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Synthesis of a Biologically Active Heptadecapeptide Related to Adrenocorticotropin[1]

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The synthesis of a heptadecapeptide, L-seryl-L-tyrosyl-L-seryl-L-methionyl-L-glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycyl-L-lysyl-L-prolyl-L-valyl-glycyl-L-lysyl-L-lysyl-L-arginine, which has an amino acid sequence identical with the first 17 residues from the NH_2– terminus of adrenocorticotropin (ACTH), is described. The synthetic heptadecapeptide has been shown to possess low adrenal-stimulating potency but high melanocyte-stimulating and lipolytic activities.

In an attempt to define the structural features of adrenocorticotropin[2a] essential for eliciting the major biological activity of this hormone, namely, the stimulation of the adrenals, we first synthesized a nonadecapeptide[2b] corresponding to the first nineteen amino acid residues of this molecule. Subsequent syntheses of the glutamine analog[3] of the nonadecapeptide, a tricosapeptide,[4] an eicosapeptide amide,[5] and a tetracosapeptide,[6] all related to the N-terminal half of ACTH, substantiated the findings[2b] that the first nineteen residues of the molecule probably represent the minimal structural requirements for steroidogenesis. Hence, we directed our efforts to the synthesis of smaller peptides in order to confirm further these results. Another objective of this approach was the preparation of peptides possessing only one of the many biological activities[7] exhibited by ACTH to a high degree. In this connection we synthesized the heptadecapeptide seryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginine.[8,9] This paper describes in detail the synthesis of this peptide and several intermediates.

For the synthesis of the protected heptadecapeptide, a new, crystalline, protected decapeptide carbobenzoxy-seryl-tyrosyl-seryl-methionyl-γ-benzyl-glutamyl-histidyl-phenylalanyl-N^G-tosyl-arginyl-tryptophyl-glycine (III) was prepared. Crystalline carbobenzoxy-seryl-tyrosyl-seryl-methionine hydrazide[2b] (I) was prepared by a modified procedure using a mixture of dimethylformamide and methanol as the solvent for the conversion of the protected tetrapeptide ester to the hydrazide. I was converted to the azide and reacted with crystalline γ-benzyl-glutamyl-histidyl-phenylalanyl-N^G-tosyl-arginyl-tryptophyl-glycine ditrifluoroacetate[10] (II) to obtain crystalline III in 78% yield. Catalytic hydrogenolysis of III in the presence of freshly prepared palladium at 50° yielded seryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-N^G-tosyl-arginyl-tryptophyl-glycine (IV). Digestion of IV with leucineaminopeptidase released the constituent amino acids in the expected amount. These data, in conjunction with the fact that the intermediates involved in the synthesis of the decapeptide were purified at every stage by crystallization, indicated that the decapeptide possessed a high degree of chemical and optical homogeneity. In addition, III was submitted to countercurrent distribution in the toluene system and the carbon tetrachloride system and found to be homogeneous.

The scheme for the synthesis of the C-terminal heptapeptide derivative N^ϵ-tosyl-lysyl-prolyl-valyl-glycyl-N^ϵ-tosyl-lysyl-N^ϵ-tosyl-lysyl-N^G-tosyl-arginine benzyl ester (VII), is presented in Fig. 1. The N-terminal tetrapeptide sequence was synthesized by two different routes. The first approach has already been described.[2b] In the second route, stepwise synthesis by the *p*-nitrophenyl ester method[11] was utilized. In this connection the *p*-nitrophenyl ester of N^α-*t*-butyloxycarbonyl-N^ϵ-tosyl-lysine was prepared. Reaction of carbobenzoxy-valine *p*-nitrophenyl ester with glycine methyl ester gave the pure crystalline dipeptide derivative in 80% yield. This was hydrogenolyzed in the presence of freshly prepared palladium and allowed to react with carbobenzoxy-proline *p*-nitrophenyl ester. Carbobenzoxy-prolyl-valyl-glycine methyl ester was formed in 67% yield. This protected tripeptide ester melted at 111–112° when crystallized from dichloromethane–petroleum ether and was found analytically pure. The optical rotation was found to be $[\alpha]^{25}D$ −90.4° (*c* 1, methanol). Hofmann and co-workers[12] have reported a melting point of 125–127° for the same tripeptide prepared by the reaction of carbobenzoxy-proline with valyl-glycine methyl ester by the anhydride procedure. The optical rotation of their product was reported to be $[\alpha]^{24}D$ −89.7° (*c* 1.05, methanol). These investigators[12] had precipitated their product from methanol–ether. In view of this, the crystallization of carbobenzoxy-prolyl-valyl-glycine methyl ester with m.p. 111–112° from different solvents was attempted. Crystallization from methanol–water yielded a product with m.p. 132–133°, which gave the correct analysis required for the protected tripeptide ester. The optical rotation of the higher melting form was identical with that of the lower melting product. When the higher melting form was recrystallized from dichloromethane–petroleum ether, the product had m.p. of 109–111°. The paper chromatographic behavior of the two products in two solvent systems was identical. Hence, the difference in melting point must be attributed to dimorphism. Examination of the two products under a microscope fitted with a polarizer

(1) This work was supported in part by a grant (G-2907) from the National Institutes of Health of the United States Public Health Service.

(2) (a) C. H. Li, *Advan. Protein Chem.*, 11, 101 (1956); (b) 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).

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

(4) K. Hofmann, H. Yajima, T-Y. Liu, and N. Yanaihara, *J. Am. Chem. Soc.*, **84**, 4475 (1962).

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

(6) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **46**, 1550 (1963).

(7) C. H. Li, *Recent Progr. Hormone Research*, **18**, 1 (1962).

(8) C. H. Li, D. Chung, J. Ramachandran, and B. Gorup, *J. Am. Chem. Soc.*, **84**, 2460 (1962).

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

(10) C. H. Li, B. Gorup, D. Chung, and J. Ramachandran, *J. Org. Chem.*, **28**, 178 (1963).

(11) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

(12) K. Hofmann, E. Sturz, G. Spuhler, H. Yajima, and E. T. Schwartz, *ibid.*, **82**, 3727 (1960).

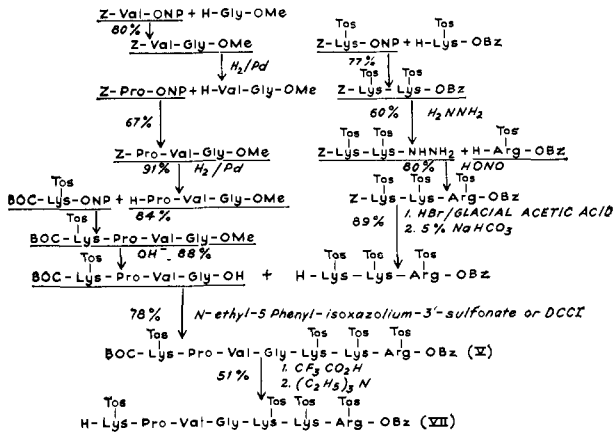

Fig. 1.—Synthetic scheme of the C-terminal heptapeptide derivative N^{ϵ}-tosyl-lysyl-prolyl-valyl-glycyl-N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginyl benzyl ester (VII): Z, carbobenzoxy; TOS, *p*-toluenesulfonyl; *t*-Bu, *t*-butyl; BOC, *t*-butyloxycarbonyl; ONP, *p*-nitrophenoxy.

revealed that the crystals of the higher melting form are needles while those of the lower melting form are platelets. Such dimorphism is not unusual and has been observed with carbobenzoxy-glycyl-phenylalanine *p*-nitrophenyl ester by Goodman and Steuben.[13]

N^{α}-*t*-Butyloxycarbonyl-N^{ϵ}-tosyl-lysyl-prolyl-valyl-glycine methyl ester was obtained in crystalline form in 84% yield from the reaction of N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-tosyl-lysine *p*-nitrophenyl ester with prolyl-valyl-glycine methyl ester. The crystalline protected tetrapeptide acid was prepared in 88% yield by saponification. This recrystallized tetrapeptide derivative was utilized in the synthesis of the heptapeptide.

The benzyl ester of N^{ϵ}-tosyl-lysine was prepared by esterification with benzyl alcohol and hydrogen chloride and allowed to react with N^{α}-carbobenzoxy-N^{ϵ}-tosyl-lysine *p*-nitrophenyl ester. The crystalline protected dipeptide benzyl ester was formed in 77% yield. This was converted to the crystalline hydrazide by treatment with hydrazine. N^{α}-carbobenzoxy-N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysine azide was prepared from the hydrazide and treated with N^{G}-tosyl-arginine benzyl ester in ethyl acetate solution. The protected tripeptide benzyl ester[14] was isolated in 80% yield from this reaction. Attempts to crystallize the peptide failed. The amorphous product was analytically pure and behaved as a homogeneous component in paper chromatography in two solvent systems and in countercurrent distribution in the toluene system. In order

(13) M. Goodman and K. C. Steuben, *J. Am. Chem. Soc.*, **84**, 1279 (1962).

