of the nonadecapeptide amide, which, as seen in Table III, exhibits an activity of 345 units/μmole when assayed *in vivo* compared with 110 units/μmole exhibited by the corresponding nonadecapeptide acid. The activities of these two peptides *in vitro* are 122 and 82 I.U./μmole, respectively. These results suggest that this region of the molecule may be involved in the binding of the hormone to its receptor and this process may involve an interaction with a negatively charged surface at the receptor site. The presence of a free carboxyl group and hence a negative charge in positions 17 or 19 may lead to decreased binding with the negatively charged receptor. If this were correct, it would be expected that the octadecapeptide amide which has the same number of positive charges as the nonadecapeptide amide should have the same *in vivo* activity as the latter. This is indeed found to be the case. Furthermore, both these peptides are seen to be more potent than X since they contain an extra arginine residue. Since alterations of the NH_2 terminus lead to drastic reduction in the activity, it may be assumed that the amino terminal decapeptide sequence contains the active site. In this sequence are present all the functional amino acids that have been found to be implicated in the active sites of a number of proteolytic enzymes. Thus we can envisage the core of basic amino acids as representing the binding site and bringing the active site in proper juxtaposition with the receptor. That the proper orientation of the decapeptide to the binding site is essential is shown by the fact that the pentadecapeptide (XXVII) with the altered sequence has negligible adrenal-stimulating potency. The omission of the tetrapeptide sequence lysylprolylvalylglycine between positions 10 and 15 results in almost total loss of steroidogenic properties. Since the presence of proline in position 12 in the molecule introduces a bend in the peptide chain, the above result suggests that the spatial arrangement of the basic core and the amino terminal decapeptide is important for the functioning of the hormone. Whether the tetrapeptide sequence found in positions 11–14 has any intrinsic role other than orientation of the binding and active sites remains to be seen.

As mentioned earlier, the steroidogenic activity manifested by the native hormone is higher than that exhibited by the synthetic fragments. This suggests that the COOH-terminal part of the molecule may play a role, albeit secondary, in the functioning of ACTH. In view of the fact that the acidic amino acids are all located near the C-terminus, it seems likely that this part of the molecule may serve to increase the binding of the hormone by electrostatic interaction with a positively charged site on the hypothetical receptor. It is evident that the elucidation of the mechanism of action of ACTH must await the elucidation of the nature of the receptor in the cell.

The melanophore-stimulating activities of the synthetic peptides have also been measured both *in vitro*[41a] and *in vivo*[41b] and are also given in Table III. It is seen that all the synthetic peptides are comparable in skin-darkening effect to α_s-ACTH with the exception of XXVII. This result is not surprising since all these peptides (except XXVII) and α_s-ACTH contain the amino acid sequence found in α-MSH. XXVII lacks the tripeptide sequence lysylprolylvaline found at the COOH terminus of α-MSH, and exhibits only 1% of the activity of other peptides both *in vitro* and *in vivo*. These results confirm the earlier findings of Hofmann and co-workers[22] that the lysylprolylvaline plays an important role in the manifestation of MSH activity.

Experimental[42]

General. Catalytic hydrogenolysis was performed in the presence of Pd freshly prepared[47] from $PdCl_2$, by means of a Vibro-Mixer[48] in the apparatus described by Meienhofer.[49] The completion of decarbobenzoxylation was always checked by means of chromatography on paper or by thin layer chromatography on silica gel. Countercurrent distribution was performed on a 100-tube, all-glass machine with a capacity of 10 cc. for each phase. The peaks were located usually by evaporating aliquots to dryness and

(41) (a) K. Shizume, A. B. Lerner, and T. B. Fitzpatrick, *Endocrinology*, **54**, 553 (1954); (b) L. T. Hogben and D. Slome, *Proc. Roy. Soc.* (London), **B108**, 10 (1931).

(42) Melting points were determined on a Fisher-Johns block and are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. All samples for microanalysis were dried in an Abderhalden drying pistol with P_2O_5 at 77° for 16 hr. at 0.3 mm. Paper chromatography was carried out on Whatman No. 1 filter paper at room temperature. The solvents used were 1-butanol–acetic acid–water (BAW) in a ratio of 4:1:1, 2-butanol–10% ammonia (SBA) in a ratio of 85:15, and 1-butanol–pyridine–acetic acid–water (BPAW) in a ratio of 30:20:6:24, all by volume. Thin layer chromatography was performed according to Stahl[43] on silica gel in chloroform–methanol (CM) in the ratio 8:2 by volume. Peptide spots were located by the ninhydrin reagent, the Pauly reagent,[44] and the Ehrlich reagent,[45] and by the chlorine method.[46] For countercurrent distribution, the following solvent systems were employed: toluene system composed of toluene–chloroform–methanol–water (5:5:8:2) by volume; carbon tetrachloride system composed of carbon tetrachloride–chloroform–methanol–water (1:3:3:1) by volume; the BAW system composed of 1-butanol–acetic acid–water (4:1:5) by volume.

(43) E. Stahl, *Chem. Ztg.*, **82**, 323 (1958).