(14) With the availability of N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-tosyl-lysine *p*-nitrophenyl ester, an attempt was made to prepare the tripeptide N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginine benzyl ester by the stepwise *p*-nitrophenyl ester method. Thus, reaction of N^{G}-tosyl-arginine benzyl ester with the *p*-nitrophenyl ester of N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-tosyl-lysine gave the protected dipeptide ester which was contaminated with *p*-nitrophenol. The *p*-nitrophenol could, however, be removed by dissolving the crude protected dipeptide in the lower phase of the toluene system and extracting with the upper phase until all the yellow color disappeared. The protected dipeptide ester was obtained in 72% yield. The *t*-butyloxycarbonyl group was removed by dissolving the peptide derivative in trifluoroacetic acid for a few minutes. The dipeptide trifluoroacetate was again allowed to react with N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-tosyl-lysine *p*-nitrophenyl ester in the presence of an equivalent of triethylamine. The protected tripeptide ester was contaminated with unreacted dipeptide base which could not be removed by washing with mild acid. Stronger acid could not be used because of the *t*-butyloxycarbonyl group. Moreover, neither the dipeptide nor the tripeptide derivatives could be crystallized. Hence this approach was abandoned.

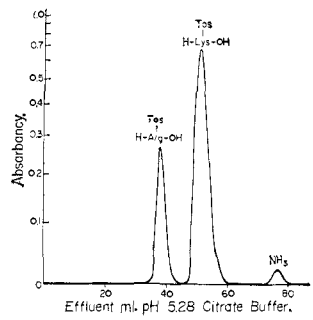

Fig. 2.—Chromatography of a leucine amino peptidase digest of N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginine on the short column of the automatic amino acid analyzer. The ordinate shows the absorbancy of the ninhydrin reaction product.

to establish the optical purity and to eliminate the possibility of contamination with Curtius rearrangement products,[15] the tripeptide was subjected to hydrogenolysis in the presence of freshly prepared palladium. N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginine was isolated and incubated with leucine aminopeptidase for 24 hr. in Tris buffer (pH 8.5), containing 50% methanol to dissolve the peptide. Paper chromatography of the digest along with authentic samples of the tripeptide, N^{ϵ}-tosyl-lysine, and N^{G}-tosyl-arginine, in 1-butanol–acetic acid–water (4:1:1) (BAW) revealed that the peptide was completely digested by the enzyme. The elution patterns and color yields with ninhydrin of N^{ϵ}-tosyl-lysine and N^{G}-tosyl-arginine in the Beckman-Spinco amino acid analyzer were also examined[16] in order to obtain quantitative information. It was found that from the short column of the analyzer (which is normally used for the separation of the basic amino acids) N^{G}-tosyl-arginine emerged first, closely followed by N^{ϵ}-tosyl-lysine using citrate buffer of pH 5.28 as the eluent. Both compounds emerge ahead of ammonia. Figure 2 represents the elution pattern of the leucineaminopeptidase digest of the tripeptide on the short column of the amino acid analyzer. This clean separation of the two tosylated derivatives enabled quantitative determination of the composition of the enzyme digest, which was found to be N^{ϵ}-tosyl-lysine:N^{G}-tosyl-arginine in a ratio of 2.03:1. The recovery of the amino acids was 97%. Thus, the optical purity and chemical homogeneity of the tripeptide was established.

The protected tripeptide benzyl ester was then treated with hydrogen bromide in glacial acetic acid for 20 min. and the hydrobromide of the tripeptide base benzyl ester was isolated by precipitation from ether. The free base N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginine benzyl ester was prepared from the hydrobromide by stirring with ice-cold 5% sodium bicarbonate solution and extracting with ethyl acetate. The tripeptide base isolated by precipitation from methanol–ether was found to be analytically pure and homogeneous in paper chromatography.

The tetrapeptide N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-tosyl-lysyl-prolyl-valyl-glycine was linked with N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginine benzyl ester by the use of either dicyclohexylcarbodiimide[17] (DCCI)

(15) E. Schnabel, *Ann.*, **659**, 168 (1962).

(16) J. Ramachandran, Dissertation, University of California, Berkeley, 1962.

(17) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

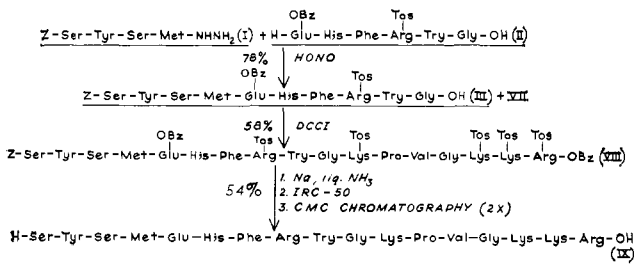

Fig. 3.—Synthetic scheme of the heptadecapeptide (α^{1-17}-ACTH).

or N-ethyl-5-phenylisoxazolium 3′-sulfonate.[18] The latter reagent has been used for combining carbobenzoxyamino acids with amino acid esters. It has not been used yet in the synthesis of long chain peptides. Since the activation of the carboxyl group of the tetrapeptide involved a glycine residue, there was no danger of racemization. Hence, the tetrapeptide was activated at 0° in acetonitrile according to Woodward, *et al.*,[18] and the tripeptide base benzyl ester was added. The reaction was allowed to proceed overnight and the protected heptapeptide ester (V) was isolated by the usual procedure of washing with acid and base. The protected heptapeptide ester, however, could not be crystallized. In order to establish the homogeneity of the peptide derivative, it was subjected to countercurrent distribution for 100 transfers in the toluene system. The peptide was located by alkaline hydrolysis of aliquots followed by reaction with ninhydrin. A major peak with $K = 0.21$, which closely followed the theoretical curve, and a small fast-moving component[19] with $K = 4.7$ were found. The material in the major peak was isolated and found by microanalysis to be the desired heptapeptide derivative. Paper chromatography in two solvent systems showed that the protected heptapeptide ester behaved as a homogeneous material. The heptapeptide derivative was obtained in 78% yield after countercurrent distribution.

The protected heptapeptide ester was treated with trifluoroacetic acid to remove the *t*-butyloxycarbonyl group. N^{ϵ}-Tosyl-lysyl-prolyl-valyl-glycyl-N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginine benzyl ester trifluoroacetate (VI) was isolated by precipitation from methanol-ether and found to be analytically pure and homogeneous in paper chromatography. Further, countercurrent distribution of this heptapeptide derivative for 100 transfers in the toluene system revealed a single peak with $K = 6$. There were no traces of material with $K = 0.21$, thus showing that the removal of the *t*-butyloxycarbonyl group was complete. The partially protected heptapeptide base VII was liberated from the trifluoroacetate by the addition of triethylamine; VII was found to be homogeneous by elemental analysis, paper chromatography in two solvents, and by countercurrent distribution in the toluene system ($K = 0.62$). Since the distribution coefficients of peptides V, VI, and VII in the toluene system are sufficiently different, countercurrent distribution has been very valuable in the purification of these intermediates. This has been especially advantageous in view of the fact that these peptide derivatives could not be obtained in crystalline form.

(18) R. B. Woodward, R. A. Olofson, and H. Mayer, *J. Am. Chem. Soc.*, **83**, 1010 (1961).

(19) The fast-moving component formed less than 15% of the crude material and could be identified with unreacted N^{α}-*t*-butoxycarbonyl-N^{ϵ}-tosyl-lysyl-prolyl-valyl-glycine by paper chromatography.

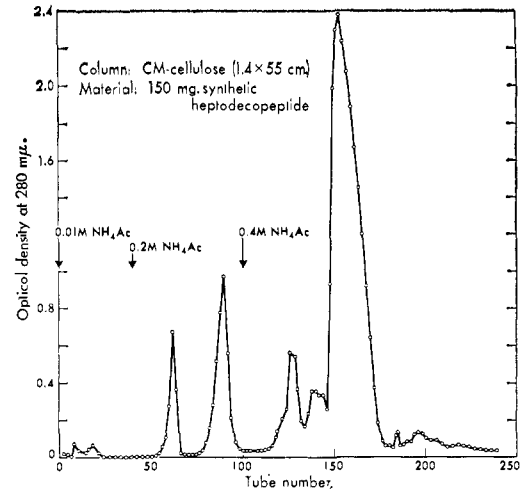

Fig. 4.—CMC chromatography of crude α^{1-17}-ACTH. The initial buffer was 0.01 *M* ammonium acetate of pH 4.5. After 3–4 hold-up volumes (4 cc./tube) had been collected, a gradient with respect to pH and concentration was started by introducing 0.2 *M* ammonium acetate buffer of pH 6.7 through a 500-ml. 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 synthesis of the protected heptadecapeptide (VIII) was achieved by treating III with VII using either dicyclohexylcarbodiimide[17] or N-ethyl-5-phenylisoxazolium 3′-sulfonate[18] (Fig. 3). The product was isolated by countercurrent distribution in the carbon tetrachloride system, followed by washing with methanol. The yields of VIII obtained by the use of the two reagents were comparable.