(44) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).

(45) J. Smith, *Nature*, **171**, 43 (1953).

(46) H. Zahn and E. Rexroth, *Z. Anal. Chem.*, **148**, 181 (1955).

(47) R. Willstätter and E. Waldschmidt-Leitz, *Ber.*, **54**, 128 (1921).

(48) Vibro-Mixer, A. G. Feuer Chemie-Appartebau, Zurich, Model E 1.

(49) J. Meienhofer, *Chimia*, **16**, 385 (1962).

weighing, by ultraviolet absorption, and by the use of the ninhydrin reagent after alkaline hydrolysis.

I. Synthesis of the Heptadecapeptide Amide and Intermediates. N^{α}-Carbobenzoxy-N^{G}-tosylarginine Amide. N^{α}-Carbobenzoxy-N^{G}-tosylarginine[11] (4.63 g., 10 mmoles) was dissolved in 150 cc. of acetonitrile and cooled to 0°. Triethylamine (1.4 cc., 10 mmoles) and N-ethyl-5-phenylisoxazolium 3′-sulfonate[12] (NEPIS) (2.54 g., 10 mmoles) were added and the mixture was stirred at 0° for 1 hr. Anhydrous ammonia was then bubbled into the solution for 15 min. at 0° and kept overnight at 4°. The solvent was removed *in vacuo*, redissolved in wet ethyl acetate, and washed with water, 5% sodium bicarbonate, and water. The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo* to yield 3.98 g. (86%) of the title compound as a glassy residue which failed to crystallize. N^{α}-Carbobenzoxy-N^{G}-tosylarginine amide was found to be homogeneous in paper chromatography; R_{fBAW} 0.81; R_{fsBA} 0.74; m.p. 83–88°; $[\alpha]^{25}D$ +1.4° (*c* 5, methanol).

Anal. Calcd. for $C_{21}H_{27}N_5O_5S$ (461.5): C, 54.6; H, 5.90; N, 15.2. Found: C, 54.8; H, 5.73; N 15.0.

N^{G}-Tosylarginine Amide. N^{α}-Carbobenzoxy-N^{G}-tosylarginine amide (2.64 g., 5.7 mmoles) was dissolved in 100 cc. of methanol and submitted to catalytic hydrogenolysis in the presence of Pd freshly prepared from 0.5 g. of $PdCl_2$, until the evolution of CO_2 ceased. The catalyst was filtered off and washed with methanol, and the filtrate and washings were evaporated to dryness. The residue crystallized upon trituration with ethyl acetate to yield 1.68 g. (90%) of N^{G}-tosylarginine amide, m.p. 140–142°; $[\alpha]^{25}D$ +10.0° (*c* 1, methanol); R_{fBAW} 0.40; R_{fsBA} 0.32.

Anal. Calcd. for $C_{13}H_{21}N_5O_3S$ (327.4): C, 47.7; H, 6.47; N, 21.4. Found: C, 47.6; H, 6.24; N, 21.3.

N^{α}-Carbobenzoxy-N^{ϵ}-butyloxycarbonyllysyl-N^{G}-tosylarginine Amide (II). N^{G}-Tosylarginine amide (1.64 g., 5 mmoles) was dissolved in 40 cc. of dimethylformamide and stirred with 2.75 g. (5 mmoles) of N^{α}-carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysine *p*-nitrophenyl ester (I)[13] at room temperature for 16 hr. The solvent was removed *in vacuo* at 40° and the residue was purified by countercurrent distribution in the carbon tetrachloride system for 100 transfers. The main component (K = 0.27) was isolated to yield 2.65 g. (67%) of II as an amorphous powder, m.p. 95–105°; $[\alpha]^{25}D$ −5.5° (*c* 1, methanol); R_{fBAW} 0.82; R_{fsBA} 0.84.

Anal. Calcd. for $C_{32}H_{47}N_7O_8S$ (689.8): C, 55.7; H, 6.81; N, 14.2. Found: C, 55.2; H, 7.03; N, 14.5.

N^{α}-Carbobenzoxy-N^{ϵ}-t-butyloxycarbonyllysyl-N^{ϵ}-t-butyloxycarbonyllysyl-N^{G}-tosylarginine Amide (III). Peptide II (2.52 g., 3.66 mmoles) was dissolved in 100 cc. of methanol and the carbobenzoxy group was removed by catalytic hydrogenolysis as described above. After removal of the methanol the product was isolated as a glassy residue in 91% yield (1.86 g.) and found to be homogeneous in paper chromatography; R_{fBAW} 0.70. This material was dissolved in 50 cc. of acetonitrile and allowed to react with 1.83 g. (3.67 mmoles) of I for 24 hr. at room temperature. The tripeptide III crystallized out of the reaction mixture and was filtered off, washed with acetonitrile, and dried to yield 1.94 g. (64%) of III, m.p. 130–132°. The mother liquor and washings were evaporated to dryness *in vacuo* and the residue was submitted to countercurrent distribution in the carbon tetrachloride system for 100 transfers. Material from the peak with K = 0.15 was isolated and crystallized from warm acetonitrile to yield 0.81 g. (26%) more of III, m.p. 130–132°; R_{fBAW} 0.88; R_{fsBA} 0.88. Total yield of III was 90%; $[\alpha]^{25}D$ −15.0° (*c* 1, methanol).