The removal of all the protecting groups, namely, one carbobenzoxy group, two benzyl groups, and five tosyl groups, was accomplished by treatment with sodium in liquid ammonia.[20] The free heptadecapeptide (IX) was desalted on an IRC-50 resin column[21] and chromatographed on carboxymethylcellulose (CMC)[22] by means of continuous gradient elution with ammonium acetate. The material in the major peak was rechromatographed on CMC. The results of typical experiments are seen in Fig. 4 and 5. The heptadecapeptide was obtained in 54% yield after rechromatography on CMC. Paper electrophoresis at pH 3.7, 7.0, and 11.0 showed that the heptadecapeptide migrated as a single component. The results of electrophoresis at pH 4.5 in polyacrylamide gel[23] are shown in Fig. 6.

The amino acid composition of an acid hydrolysate of the heptadecapeptide was determined by the chromatographic method[24] as well as by microbiological means.[25] These data are summarized in Table I. Tryptophan content was determined spectrophotometrically.[26]

In order to establish that all the protecting groups had been removed from this homogeneous preparation, enzymatic digestion was attempted. Digestion of the

(20) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937).

(21) H. B. F. Dixon and M. B. Stack-Dunne, *Biochem. J.*, **61**, 483 (1955).

(22) E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.*, **78**, 751 (1956).

(23) S. Raymond and L. Weintraub, *Science*, **130**, 711 (1959); R. A. Reisfeld, U. J. Lewis, and D. E. Williams, *Nature*, **195**, 281 (1962).

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

(25) The microbiological assay was carried out by the Shankman Laboratories, Los Angeles, Calif.

(26) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).

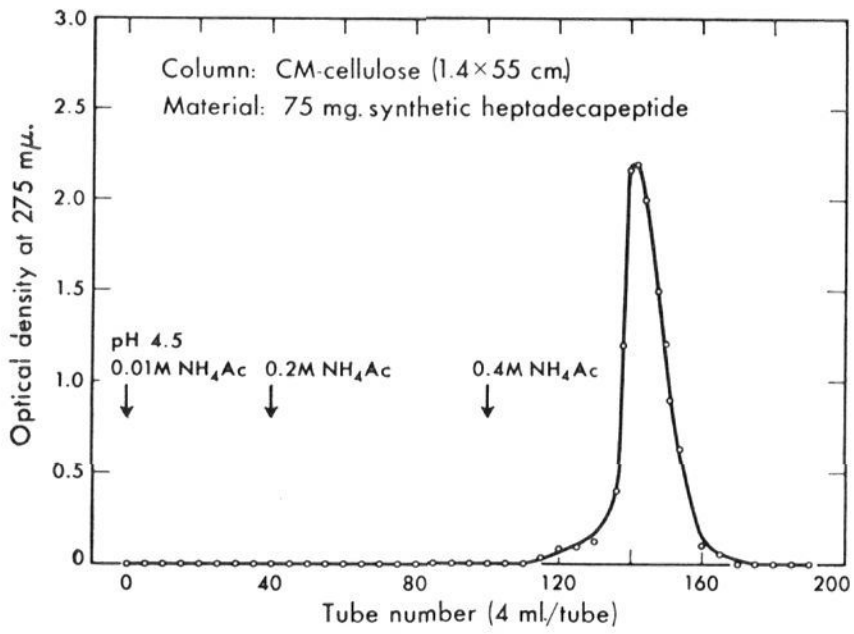

Fig. 5.—Rechromatography of purified α^{1-17}-ACTH on CMC; conditions as in Fig. 4.

heptadecapeptide with trypsin and chymotrypsin followed by leucineaminopeptidase gave the results shown in Table I. It is apparent that the pentapeptide lysyl-

TABLE I

AMINO ACID CONTENT OF THE SYNTHETIC HEPTADECAPEPTIDE

Amino acid	Theoretical	Acid hydrolysis: Chromatographic	Acid hydrolysis: Microbiological	Enzymic digest[a]
Serine	2	1.85	2.24	2.02
Tyrosine	1	0.97	0.96	1.05
Methionine	1	0.91	0.92	0.89
Glutamic acid	1	1.07	1.22	1.00
Histidine	1	1.04	0.90	1.20
Phenylalanine	1	1.00	1.01	0.99
Arginine	2	2.07	1.95	2.13
Tryptophan	1	1.00[b]	..	1.05
Glycine	2	2.10	2.00	0.78
Lysine	3	3.22	2.88	1.18
Proline	1	0.94	1.09	0.00
Valine	1	1.15	1.06	0.27

[a] Obtained from trypsin, chymotrypsin, and leucineaminopeptidase digestion and analyzed chromatographically.[24] [b] Determined spectrophotometrically.

prolyl-valyl-glycyl-lysine is not attacked by these enzymes. The resistance of the arginyl-proline bond to attack by trypsin or leucineaminopeptidase has been reported[27] previously. To show that the lysyl-proline bond is also resistant to LAP, the tetrapeptide N^{ϵ}-tosyl-lysyl-prolyl-valyl-glycine was prepared by hydrogenolysis of N^{α}-carbobenzoxy-N^{ϵ}-tosyl-lysyl-prolyl-valyl-glycine in the presence of palladium. A 24-hr. digest of the tetrapeptide with one preparation of LAP (Worthington lot no. 5913A) gave the ratio of amino acids N^{ϵ}-tosyl-lysine:proline:valine:glycine = 0.8:0.75:1.0:1.0. Although these results indicated considerable splitting of the lysyl-proline bond, the higher amounts of valine and glycine indicated the presence of some enzyme with carboxypeptidase-like activity.[28] Hence, another preparation (Worthington lot no. 5917) was tried. This enzyme preparation was found to be fully effective in digesting the tripeptide N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginine completely in 24 hr. However, when the tetrapeptide was incubated with this preparation, no traces of proline could be seen

(27) J. Meienhofer and C. H. Li, *J. Am. Chem. Soc.*, **84**, 2434 (1962).

(28) This suspicion was confirmed by other experiments (B. T. Pickering, unpublished) with ACTH in which considerable amounts of phenylalanine from the C-terminal were released.

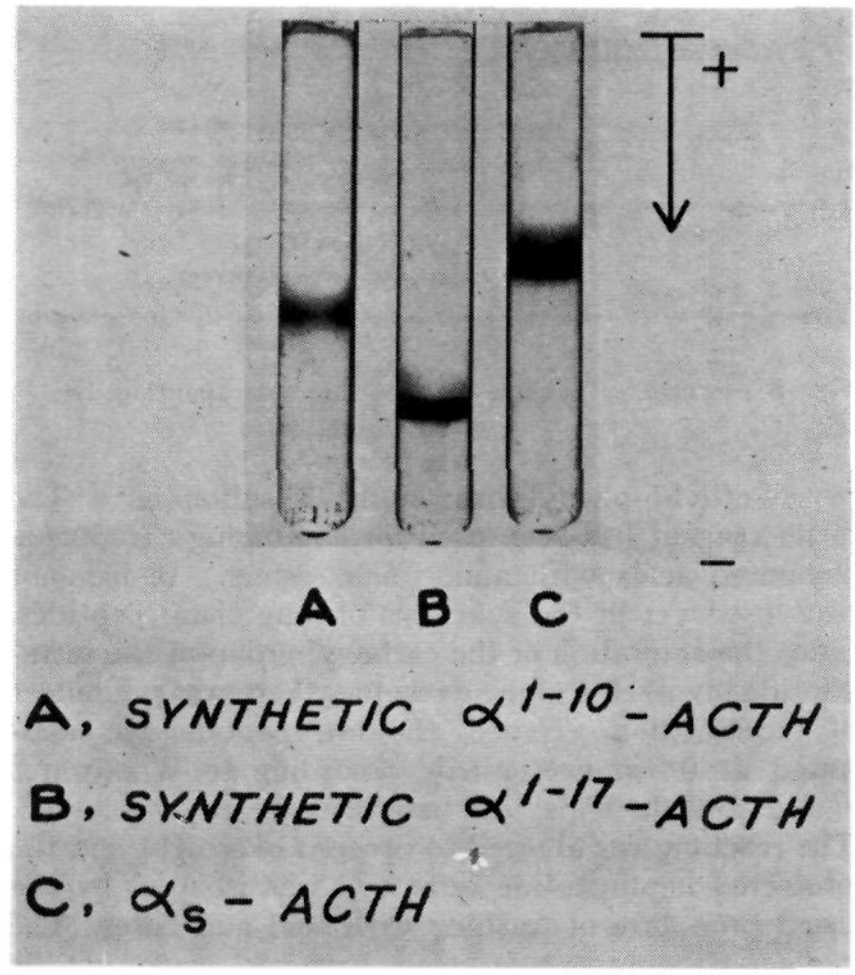

Fig. 6.—Disk electrophoresis of α^{1-17}-ACTH on polyacrylamide gel (0.8 × 6.5 cm.) at pH 4.5, 220 v., for 30 min.