Anal. Calcd. for $C_{43}H_{67}N_9O_{11}S$ (918.1): C, 56.3; H, 7.36; N, 13.7. Found: C, 56.7; H, 7.27; N; 13.9.

N^{α}-Carbobenzoxy-N^{ϵ}-t-butyloxycarbonyllysylprolylvalylglycyl-N^{ϵ}-t-butyloxycarbonyllysyl-N^{ϵ}-t-butyloxycarbonyllysyl-N^{G}-tosylarginine Amide (VI). Peptide III (1.93 g., 2.1 mmoles) was dissolved in 50 cc. of methanol and decarbobenzoxylated by means of catalytic hydrogenolysis. The product was isolated and submitted to countercurrent distribution in the carbon tetrachloride system (100 transfers). N^{ϵ}-*t*-Butyloxycarbonyllysyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{G}-tosylarginine amide (IV) was isolated from the main peak, with K = 0.33, in 83% yield (1.37 g), and was found to be homogeneous in paper chromatography, R_{fBAW} 0.77.

N^{α}-Carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysylprolylvalylglycine[7] (V) (0.634 g., 1 mmole) was dissolved in 30 cc. of acetonitrile and cooled to 0°. Triethylamine (0.14 cc., 1 mmole) and 2.54 g. (1 mmole) of NEPIS were added, and the mixture was stirred at 0° for 1 hr. Peptide IV (0.784 g., 1 mmole) was added, and the mixture was stirred at room temperature for 16 hr. The solvent was removed *in vacuo* and the residue was purified by countercurrent distribution in the carbon tetrachloride system for 100 transfers. The heptapeptide VI could be isolated from the peak with K = 0.08. Upon treatment with warm acetonitrile, VI crystallized in the form of platelets. The crystalline product was filtered, washed with cold acetonitrile, and dried to yield 0.991 g. (71%) of VI; m.p. 172–174°; $[\alpha]^{25}D$ −38.7° (*c* 1, methanol); R_{fBAW} 0.91; R_{fsBA} 0.87.

Anal. Calcd. for $C_{66}H_{106}N_{14}O_{17}S$ (1400): C, 56.6; H, 7.63; N, 14.0. Found: C, 56.8; H, 7.39; N, 13.7.

N^{ϵ}-t-Butyloxycarbonyllysylprolylvalylglycyl-N^{ϵ}-t-butyloxycarbonyllysyl-N^{ϵ}-t-butyloxycarbonyllysyl-N^{G}-tosylarginine Amide (VII). Peptide VI (0.869 g., 0.62 mmole) was dissolved in 50 cc. of methanol and the carbobenzoxy group was removed by catalytic hydrogenolysis in the presence of freshly prepared Pd as described earlier. The product was isolated and purified by means of countercurrent distribution in the carbon tetrachloride system (100 transfers). The material from the peak with K = 0.16 was isolated to yield 0.706 g. (90%) of VII. Peptide VII was found to be homogeneous in paper chromatography; R_{fBAW} 0.76; R_{fsBA} 0.85; m.p. 128–134°; $[\alpha]^{25}D$ −32.4° (*c* 1, methanol).

Anal. Calcd. for $C_{58}H_{100}N_{14}O_{15}S$ (1226): C, 54.3; H, 8.01; N, 15.3. Found: C, 54.2; H, 7.71; N, 15.1.

Seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginine Amide (X). Carbobenzoxyseryltyrosylserylmethionyl-γ-benzylglutamylhistidylphenylalanyl-N^{G}-tosylarginyltryptophylglycine[5] (VIII) (0.168 g.,

0.1 mmole) and VII (0.127 g., 0.1 mmole) were dissolved in 2 cc. of dimethylformamide. The solution was cooled to 0°, 0.023 g. (0.11 mmole) of dicyclohexylcarbodiimide (DCCI) was added, and the mixture was stirred at 0° for 1 hr. and kept at 4° for 4 days. Glacial acetic acid (0.5 cc.) was added and the solvent was removed *in vacuo*. The residue was purified by countercurrent distribution in the carbon tetrachloride system (containing 0.01 *M* ammonium acetate instead of water) for 150 transfers. The material with K = 0.03 was pooled, evaporated to dryness *in vacuo*, and dissolved in 15 cc. of trifluoroacetic acid. After the solution had been kept at room temperature for 2 hr. the solvent was removed *in vacuo*, and further purification was achieved at this stage by means of countercurrent distribution in the system composed of 1-butanol–acetic acid–water (4:1:5) (BAW) for 100 transfers. The material from the peak with K = 1.7 was isolated to yield 0.121 g. (37%) of carbobenzoxyseryltyrosylserylmethionyl-γ-benzylglutamylhistidylphenylalanyl-N^G-tosylarginyltryptophylglycyllysylprolylvalylglycyllysyllysyl-N^G-tosylarginine amide (IX).

The partially protected heptapeptide amide IX (0.121 g.) was dissolved in 150 cc. of freshly distilled liquid ammonia and treated with small pieces of sodium until a permanent blue color was maintained for 0.5 hr. Ammonia was allowed to evaporate, and the residue was dried overnight over P_2O_5 and concentrated H_2SO_4, desalted on IRC-50 resin, and eluted with pyridine–acetic acid–water (30:4.66). The lyophilized product (0.083 g.) was purified by chromatography on carboxymethylcellulose using gradient elution with ammonium acetate (Figure 5). The material in the major peak was isolated by lyophilization and rechromatographed on carboxymethylcellulose to yield 0.039 g. (50%) of the heptadecapeptide amide X. Peptide X was found to be homogeneous by electrophoresis on paper (pH 3.7, mobility relative to lysine 0.95) as well as on polyacrylamide gel. The amino acid composition of an acid hydrolysate of X is given in Table I and is seen to be in good agreement with the expected values; $[\alpha]^{25}D$ −77.1° (*c* 0.5, 0.1 *N* acetic acid).

II. Synthesis of the Octadecapeptide Amide and Intermediates. N^α-Carbobenzoxy-N^G-tosylarginyl-N^G-tosylarginine Amide (XI). N^α-Carbobenzoxy-N^G-tosylarginine (2.31 g., 5 mmoles) was dissolved in 50 cc. of acetonitrile and cooled to 0°. Triethylamine (0.7 cc., 5 mmoles) and NEPIS (1.27 g., 5 mmoles) were added and the mixture stirred for 1 hr. at 0°. A solution of 1.63 g. (5 mmoles) of N^G-tosylarginine amide in 10 cc. of dimethylformamide was added and the mixture was stirred overnight at room temperature. The solvent was removed *in vacuo* and the residue was purified by countercurrent distribution in the carbon tetrachloride system (100 transfers). The main component (K = 0.37) was pooled and evaporated to dryness *in vacuo* to yield 2.8 g. (73%) of XI. Peptide XI was found to be homogeneous in chromatography, R_{fBAW} 0.73; R_{fSBA} 0.70; m.p. 110–120°; $[\alpha]^{25}D$ +1.1° (*c* 1, methanol).

Anal. Calcd. for $C_{32}H_{45}N_9O_8S_2$ (771.9): C, 52.9; H, 5.88; N, 16.3. Found: C, 52.7; H, 5.61; N, 16.1.

N^α-Carbobenzoxy-N^ϵ-t-butyloxycarbonyllysyl-N^G-tosylarginyl-N^G-tosylarginine Amide (XII). Peptide XI (2.74 g., 3.55 mmoles) was dissolved in 50 cc. of methanol and decarbobenzoxylated by means of catalytic hydrogenolysis. N^G-Tosylarginyl-N^G-tosylarginine amide was obtained as an oil but was found to be homogeneous in paper chromatography; R_{fBAW} 0.35. This product was dissolved in 50 cc. of dimethylformamide and stirred with 1.77 g. (3.55 mmoles) of I for 24 hr. at room temperature. The solvent was removed *in vacuo* and the residue was submitted to countercurrent distribution in the carbon tetrachloride system for 100 transfers. The desired tripeptide XII was isolated from the main peak (K = 0.18) in 87% yield (3.07 g) by means of precipitation from methanol–ether; m.p. 112–118°; $[\alpha]^{25}D$ −12.6° (*c* 1, methanol); R_{fBAW} 0.79; R_{fSBA} 0.83.

Anal. Calcd. for $C_{45}H_{65}N_{11}O_{11}S_2$ (1000): C, 54.0; H, 6.55; N, 15.4. Found: C, 53.7; H, 6.37; N, 15.4.

N^α-Carbobenzoxy-N^ϵ-t-butyloxycarbonyllysyl-N^ϵ-t-butyloxycarbonyllysyl-N^G-tosylarginyl-N^G-tosylarginine Amide (XIII). Peptide XII (2.9 g., 2.9 mmoles) was dissolved in 50 cc. of methanol and submitted to catalytic hydrogenolysis. The decarbobenzoxylated tripeptide was found to be homogeneous in paper chromatography (R_{fBAW} 0.62); hence, it was dissolved in 40 cc. of dimethylformamide and stirred with 1.45 g. (2.9 mmoles) of I at room temperature for 24 hr. Countercurrent distribution in the carbon tetrachloride system (100 transfers) was again employed for isolating the protected tetrapeptide XIII in 86% yield (3.07 g., K = 0.20); m.p. 115–125°; $[\alpha]^{25}D$ −10.7° (*c* 1, methanol); R_{fBAW} 0.81; R_{fSBA} 0.84.

Anal. Calcd. for $C_{56}H_{85}N_{13}O_{14}S_2$ (1229): C, 54.8; H, 6.97; N, 14.8. Found: C, 55.1; H, 6.78; N, 15.0.