on the chromatograms of the digest after 24 hr. After prolonged digestion (96 hr. at 37°) using one-fourth by weight of the enzyme as the substrate, small amounts of the constituent amino acids were seen, but a considerable amount of the tetrapeptide remained undigested. Apparently, the second preparation was relatively free of contaminating enzymes. The results of the enzymic digestion (Table I) showed that the tosyl groups had been completely removed from the arginine side chains. That the ϵ-amino groups of the lysine residues were also free in the synthetic heptadecapeptide was established by determining the alkali uptake in the pH stat during dinitrophenylation.[29] The results showed that the theoretical quantity of alkali was utilized.

The steroidogenic activities of IX as determined by assays *in vitro*[30] and *in vivo*[31] are, respectively: 6.4 and 1.7 I.U. per mg. In comparison (on the weight basis) with the nonadecapeptide,[2] IX exhibits a low degree of potency in stimulating the adrenals. This shows that the integrity of the basic amino acid core lysyl-lysyl-arginyl-arginyl-proline is probably important for the manifestation of high adrenal-stimulating activity.

The *in vitro* lipolytic activity of the synthetic heptadecapeptide has been found to be higher than that of the natural hormone when rabbit adipose tissues were used.[32] By *in vitro* frog skin assay,[33] the melanocyte-stimulating activity of IX was found to be 2×10^8 units/g. A detailed comparison of all the biological activities of the synthetic peptides prepared in our laboratory will be published elsewhere.

(29) We wish to thank Dr. G. Samuelsson of this laboratory for these results.

(30) M. Saffran and A. V. Schally, *Endocrinology*, **56**, 523 (1955).

(31) H. Lipscomb and D. H. Nelson, *ibid.*, **71**, 13 (1962).

(32) A. Tanaka, B. T. Pickering, and C. H. Li, *Arch. Biochem. Biophys.*, **99**, 294 (1962).

(33) K. Shizume, A. B. Lerner, and T. B. Fitzpatrick, *Endocrinology*, **54**, 553 (1954).

The data presented above show that this biologically active heptadecapeptide is homogeneous by several criteria and can be obtained in good yields. The use of the tosyl group for masking basic side chain functions in the synthesis of ACTH peptides has been criticized recently[6,34] since treatment with sodium in liquid ammonia is required for the removal of the tosyl groups. In the present work the removal of eight protecting groups from peptide VIII by treatment with sodium in liquid ammonia and purification by two chromatographic runs on CMC resulted in a 54% yield of free heptadecapeptide which has been found homogeneous by several criteria. Similar results were obtained in the removal of tosyl and other protecting groups from the nonadecapeptide[35] and pentadecapeptide.[36] Hence, it is apparent that peptide chain fragmentation[34] resulting from this treatment, if any, must be minor. The presence of methionine in the heptadecapeptide in the expected amount shows that cleavage of the thioether link of methionine by sodium in liquid ammonia cannot be a major hazard under the conditions employed here. There is abundant evidence in the literature[37-40] to show that protecting groups can be removed from methionine-containing peptides to give products in high yield by the use of sodium in liquid ammonia, if undue excess of sodium is avoided.

Experimental[41]

Carbobenzoxy-valyl-glycine Methyl Ester.—Carbobenzoxy-valine *p*-nitrophenyl ester[45] was prepared in 90% yield by the DCCI procedure[17,46] (m.p. 66°, $[\alpha]^{25}D$ −70° (*c* 1.5, methanol)). The nitrophenyl ester (26.04 g., 70 mmoles) was added to an ice-cold mixture of glycine methyl ester hydrochloride (12 g., 96 mmoles) and triethylamine (14 cc., 100 mmoles) in 120 cc. of ethyl acetate. The mixture was stirred at room temperature for 48 hr. The protected dipeptide crystallized from the reaction mixture. The crystals were filtered, washed with water and ether, and dried to yield 17 g. of dipeptide derivative, m.p. 157–159°. The combined ethyl acetate and ether filtrates were concentrated *in vacuo*, the residue was dissolved in hot methanol, and the solution was allowed to crystallize, yielding an additional 3 g. of the product, m.p. 155–156°, total yield 89%. Recrystallization of the combined product from hot methanol gave 18 g. (80%) of carbobenzoxy-valyl-glycine methyl ester, m.p. 159–160°, $[\alpha]^{25}D$ −25.5° (*c* 1, methanol); lit.[12] m.p. 160–161°, $[\alpha]^{27}D$ −24.3° (*c* 1.6, absolute ethanol), $[\alpha]^{24}D$ −30.0° (*c* 1.85, methanol). The dipeptide derivative was found to be homogeneous in paper chromatography in two solvent systems: $R_{f\ BAW}$ 0.87, $R_{f\ SBA}$ 0.85.

Carbobenzoxy-propyl-valyl-glycine Methyl Ester.—Carbobenzoxy-valyl-glycine methyl ester (1.61 g., 5 mmoles) was dissolved in 20 cc. of methanol and hydrogenolyzed in the presence of freshly prepared Pd. The catalyst was filtered and washed with methanol. The filtrate and washings were evaporated to dryness *in vacuo* at 20°. The residue was dissolved in 25 cc. of ethyl acetate, and 1.85 g. of carbobenzoxy-proline *p*-nitrophenyl ester[11] was added. A transparent, gelatinous mass was formed after 2 days. Ethyl acetate, 20 cc., was added to dissolve the material, and the solution was washed with water, 5% sodium bicarbonate, and water. The organic layer was dried over anhydrous sodium sulfate. The solvent was then removed and the residue was crystallized from methanol–water to give 1.4 g. (67%) of protected tripeptide ester, m.p. 130–132°, $[\alpha]^{25}D$ −90° (*c* 1, methanol). A sample was recrystallized from methanol, for analysis; m.p. 132–133°, $[\alpha]^{25}D$ −91° (*c* 1, methanol); lit.[12] m.p. 125–127°, $[\alpha]^{24}D$ −89.7° (*c* 1.05, methanol).

Anal. Calcd. for $C_{21}H_{29}N_3O_6$ (419.5): C, 60.1; H, 6.97; N, 10.0. Found: C, 60.0; H, 6.84; N, 10.1.

The tripeptide derivative was found to be homogeneous in paper chromatography in two solvents: $R_{f\ BAW}$ 0.87, $R_{f\ SBA}$ 0.87.

Prolyl-valyl-glycine Methyl Ester.—Carbobenzoxy-prolyl-valyl-glycine methyl ester (4.2 g., 10 mmoles) was dissolved in 80 cc. of methanol and hydrogenolyzed in the presence of Pd until evolution of CO_2 ceased. The catalyst was filtered and washed with methanol. The filtrate and washings were evaporated to dryness *in vacuo*. A crystalline residue was obtained; yield 2.6 g. (91%), m.p. 143–144°, $[\alpha]^{25}D$ −76° (*c* 1, methanol).

The tripeptide base was found to be homogeneous in two solvents in paper chromatography. It gave a yellow color with ninhydrin and a blue color in the isatin test; $R_{f\ BAW}$ 0.56, $R_{f\ SBA}$ 0.66.

Anal. Calcd. for $C_{18}H_{28}N_4O_4$ (285.3): C, 54.7; H, 8.13; N, 14.7. Found: C, 55.0; H, 8.29; N, 14.5.

N^{α}-*t*-Butyloxycarbonyl-N^{ϵ}-tosyl-lysine *p*-Nitrophenyl Ester.—N^{ϵ}-tosyl-lysine[47] (3.01 g., 10 mmoles) and MgO (0.8 g., 20 mmoles) were mixed in a mortar and stirred for 1 hr. at 45–50° in 40 cc. of 50% aqueous dioxane. *t*-Butyloxycarbonyl azide[48,49] (3 g., 21 mmoles) was added and the mixture was stirred at 45–50° for 36 hr. It was then poured into 150 cc. of cold water, and the solution was filtered free of any insoluble matter and extracted with ethyl acetate (2 × 50 cc.). The aqueous extract was cooled in ice and brought to pH 4 with ice-cold 10% citric acid. The solution was then saturated with NaCl and extracted with ethyl acetate (4 × 80 cc.). The organic phase was washed with saturated NaCl and dried. The solvent was removed *in vacuo* to give N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-tosyl-lysine, 4 g., in the form of an oil. This material was ninhydrin-negative and was found to be homogeneous in paper chromatography in two solvents: $R_{f\ BAW}$ 0.76, $R_{f\ SBA}$ 0.50.