N^α-Carbobenzoxy-N^ϵ-t-butyloxycarbonyllysylprolylvalylglycyl-N^ϵ-t-butyloxycarbonyllysyl-N^ϵ-t-butyloxycarbonyllysyl-N^G-tosylarginyl-N^G-tosylarginine Amide (XIV). The above protected tetrapeptide amide XIII (2.45 g., 2 mmoles) was decarbobenzoxylated by means of catalytic hydrogenolysis in methanol. The product was isolated in 93% yield (2.03 g.) and was found to be homogeneous in paper chromatography; R_{fBAW} 0.71. Peptide V[7] (0.444 g., 0.7 mmole) was dissolved in 30 cc. of acetonitrile and cooled to 0°. Triethylamine (0.1 cc., 0.7 mmole) and NEPIS (0.178 g., 0.7 mmole) were added and the mixture was stirred at 0° for 1 hr. The decarbobenzoxylated tetrapeptide amide prepared above (0.766 g., 0.7 mmole) was dissolved in 20 cc. of dimethylformamide and added to the reaction mixture, which was stirred at room temperature for 24 hr. The solvent was then removed *in vacuo* and the residue purified by means of countercurrent distribution in the carbon tetrachloride system for 150 transfers. The desired octapeptide amide XIV was isolated from the main peak (K = 0.05) and could be crystallized by triturating with methanol. Recrystallization from warm methanol yielded 0.773 g. (65%) of XIV in the form of needles; m.p. 165–167°; $[\alpha]^{25}D$ −24.8° (*c* 1, dimethylformamide). The crystalline product was found to be homogeneous in paper chromatography, R_{fBAW} 0.85; R_{fSBA} 0.92; as well as thin layer chromatography on silica gel; R_{fBAW} 0.48.

Anal. Calcd. for $C_{79}H_{124}N_{18}O_{20}S_2$ (1710): C, 55.5; H, 7.31; N, 14.7. Found: C, 55.3; H, 7.06; N, 14.7.

Seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginine Amide (XV). Peptide XIV (0.64 g., 0.37 mmole) was dissolved in 30 cc. of dimethylformamide and the carbobenzoxy group was removed by catalytic hydrogenolysis. After removal of the catalyst and solvent, the decarbobenzoxylated octapeptide amide (XIVa) was isolated by precipitation from methanol–ether in 99% yield (0.582 g.). This peptide was found to be homogeneous in thin layer chromatography; R_{fCM} 0.16.

Peptide XIVa (0.158 g., 0.1 mmole) and 0.168 g. (0.1 mmole) of VIII[5] were dissolved in 2 cc. of dimethylformamide, cooled to 0°, and stirred with 0.023 g. (0.11 mmole) of DCCI for 1 hr. The mixture was then stored at 4° for 4 days. Glacial acetic acid, 0.5 cc., was added and the solvent was removed *in vacuo*. The residue was submitted to countercurrent distribution in the carbon tetrachloride system (containing 0.01 *M* ammonium acetate instead of water) for 150 transfers. The material from tubes 0–19 was isolated and dissolved in 15 cc. of trifluoroacetic acid and kept at room temperature for 2 hr. The solvent was removed *in vacuo* and the residue was further purified by means of countercurrent distribution in the BAW system for 100 transfers. The material from the peak with $K = 1.9$ was isolated to yield 0.119 g. (36%) of the partially deblocked octadecapeptide amide.

This product was dried thoroughly, dissolved in 150 cc. of freshly distilled liquid ammonia, and treated with small pieces of sodium until a permanent blue color was maintained for 0.5 hr. Ammonia was allowed to evaporate, and the residue was dried overnight over P_2O_5 and concentrated H_2SO_4. The residue was desalted on IRC-50 resin to yield 0.076 g. of the crude product. Purification was achieved by means of chromatography on carboxymethylcellulose using gradient elution with ammonium acetate under conditions identical with those described in Figure 5. The free octadecapeptide XV was obtained in 44% yield (0.043 g.) and found homogeneous in paper electrophoresis (pH 3.7, 400 volts, 6 hr., mobility relative to lysine 0.93). The amino acid composition of an acid hydrolysate of XV is given in Table I and is seen to agree well with the theoretical values; $[\alpha]^{25}D$ −92.7° (*c* 0.1, 0.1 *N* acetic acid).

III. Synthesis of the Nonadecapeptide Amide and Intermediates. N^{α}-Carbobenzoxy-N^{G}-tosylarginyl-N^{G}-tosylarginylproline (XVI). N^{α}-Carbobenzoxy-N^{G}-tosylarginyl-N^{G}-tosylarginylproline *t*-butyl ester[9] (3.8 g., 4.1 mmoles) was dissolved in 20 cc. of trifluoroacetic acid and stirred at room temperature for 1.5 hr. The solvent was subsequently removed *in vacuo*, and the residue was dried overnight over P_2O_5 and NaOH. Peptide XVI was isolated by precipitation from methanol–ether in 98% yield (3.5 g.) and found to be homogeneous in paper chromatography; R_{fBAW} 0.88; R_{fSBA} 0.26; m.p. 142–146°; $[\alpha]^{25}D$ −23.7° (*c* 0.8, methanol).