This oil was dissolved in 25 cc. of ethyl acetate, and the solution was cooled in ice and then stirred with 1.39 g. of *p*-nitrophenol and 2.06 g. of dicyclohexylcarbodiimide.[17,46] The mixture was stirred at 5° for 24 hr.; dicyclohexylurea was then removed by filtration and washed with ethyl acetate. The filtrate and washings were acidified with 10 drops of glacial acetic acid and kept at 5° for 2 hr., and was then filtered and the filtrate evaporated to dryness. The residue was crystallized from ethyl acetate–petroleum ether to give 3.2 g. (61.2%) of N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-tosyl-lysine *p*-nitrophenyl ester, m.p. 110–112°. A sample was recrystallized from ethyl acetate–petroleum ether for analysis; m.p. 113–114°, $[\alpha]^{25}D$ −22.5° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{24}H_{31}N_3O_7S$ (521.6): C, 55.3; H, 5.99; N, 8.06; S, 6.15. Found: C, 55.0; H, 6.19; N, 7.96; S, 6.01.

N^{α}-*t*-Butyloxycarbonyl-N^{ϵ}-tosyl-lysyl-prolyl-valyl-glycine Methyl Ester.—Prolyl-valyl-glycine methyl ester (1.42 g., 5 mmoles) was dissolved in 100 cc. of ethyl acetate with 2.61 g. of N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-tosyl-lysine *p*-nitrophenyl ester (5

(34) K. Hofmann and H. Yajima, *J. Am. Chem. Soc.*, **83**, 2289 (1961).
(35) C. H. Li, D. Chung, and J. Ramachandran, *ibid.*, **86**, 2715 (1964).
(36) C. H. Li, J. Ramachandran and D. Chung, *ibid.*, **86**, 2711 (1964).
(37) C. M. Stevens and R. Watanabe, *ibid.*, **72**, 725 (1950).
(38) K. Hofmann, A. Jöhl, A. E. Furlenmeier, and H. Kappeler, *ibid.*, **79**, 1636 (1957).
(39) H. B. Milne and C-H. Peng, *ibid.*, **79**, 639 (1957).
(40) K. Hofmann, T. A. Thompson, H. Yajima, E. T. Schwartz, and H. Inouye, *ibid.*, **82**, 3715 (1960).
(41) 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. pressure. 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, 3% ammonia–2-butanol (ASB) in a ratio of 44:100, and 1-butanol–pyridine–acetic acid–water (BPAW) in a ratio of 30:20:6:24, all by volume. Peptide spots were located by the ninhydrin reagent, Pauly reagent[42] and the Ehrlich reagent,[43] and by the chlorine method.[44] For countercurrent distribution (CCD), the following solvent systems were employed: toluene system: toluene–chloroform–methanol–water (5:5:8:2) by volume; carbon tetrachloride system: carbon tetrachloride–chloroform–methanol–0.01 *M* ammonium acetate (1:3:3:1) by volume.
(42) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).
(43) J. Smith, *Nature*, **171**, 43 (1953).
(44) H. Zahn and E. Rexroth, *Z. Anal. Chem.*, **148**, 181 (1955).
(45) B. Iselin, W. Rittel, P. Sieber, and R. Schwyzer, *Helv. Chim. Acta*, **40**, 373 (1957).
(46) M. Rothe and F. W. Kunitz, *Ann.*, **609**, 88 (1957).
(47) R. Roeske, F. H. C. Stewart, R. J. Stedman, and V. du Vigneaud, *J. Am. Chem. Soc.*, **78**, 5803 (1956).
(48) L. A. Carpino, *ibid.*, **79**, 98, 4427 (1957); **81**, 955 (1959); **82**, 2725 (1960).
(49) R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).

mmoles). The reaction mixture was kept 3 days at room temperature and then evaporated to dryness *in vacuo*. The residue was redissolved in 60 cc. of ethyl acetate, washed, successively, with ice-cold 1% citric acid, water, 5% sodium bicarbonate, and water, and dried over anhydrous sodium sulfate. This solution was concentrated and the tetrapeptide derivative was crystallized from ethyl acetate–petroleum ether; m.p. 106–108°, yield 2.8 g. (84%). A sample was recrystallized from ethyl acetate–petroleum ether; m.p. 109–110°, $[\alpha]^{25}D$ −73.6° (*c* 1, methanol), $R_{f\ BAW}$ 0.91, $R_{f\ SBA}$ 0.88.

Anal. Calcd. for $C_{31}H_{49}N_5O_9S$ (667.8): C, 55.8; H, 7.40; N, 10.5; S, 4.50. Found: C, 55.7; H, 7.37; N, 10.6; S, 4.46.

N$^{\alpha}$-*t*-Butyloxycarbonyl-N$^{\epsilon}$-tosyl-lysyl-prolyl-valyl-glycine.—The protected tetrapeptide ester described above (4.55 g., 6.8 mmoles) was dissolved in 60 cc. of acetone by warming and the solution was then cooled in ice with stirring. The solution became cloudy, but cleared with the addition of 15 cc. of 1 *N* NaOH. The mixture was stirred at room temperature for 1 hr. and then diluted with 300 cc. of cold water. The clear solution was cooled in ice and brought to pH 3 with ice-cold 10% citric acid. The solution was saturated with NaCl and extracted with ethyl acetate (3 × 60 cc.). The ethyl acetate layer was washed with a solution of 10% NaCl until neutral, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was crystallized from ethyl acetate to give 3.9 g. (87.6%) of protected tetrapeptide acid, m.p. 104–106°. A sample was recrystallized from ethyl acetate; m.p. 104–106°, $[\alpha]^{25}D$ −68.3° (*c* 1, methanol), $R_{f\ BAW}$ 0.91, $R_{f\ SBA}$ 0.37.

Anal. Calcd. for $C_{30}H_{47}N_5O_9S$ (653.8): C, 55.1; H, 7.25; N, 10.7; S, 4.91. Found: C, 54.9; H, 7.21; N, 10.6; S, 4.72.

N$^{\epsilon}$-Tosyl-lysyl-prolyl-valyl-glycine.—N$^{\alpha}$-Carbobenzoxy-N$^{\epsilon}$-tosyl-lysyl-prolyl-valyl-glycine[2b] (1.03 g., 1.5 mmoles) was dissolved in 60 cc. of methanol and hydrogenolyzed in the presence of Pd freshly prepared from 1 g. of $PdCl_2$ until evolution of CO_2 ceased. The catalyst was filtered and washed with methanol. The filtrate and washings were evaporated to dryness to give N$^{\epsilon}$-tosyl-lysyl-prolyl-valyl-glycine, 0.75 g. (90.3%), m.p. 135–138°, $[\alpha]^{25}D$ −54° (*c* 0.5, methanol). The peptide was found to be homogeneous in paper chromatography in four solvents: $R_{f\ BAW}$ 0.59, $R_{f\ SBA}$ 0.09, $R_{f\ BPAW}$ 0.71, $R_{f\ ASB}$ 0.48.

Anal. Calcd. for $C_{25}H_{39}N_5O_7S$ (553.7): C, 54.2; H, 7.12: N, 12.7. Found: C, 53.6; H, 7.19; N, 12.8.

N$^{\epsilon}$-Tosyl-lysine-Benzyl Ester Hydrochloride.—N$^{\epsilon}$-Tosyl-lysine[47] (6.93 g., 23 mmoles) was dissolved in benzyl alcohol, 80 cc., saturated with hydrogen chloride. The mixture was stirred and fresh hydrogen chloride was passed into the solution after 12 hr. Crystals appeared after 48 hr., and paper chromatography in BAW indicated that esterification was practically complete. The mixture was poured into 500 cc. of ether, and white needles (9.8 g.) were collected by filtration; m.p. 178–179°. Recrystallization from methanol–ether yielded 8 g. (83%) of N$^{\epsilon}$-tosyl-lysine benzyl ester hydrochloride, m.p. 180°, $[\alpha]^{25}D$ −2.1° (*c* 2, methanol), $R_{f\ BAW}$ 0.76, $R_{f\ SBA}$ 0.82.

Anal. Calcd. for $C_{20}H_{25}N_2O_4SCl$ (428): C, 56.1; H, 6.59; N, 6.55. Found: C, 55.8; H, 6.45; N, 6.95.