Anal. Calcd. for $C_{39}H_5N_9O_{10}S_2$ (870): C, 53.8; H, 5.91; N, 14.5. Found: C, 53.7; H, 5.72; N, 13.8.

N^{α}-Carbobenzoxy-N^{G}-tosylarginyl-N^{G}-tosylarginylproline Amide (XVII). Peptide XVI (2.6 g., 3 mmoles) was dissolved in a mixture of 25 cc. of acetonitrile and 10 cc. of dimethylformamide and cooled to 0°. Triethylamine (0.42 cc., 3 mmoles) and NEPIS (0.762 g., 3 mmoles) were added and the mixture was stirred at 0° for 1 hr. Dry ammonia was passed into this mixture for 15 min. and the mixture was stirred for 30 hr. at room temperature. The solvent was removed *in vacuo*, and the residue was dissolved in a mixture of 1-butanol and wet ethyl acetate (1:1) and washed with water, 5% sodium bicarbonate, and water. The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The residue was dissolved in methanol and precipitated from ether to yield 2.5 g. (96%) of XVII; m.p. 125–130°; R_{fBAW} 0.79; R_{fSBA} 0.70; $[\alpha]^{25}D$ −25.2° (*c* 1, methanol).

Anal. Calcd. for $C_{39}H_{52}N_{10}O_9S_2$ (869): C, 53.9; H, 6.03; N, 16.2. Found: C, 53.4; H, 5.86; N, 16.1.

N^{α}-Carbobenzoxy-N^{ϵ}-t-butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginylproline Amide (XVIII). Peptide XVII (2.4 g., 2.76 mmoles) was dissolved in 60 cc. of methanol and decarbobenzoxylated by means of catalytic hydrogenolysis. N^{G}-Tosylarginyl-N^{G}-tosylarginylproline amide was isolated and purified by countercurrent distribution in the toluene system ($K = 5$). This tripeptide was found homogeneous in paper chromatography; R_{fBAW} 0.48; R_{fSBA} 0.40; yield, 1.9 g. (94%).

The decarbobenzoxylated tripeptide was dissolved in a mixture of 15 cc. of dimethylformamide and 20 cc. of acetonitrile and allowed to react with 1.5 g. (3 mmoles) of I at room temperature for 4 days. The solvent was removed *in vacuo*, and the residue was purified by means of countercurrent distribution in the carbon tetrachloride system (100 transfers). The desired tetrapeptide amide XVIII was isolated from the main peak with $K = 0.2$ in 71% yield (2 g); m.p. 120–126°; R_{fBAW} 0.86; R_{fSBA} 0.81; $[\alpha]^{25}D$ −26.2° (*c* 1, methanol).

Anal. Calcd. for $C_{50}H_{72}N_{12}O_{12}S_2$ (1097): C, 54.8; H, 6.62; N, 15.3. Found: C, 54.5; H, 6.42; N, 15.5.

N^{α}-Carbobenzoxy-N^{ϵ}-t-butyloxycarbonyllysyl-N^{ϵ}-t-butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginylproline Amide (XIX). Peptide XVIII (1.9 g., 1.73 mmoles) was dissolved in 50 cc. of methanol and the carbobenzoxy group was removed by means of catalytic hydrogenolysis. The product was isolated in homogeneous form, R_{fBAW} 0.64, yield, 1.64 g., and was dissolved in a mixture of 5 cc. of dimethylformamide and 20 cc. of acetonitrile and stirred with 1 g. (2 mmoles) of I for 3 days at room temperature and 2 days at 37°. The solvent was removed *in vacuo* and the residue was purified by means of countercurrent distribution in the carbon tetrachloride system (containing 0.01 *M* ammonium acetate instead of water) for 100 transfers. The main component ($K = 0.1$) was isolated by precipitation from methanol–ether in 89% yield (2 g.); m.p. 118–120°. Peptide XIX was found to be homogeneous in paper chromatography; R_{fBAW} 0.90; R_{fSBA} 0.89; $[\alpha]^{25}D$ −17.1° (*c* 1, methanol).

Anal. Calcd. for $C_{61}H_{92}N_{14}O_{15}S_2$ (1326): C, 55.3; H, 7.00; N, 14.8. Found: C, 55.1; H, 6.89; N, 14.5.

N^{ϵ}-t-Butyloxycarbonyllysyl-N^{ϵ}-t-butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginylproline Amide (XX). Peptide XIX (1.9 g., 143 mmoles) was dissolved in 40 cc. of methanol and the carbobenzoxy group was removed by means of catalytic hydrogenolysis. The product was submitted to countercurrent distribution in the toluene system (100 transfers). A single peak with $K = 2.7$ was observed. The material from this peak was isolated to yield 1.55 g. (91%) of XX; m.p. 120–124°; $[\alpha]^{25}D$ −21.9° (*c* 0.8, methanol); R_{fBAW} 0.79; R_{fSBA} 0.77.

Anal. Calcd. for $C_{53}H_{86}N_{14}O_{13}S_2$ (1192): C, 53.4; H, 7.24; N, 16.5. Found: C, 52.6; H, 7.29; N, 16.3.

N^{α}-Carbobenzoxy-N^{ϵ}-t-butyloxycarbonyllysylprolylvalylglycyl-N^{ϵ}-t-butyloxycarbonyllysyl-N^{ϵ}-t-butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginylproline Amide (XXI). Peptide[7] V (0.65 g., 1 mmole) was dissolved in 25 cc. of acetonitrile, cooled to 0°, and stirred with 0.14 cc. (1 mmole) of triethylamine and 0.254 g. (1 mmole) of NEPIS for 1 hr. at 0°. Peptide XX (1.192 g., 1 mmole), dissolved in 15 cc. of dimethylformamide, was added and the mixture was stirred at room temperature for 48 hr. The solvents were removed *in vacuo* and the residue was purified by countercurrent distribution in the toluene system (100 transfers). The desired nonapeptide amide XXI was isolated from the main peak, with $K = 1.0$, in 72% yield (1.3 g.). XXI was found homogeneous by paper chromatography (R_{fBAW} 0.91; R_{fSBA} 0.90) as well as by thin layer chromatography on silica gel (R_{fCM} 0.60); m.p. 128–134°; $[\alpha]^{25}D$ −44.6° (*c* 1, methanol).

Anal. Calcd. for $C_{84}H_{131}N_{19}O_{21}S_2$ (1807): C, 55.8; H, 7.31; N, 14.7. Found: C, 55.9; H, 7.16; N, 14.5.

N^{ϵ}-t-Butyloxycarbonyllysylprolylvalylglycyl-N^{ϵ}-t-butyloxycarbonyllysyl-N^{ϵ}-t-butyloxycarbonyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginylproline Amide (XXII). The protected nonapeptide amide XXI (1.2 g., 0.66 mmole) was dissolved in 40 cc. of methanol and submitted to catalytic hydrogenolysis. The product was isolated in the usual manner and submitted to countercurrent distribution in the toluene system for 100 transfers. A single peak with $K = 2.0$ was seen. The decarbobenzoxylated nonapeptide amide XII was isolated from this peak in 83% yield (0.92 g.). Peptide XXII readily crystallized upon trituration with ethyl acetate; m.p. 165–167°; $[\alpha]^{25}D$ −19.9° (*c* 1, dimethylformamide); R_{fBAW} 0.85; R_{fSBA} 0.87; thin layer chromatography, R_{fCM} 0.17.

Anal. Calcd. for $C_{76}H_{125}N_{19}O_{19}S_2$ (1673): C, 54.6; H, 7.53; N, 15.9. Found: C, 54.6; H, 7.33; N, 15.4.

Seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginylproline Amide (XXIII). Peptide XXII (0.167 g., 0.1 mmole) and 0.17 g. (0.1 mmole) of VIII[5] were dissolved in 2 cc. of dimethylformamide, and the solution was cooled to 0° and stirred with 0.023 g. of DCCI for 1 hr. The mixture was allowed to react for 6 days at 4°. Glacial acetic acid (0.5 cc.) was added and the solvent was removed *in vacuo*. The residue was submitted to countercurrent distribution in the carbon tetrachloride system (containing 0.01 *M* ammonium acetate instead of water) for 150 transfers. Tubes 0–13 were pooled and evaporated to dryness to yield 0.25 g. of material, which was dissolved in 15 cc. of anhydrous trifluoroacetic acid and kept at room temperature for 1.5 hr. The solvent was removed *in vacuo* and the residue was purified by means of countercurrent distribution in the BAW system for 120 transfers. The partially deblocked nonadecapeptide amide was isolated from the peak with $K = 2.0$; yield 0.15 g. (44%). This product was dissolved in 200 cc. of freshly distilled liquid ammonia and treated with small pieces of sodium until the blue color was maintained for 0.5 hr. Ammonia was allowed to evaporate and the residue was desalted as before on IRC-50 resin to yield 0.1 g. of crude nonadecapeptide amide. Further purification was achieved by means of chromatography on carboxymethylcellulose using ammonium acetate under conditions identical with those given in Figure 5. The nonadecapeptide amide XXIII was obtained in 20% yield (0.025 g.) after four lyophilizations. Peptide XXIII was found to be homogeneous in paper electrophoresis (pH 3.7, 400 volts, 6 hr., mobility relative to lysine 0.92) (Figure 6). The amino acid composition of an acid hydrolysate of XXIII was determined by the chromatographic method and is given in Table I. It is evident that the values found are in excellent agreement with the theoretical ones, $[\alpha]^{25}D$ −102.6° (*c* 0.5, 0.1 *N* acetic acid).

When the sequence of deblocking was reversed by treating 0.25 g. of the fully protected nonadecapeptide amide first with sodium in liquid ammonia and then with trifluoroacetic acid, 0.038 g. of XXIII was isolated following the usual purification.