N$^{\alpha}$-Carbobenzoxy-N$^{\epsilon}$-tosyl-lysyl-N$^{\epsilon}$-tosyl-lysine Benzyl Ester.—N$^{\alpha}$-Carbobenzoxy-N$^{\epsilon}$-tosyl-lysine *p*-nitrophenyl ester[50] (m.p. 110°) (2.78 g., 5 mmoles) was added to a solution of N$^{\epsilon}$-tosyl-lysine benzyl ester, prepared by stirring finely powdered N$^{\epsilon}$-tosyl-lysine benzyl ester hydrochloride (2.14 g., 5 mmoles) with 0.7 cc. of triethylamine (5 mmoles) in ethyl acetate (30 cc.), at 0° for 30 min., filtering, and washing with 15 cc. of ethyl acetate. The mixture was stirred at room temperature for 4 days. The protected dipeptide ester started to crystallize after 8 hr. Ether (50 cc.) was added and the crystals were filtered and washed with ether; yield 3.1 g. (77%), m.p. 112–114°. A sample was recrystallized from ethyl acetate; m.p. 116°, $[\alpha]^{25}D$ −7.5° (*c* 1, dimethylformamide), $R_{f\ BAW}$ 0.89, $R_{f\ SBA}$ 0.88.

Anal. Calcd. for $C_{41}H_{50}N_4O_9S_2$ (807): C, 61.0; H, 6.24; N, 6.94; S, 7.95. Found: C, 60.7; H, 6.17; N, 7.11; S, 7.81.

N$^{\alpha}$-Carbobenzoxy-N$^{\epsilon}$-tosyl-lysyl-N$^{\epsilon}$-tosyl-lysine Hydrazide.—The protected dipeptide ester described above (6.05 g., 7.5 mmoles) was dissolved in warm methanol, 50 cc., and treated with hydrazine, 0.5 cc., for 24 hr. The solvent was removed *in vacuo* and the residue was crystallized from ethyl acetate to give 3.3 g. (60%) of the protected dipeptide hydrazide, m.p. 143–144°. Recrystallization from ethyl acetate yielded a product melting at 145–146°, $[\alpha]^{25}D$ −7.2° (*c* 2, acetic acid).

Anal. Calcd. for $C_{36}H_{46}N_6O_8S_2$ (730.9): C, 55.9; H, 6.34; N, 11.5. Found: C, 56.2; H, 6.39; N, 11.8.

N$^{\alpha}$-Carbobenzoxy-N$^{\epsilon}$-tosyl-lysyl-N$^{\epsilon}$-tosyl-lysyl-N^{G}-tosyl-arginine Benzyl Ester.—N$^{\alpha}$-Carbobenzoxy-N$^{\epsilon}$-tosyl-lysyl-N$^{\epsilon}$-tosyl-lysine hydrazide (3.66 g., 5 mmoles) was dissolved in a mixture consisting of 20 cc. of 1 *N* HCl and 15 cc. of glacial acetic acid containing a few drops of ethyl acetate. This solution was cooled to −2° and 0.42 g. of sodium nitrite (6 mmoles) was added in small portions while stirring vigorously with a Vibromixer.[51] The mixture was stirred at 0° for 30 min. more, and diluted with 50 cc. of ice water. Further operations were carried out in the cold room with reagents and glassware that had been kept at 0° for at least 2 hr. The azide was extracted into 60 cc. of ethyl acetate, and the extract was washed with water. The organic phase was washed with 5% sodium bicarbonate until neutral, then with water and with saturated sodium chloride solution. The ethyl acetate extract was dried over anhydrous sodium sulfate and added to a suspension of 2.1 g. of N^{G}-tosyl-arginine benzyl ester[52] (5 mmoles) in 20 cc. of ethyl acetate at 0°. The mixture was stirred in the cold room for 3 days. The ester dissolved completely after 12 hr. The reaction mixture was allowed to warm up to room temperature, and was evaporated to dryness *in vacuo*. The residue was redissolved in 80 cc. of ethyl acetate and washed with 1 *N* HCl until the ethyl acetate layer no longer gave any reaction with ninhydrin. The organic phase was then washed with water, 5% sodium bicarbonate, and water, and was then dried over anhydrous sodium sulfate. The solvent was removed *in vacuo* and the residue was redissolved in a small volume (20 cc.) of ethyl acetate. The protected tripeptide ester was precipitated from ether (300 cc.), filtered, and dried; yield 4.5 g. (80%), m.p. 90–95°, $[\alpha]^{25}D$ −13.3° (*c* 2, methanol), $R_{f\ BAW}$ 0.89, $R_{f\ SBA}$ 0.88. Countercurrent distribution in the toluene system for 40 transfers showed a single peak with K = 0.29.

Anal. Calcd. for $C_{64}H_{68}N_8O_{12}S_3$ (1117.3): C, 58.1; H, 6.14; N, 10.0; S, 8.61. Found: C, 57.9; H, 6.22; N, 10.1; S, 8.52.

N$^{\epsilon}$-Tosyl-lysyl-N$^{\epsilon}$-tosyl-lysyl-N^{G}-tosyl-arginine Benzyl Ester.—The protected tripeptide ester described above (4.5 g., 4 mmoles) was dissolved in 5 cc. of glacial acetic acid and stirred vigorously with 15 cc. of 4 *N* HBr in glacial acetic acid (60 mmoles) for 20 min. The tripeptide ester hydrobromide was precipitated by the addition of 250 cc. of dry ether. The supernatant fluid was decanted off and the precipitate was washed twice with fresh ether. The residue was dried overnight over P_2O_5 and NaOH *in vacuo*. It was then suspended in ethyl acetate (60 cc.) and stirred with 50 cc. of cold 5% sodium bicarbonate for 30 min. The organic phase was separated and the aqueous layer was extracted with fresh ethyl acetate (3 × 40 cc.). The combined ethyl acetate extracts were washed with fresh 5% sodium bicarbonate and water, and dried over anhydrous sodium sulfate. Ethyl acetate was then removed *in vacuo*, the residue was dissolved in 15 cc. of methanol, and the solution precipitated into 300 cc. of anhydrous ether. The precipitate was filtered and dried to give 3.5 g. (89%) of the tripeptide derivative, N$^{\epsilon}$-tosyl-lysyl-N$^{\epsilon}$-tosyl-lysyl-N^{G}-tosyl-arginine benzyl ester, m.p. 82–86°, $[\alpha]^{25}D$ −4° (*c* 1, dimethylformamide), $[\alpha]^{25}D$ −4.3° (*c* 2, methanol), $R_{f\ BAW}$ 0.80, $R_{f\ SBA}$ 0.82.

Anal. Calcd. for $C_{46}H_{62}N_8O_{10}S_3$ (983.2): C, 56.2; H, 6.36; N, 11.4. Found: C, 56.5; H, 6.42; N, 11.5.

N$^{\alpha}$-*t*-Butyloxycarbonyl-N$^{\epsilon}$-tosyl-lysyl-N^{G}-tosyl-arginine Benzyl Ester.—N$^{\alpha}$-*t*-Butyloxycarbonyl-N$^{\epsilon}$-tosyl-lysine *p*-nitrophenyl ester (2.61 g., 5 mmoles) was added to a solution of N^{G}-tosyl-arginine benzyl ester[52] (2.1 g., 5 mmoles) in ethyl acetate, 60 cc., and stirred at room temperature for 2 days. The reaction mixture was washed successively with ice-cold 1% citric acid (3 × 40 cc.), water (2 × 30 cc.), 5% sodium bicarbonate (6 × 30 cc.), and water (2 × 30 cc.). The organic layer was dried over anhydrous sodium sulfate concentrated to a small volume and precipitated from petroleum ether (b.p. 30–60°) to give 3.2 g. of faintly yellow material. Paper chromatography in SBA revealed the presence of *p*-nitrophenol in addition to the protected peptide ester. The impure peptide derivative was dissolved

(50) M. Bodanszky, J. Meienhofer, and V. du Vigneaud, *J. Am. Chem. Soc.*, **82**, 3195 (1960).

(51) Vibro-mixer, A. G. Fuer Chemie-Apparatebau, Zurich, Model E1.

(52) J. Ramachandran and C. H. Li, *J. Org. Chem.*, **27**, 4006 (1962).

in the lower phase of the toluene system and extracted with the upper phase until the yellow color disappeared. The lower phase was then evaporated to dryness; the residue was dissolved in ethyl acetate and precipitated into petroleum ether to give 2.9 g. (72%) of the protected dipeptide ester which was homogeneous in two solvents in paper chromatography; $R_{f\ BAW}$ 0.87, $R_{f\ SBA}$ 0.88, m.p. 75–85°, $[\alpha]^{25}D$ −10.9° (*c* 4, methanol).

Anal. Calcd. for $C_{38}H_{52}N_6O_9S_2$ (800.9): C, 57.0; H, 6.54; N, 10.5; S, 7.99. Found: C, 56.8; H, 6.31; N, 10.5; S, 8.19.