Biological Assays. The adrenal-stimulating activity was measured *in vitro* by a modification of the Saffran and Schally procedure.[35] Quartered adrenals of 16 60-day-old rats of the Long–Evans strain were used for each assay. The corticosteroids were extracted into chloroform (Spectroquality) after the incubation. Chloroform was removed *in vacuo*, the residue was dissolved in 95% alcohol,[50] and the amount of corticoids was determined by ultraviolet absorption. A four-point design with quadruplicate observations was employed and the relative potency was computed by variance analysis[51] using the Second International Standard. Each peptide was assayed at least three times. The steroidogenic potency *in vivo* was measured[39] by the method of Lipscomb and Nelson.[36] The details of this procedure have been described.[52]

The melanocyte-stimulating activities were determined by the procedure described by Shizume, *et al.*,[41a] using isolated skins of *Rana pipiens*. *In vivo* activities were measured with hypophysectomized *Rana pipiens* (not more than 4 days after operation) as described by Hogben and Slome.[41b]

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The Synthesis of Selenocoenzyme A[1,2]

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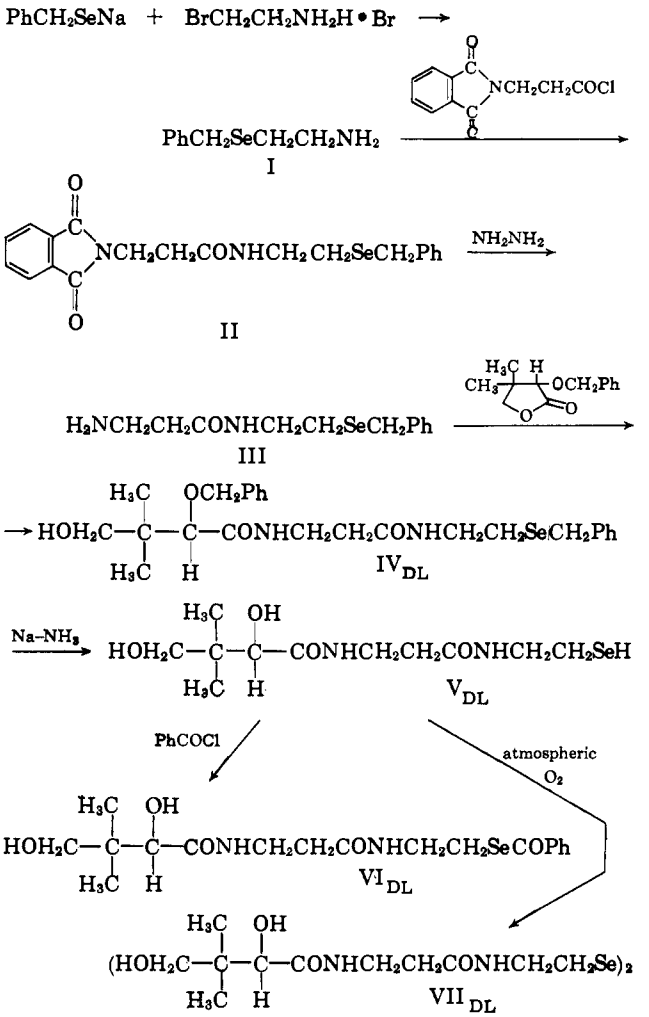
The syntheses of 4'-phosphoselenopantethine, 3'-dephosphoselenocoenzyme A, isoselenocoenzyme A, selenocoenzyme A, and related compounds are described.

The numerous roles played by coenzyme A in metabolic pathways have been the subject of intensive research since the discovery of the requirement for a cofactor for the acetylation of choline in extracts of rabbit brain[3] and the acetylation of sulfanilamide in extracts of pigeon liver.[4] The structure of this cofactor, named coenzyme A by Lipmann, was established in the laboratories of Lipmann,[5-7] Baddiley,[8] and Snell.[9-12] Lynen and his co-workers[13,14] succeeded in characterizing "active acetate" as the acetylthiol ester of coenzyme A, thus demonstrating the vital nature of the single sulfur atom of this molecule to the functions of coenzyme A either in transferring acyl groups to suitable acceptors or in activating a methylene group adjacent to the thioacyl carbon to condensation reactions.[15]

Since the atomic radii of sulfur and selenium are very similar,[16] replacement of the sulfur of coenzyme A with selenium should not affect appreciably either the size of this complex molecule or its ability to fit enzymic receptor sites. However, while acyl derivatives of coenzyme A or of selenocoenzyme A may be considered as being isosteric, their reactivities and electron distributions might be expected to differ. Thus, it has been shown by the use of model compounds that selenol esters will undergo aminolysis much more readily than thiol esters[17,18] although hydrolysis proceeds at similar rates.[2,19] While selenocoenzyme A should have an ability to fit receptor sites similar to that of its sulfur analog, the possibility exists that the strength of binding to receptor sites, or the ability to induce conformational changes in enzymes subsequent to binding, might be different for isologs of this type. In the hope of gaining additional information about

Scheme I



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