N^{ϵ}-Tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginine.—N^{α}-Carbobenzoxy-N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginine benzyl ester (0.67 g., 0.6 mmole) was dissolved in 30 cc. of methanol and hydrogenolyzed in the presence of Pd freshly prepared from 1 g. of $PdCl_2$ for 6 hr. The catalyst was filtered off and washed with methanol. The combined filtrate and washings were concentrated and the tripeptide was isolated by precipitation from ether. The precipitate was filtered and dried; yield 0.48 g. (89%), m.p. 120–125° $[\alpha]^{25}D$ +22.2° (*c* 1, methanol), $R_{f\ BAW}$ 0.67, $R_{f\ SBA}$ 0.22.

Anal. Calcd. for $C_{39}H_{56}N_8O_{10}S_3$ (893.1): C, 52.4; H, 6.32; N, 12.6. Found: C, 52.0; H, 6.07; N, 12.8.

Four micromoles (3.57 mg.) of the above peptide was dissolved in 0.2 cc. of methanol, and 0.2 cc. of 0.01 *M* Tris (tris(hydroxymethyl)aminomethane) buffer (pH 8.5) containing 0.01 *M* Mg^{++} was added. The mixture was incubated at 37° with 0.05 cc. of a solution of 0.5% leucineaminopeptidase (Worthington lot no. 5917) in water. The digestion was complete in 24 hr., as shown by paper chromatography in BAW. Quantitative analysis of an aliquot in the amino acid analyzer[24] gave a ratio of N^{ϵ}-tosyl-lysine:N^{G}-tosyl-arginine = 2.03:1. The recovery of the amino acids was 97%.

N^{α}-*t*-Butyloxycarbonyl-N^{ϵ}-tosyl-lysyl-prolyl-valyl-glycyl-N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginine Benzyl Ester (V). (a) By the Use of N-Ethyl-5-phenylisoxazolium 3′-Sulfonate.—N^{α}-*t*-Butyloxycarbonyl-N^{ϵ}-tosyl-lysyl-prolyl-valyl-glycine (1.96 g., 3 mmoles) was dissolved in acetonitrile, 50 cc., and the solution was cooled to 0°. Triethylamine (0.42 cc., 3 mmoles) and 0.76 g. (3 mmoles) of N-ethyl-5-phenylisoxazolium 3′-sulfonate (Woodward reagent K) were added and the mixture was stirred at 0°. The solution was clear at the end of 1 hr. N^{ϵ}-Tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginine benzyl ester (3 g., 3 mmoles), dissolved in 25 cc. of acetonitrile, was added and the mixture was stirred for 20 hr. at room temperature. The solvent was removed *in vacuo*, and the residue was dissolved in wet ethyl acetate (150 cc.) and washed with water, ice-cold 1% citric acid, and 5% sodium bicarbonate. The organic phase was finally washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness *in vacuo*. The residue was dissolved in 15 cc. of methanol and precipitated from 300 cc. of ether. The precipitate was filtered and dried over P_2O_5 to give 4.5 g. of protected heptapeptide benzyl ester, m.p. 110–114°. Paper chromatography in SBA indicated contamination with some acidic impurity. The impure material was purified by a 100-transfer countercurrent distribution in a 100-tube all-glass apparatus in the toluene system. The peptide was located by alkaline hydrolysis of aliquots, followed by reaction with ninhydrin. Two peaks were located. The material in the major peak (K = 0.23), which closely followed the theoretical curve, was isolated, evaporated to dryness, and precipitated from methanol–ether to give 3.8 g. of protected heptapeptide ester (78%), m.p. 120–125°, $[\alpha]^{25}D$ −33.8° (*c* 1, methanol). This product was found to be homogeneous in paper chromatography in two solvents; $R_{f\ BAW}$ 0.89, $R_{f\ SBA}$ 0.86.

Anal. Calcd. for $C_{76}H_{107}N_{13}O_{18}S_4$ (1619): C, 56.4; H, 6.67; N, 11.3; S, 7.93. Found: C, 56.0; H, 6.68; N, 11.2; S, 8.01.

(b) *Via* **the DCCI Method.**—N^{α}-*t*-Butyloxycarbonyl-N^{ϵ}-tosyl-lysyl-prolyl-valyl-glycine (0.327 g., 0.5 mmole) was dissolved in 10 cc. of acetonitrile together with 0.492 g. (0.5 mmole) of N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginine benzyl ester. The mixture was stirred at 0° and 0.103 g. of DCCI[17] (0.5 mmole) was added. Stirring was continued for 8 hr. at 0° and the mixture was kept at 4° for 48 hr. The dicyclohexylurea was removed by filtration (0.090 g., m.p. 234°), and the filtrate was evaporated to dryness *in vacuo*, redissolved in wet ethyl acetate, and washed successively with water, 0.1 *N* HCl, water, 5% $NaHCO_3$, and water. The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was redissolved in methanol and precipitated from ether to yield 0.66 g. This material was submitted to countercurrent distribution in the toluene system for 85 transfers. The main peak with K = 0.27 was isolated to yield 0.5 g of protected heptapeptide, $[\alpha]^{25}D$ −32.8° (*c* 1, methanol), yield 63%.

Anal. Calcd. C, 56.4; H, 6.67; N, 11.3. Found: C, 56.1; H, 6.46; N, 11.4.

N^{ϵ}-Tosyl-lysyl-prolyl-valyl-glycyl-N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginine Benzyl Ester Trifluoroacetate (VI).—The protected heptapeptide benzyl ester (3.24 g., 2 mmoles) was dissolved in 20 cc. of trifluoroacetic acid and stirred at room temperature for 20 min. The trifluoroacetic acid was removed *in vacuo*. The residue was dissolved in 20 cc. of methanol and precipitated from 500 cc. of ether; yield 2.78 g. (85%), m.p. 118–122°, $[\alpha]^{25}D$ −24.8° (*c* 1, methanol), $R_{f\ BAW}$ 0.76, $R_{f\ SBA}$ 0.84. Countercurrent distribution of a sample in the toluene system for 100 transfers gave a single peak with K = 6.

Anal. Calcd. for $C_{73}H_{100}N_{13}O_{18}S_4F_3$ (1632.9): C, 53.7; H, 6.17; N, 11.2. Found: C, 53.4; H, 6.09; N, 11.0.

N^{ϵ}-Tosyl-lysyl-prolyl-valyl-glycyl-N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginine Benzyl Ester. (VII).—The heptapeptide benzyl ester trifluoroacetate (2.45 g., 1.5 mmoles) was dissolved in 10 cc. of the lower phase of the toluene system together with 0.5 cc. of triethylamine. The mixture was submitted to CCD for 60 transfers using the toluene system. A main peak with K = 0.62 and a fast moving peak (K = 9) were detected. Tubes 15–32 (K = 0.62) were pooled and evaporated to dryness *in vacuo* to yield 1.4 g. (61%) of heptapeptide benzyl ester. This was found to be homogeneous in paper chromatography in two solvent systems; $R_{f\ BAW}$ 0.78, $R_{f\ SBA}$ 0.75, $[\alpha]^{25}D$ −28.9° (*c* 1, methanol), m.p. 100–105°.

Anal. Calcd. for $C_{71}H_{99}N_{13}O_{16}S_4$ (1519); C, 56.2; H, 6.57; N, 12.0. Found: C, 56.3; H, 6.78; N, 11.9.

Carbobenzoxy-seryl-tyrosyl-seryl-methionine Hydrazide (I).—Carbobenzoxy-seryl-tyrosyl-seryl-methionine methyl ester[38] (8.34 g., 13.2 mmoles) was dissolved in a mixture of 50 ml. of dimethylformamide and 200 ml. of methanol. Hydrazine (5 ml.) was added and the solution stirred overnight at room temperature. The solution was then kept at 0° for 24 hr. The hydrazide crystallized and was filtered from the mother liquor and dried; 6.66 g. (80%), m.p. 225–228°. The hydrazide was then recrystallized from dimethylformamide–methanol (1:4 by volume) to yield 5.25 g. (63%), m.p. 245–247°, $[\alpha]^{25}D$ −4° (*c* 1, dimethylformamide).[53]

Carbobenzoxy-seryl-tyrosyl-seryl-methionyl-γ-benzyl-glutamyl-histidyl-phenylalanyl-N^{G}-tosyl-arginyl-tryptophyl-glycine (III).—Carbobenzoxy-seryl-tyrosyl-seryl-methionine hydrazide (1.90 g., 3.0 mmoles) was dissolved in a mixture of 20 ml. of glacial acetic acid, 2 ml. of concentrated hydrochloric acid, and 20 ml. of water. The solution of the hydrazide was then cooled to −5° in an ice–salt bath and then sodium nitrite (0.25 g., 3.6 mmoles) was added with vigorous stirring with a Vibro-mixer[61] for 30 min. The tetrapeptide azide formed in this manner was extracted into ethyl acetate (200 cc., precooled to 4°). All subsequent extractions and washings were done in a cold room at 4°. The ethyl acetate solution of the azide was washed repeatedly with cold (4°) 5% sodium bicarbonate until the aqueous washing was no longer acidic. Then the ethyl acetate solution was dried by extraction with a saturated sodium chloride solution and was then allowed to stand over anhydrous sodium sulfate. The dried azide solution was filtered and then added to an ice-cold solution of 1.95 g. (1.5 mmoles) of γ-benzyl-glutamyl-histidyl-phenylalanyl-N^{G}-tosyl-arginyl-tryptophyl-glycine ditrifluoroacetate[10] and 0.5 ml. of triethylamine in 30 ml. of dimethylformamide. Upon removal of ethyl acetate *in vacuo* at 0°, the reactants went into solution, which was kept at 0° for 3 days. Paper chromatography of an aliquot of the reaction mixture (approximately 1 μmole) in the BAW and SBA systems indicated that no more hexapeptide base remained after 3 days at 0°. The reaction mixture was then acidified with 0.5 ml. of 6 *N* hydrochloric acid and the solvents removed *in vacuo* at 30°. The residue was redissolved in 10 ml. of dimethylformamide, filtered to remove triethylamine hydrochloride, and then the dimethylformamide solution was added to 200 ml. of ethyl acetate to precipitate the desired protected decapeptide. Paper chromatography of the filtered, washed (ethyl acetate), dried decapeptide indicated that the product was now free of tetrapeptide azide decomposition products. The weight of crude protected decapeptide was 2.66 g. (100%). The peptide was dissolved in 30 cc. of dimethylform-

(53) The rotation of this tetrapeptide hydrazide was previously reported (see ref. 2b) as −12°.

amide, and the solution was diluted with 30 cc. of water and kept at 0° overnight to yield 1.97 g. (78%) of crystalline decapeptide, m.p. 214–216°, $[\alpha]^{25}D$ −22.3° (*c* 1, dimethylformamide). This protected decapeptide had a partition coefficient of 6.0 in the toluene system. In the carbon tetrachloride system it had a partition coefficient of 1.0.

Anal. Calcd. for $C_{81}H_{96}O_{20}N_{16}S_2$ (1677.84): C, 56.8; H, 5.88; N, 13.1. Found: C, 56.9; H, 6.13; N, 12.7.

Seryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-N^G-tosyl-arginyl-tryptophyl-glycine (IV).—The above protected decapeptide (168 mg., 0.1 mmole) was dissolved in 20 ml. of dimethylformamide, and palladium from 0.5 g. of $PdCl_2$ was added. The mixture was stirred vigorously with a Vibro-mixer and hydrogen was bubbled in for 6 hr. The temperature was maintained at 50° during hydrogenolysis. The palladium was removed by filtration and the dimethylformamide solution was evaporated to dryness *in vacuo*. Paper chromatography of the residue in the system BPAW revealed a single spot whose R_f was 0.65, indicating the removal of the carbobenzoxy and benzyl ester groups. The residue was then dissolved in 30 ml. of 0.1 *N* ammonium hydroxide and lyophylized to yield 118 mg. (81%) of the tosylated decapeptide. This peptide (1.0 mg.) was dissolved in 0.1 ml. of dimethylformamide and 0.4 ml. of Tris buffer of pH 8.5 and 0.01 ml. of a solution of leucineaminopeptidase (Worthington Biochemical, lot no. 5917, 1.0 mg. in 0.2 ml. of water) was added. The solution was incubated for 24 hr. at 37° and then analyzed in the automatic Spinco amino acid analyzer.[24] The results were:

	Ser	Tyr	Met	Glu	His	Phe	Arg(Tos)	Try	Gly
Theor.	2	1	1	1	1	1	1	1	1
Found	2.02	1.08	1.00	1.01	0.90	0.96	0.93	0.80	0.80

Seryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycine.—The protected decapeptide III (0.168 g., 0.1 mmole), was dissolved in 150 cc. of liquid ammonia. Small pieces of sodium were added until the blue color persisted for 1 hr. at the boiling point of liquid ammonia. The ammonia was then allowed to evaporate and the residue was dried *in vacuo* over concentrated sulfuric acid. The residue was then desalted using IRC-50 resin.[21] The eluate was lyophylized to yield 0.120 g. (92%) of the crude decapeptide. Chromatography using carboxymethylcellulose[22] (1.5 × 50 cm. column) with an ammonium acetate gradient yielded one major peak, which when lyophilized twice yielded 0.84 g. (65%) of the free decapeptide. This peptide behaved as a homogeneous substance in paper chromatography (BAW, BPAW systems), paper electrophoresis at pH 3.7, and polyacrylamide gel electrophoresis (Fig. 6).

A sample of the above (0.73 mg.) was dissolved in 0.1 ml. of dimethylformamide and 0.5 ml. of pH 8.5 Tris buffer (0.01 *M* with respect to magnesium chloride). Twenty microliters of a solution containing 1 mg. of trypsin (Armour lot no. 904CL3) and 1 mg. of chymotrypsin (Armour lot no. 381092) in 1.0 cc. of Tris buffer was added and the digest (1 to 50, enzyme to substrate ratio) incubated at 37° for 24 hr. The enzymes were inactivated by heating the solution at 100° for 15 min., followed by slow cooling to room temperature. Forty microliters of a leucineaminopeptidase solution (1 mg. of Worthington lot no. 5917 in 1.0 cc. of buffer) was then added to the solution (1 to 25, enzyme to substrate ratio) and the solution incubated at 37° for 24 hr. The digest was then analyzed in the Spinco amino acid analyzer. The results were:

	Ser	Tyr	Met	Glu	His	Phe	Arg	Try	Gly
Theor.	2	1	1	1	1	1	1	1	1
Found	2.12	1.10	0.98	1.10	0.93	1.05	0.86	0.79	1.06

Average recovery of amino acids was 99.2%.

Carbobenzoxy-seryl-tyrosyl-seryl-methionyl-γ-benzyl-glutamyl-histidyl-phenylalanyl-N^G-tosyl-arginyl-tryptophyl-glycyl-N^ϵ-tosyl-lysyl-prolyl-valyl-glycyl-N^ϵ-tosyl-lysyl-N^ϵ-tosyl-lysyl-N^G-tosyl-arginine Benzyl Ester (VIII). (a) *Via* the **DCCI Method.**—N^G-Tosyl-lysyl-prolyl-valyl-glycyl-N^ϵ-tosyl-lysyl-N^ϵ-tosyl-lysyl-N^G-tosyl-arginine benzyl ester (0.52 g., 0.345 mmoles) was dissolved in 5 ml. of dimethylformamide together with 0.580 g. (0.345 mmole) of carbobenzoxy-seryl-tyrosyl-seryl-methionyl-γ-benzyl-glutamyl-histidyl-phenylalanyl-N^G-tosyl-arginyl-tryptophyl-glycine. The solution was cooled to 0° and DCCI[17] (0.078 g., 0.380 mmoles) was added and the mixture was stirred for 2 hr. at 0° and then placed in a refrigerator (0°) for 5 days. Glacial acetic acid (0.5 ml.) was added and the solvents were removed *in vacuo* (bath temperature 30°) after the removal by filtration of some dicyclohexylurea. The residue was then redissolved in the lower phase of the carbon tetrachloride system and submitted to countercurrent distribution (upper and lower phases, 10 ml.) for 100 transfers. Tubes 0 to 21 were pooled and evaporated to dryness (unreacted decapeptide has a partition coefficient of 1.0) and then washed thoroughly with methanol to remove all traces of unreacted heptapeptide base to yield 0.658 g. (58%) of the desired protected heptadecapeptide, $[\alpha]^{25}D$ −17.4° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{152}H_{193}O_{35}N_{29}S_6$ (3177.7): C, 57.4; H, 6.12; N, 12.8. Found: C, 56.9; H, 5.98; N, 12.4.

(b) **By the Use of N-Ethyl-5-phenylisoxazolium 3′-Sulfonate.** The decapeptide III (0.175 g., 100 μmoles) was dissolved in 2 ml. of DMF, and the solution was cooled to 0° and stirred for 1 hr. with triethylamine (0.05 cc.) and N-ethyl-5-phenylisoxazolium 3′-sulfonate[18] (0.05 g., 200 μmoles); VII (0.160 g.) was then added. The reaction mixture was kept at 37° for 72 hr. The solvent was removed *in vacuo* and the residue submitted to countercurrent distribution in the carbon tetrachloride system. Tubes 0–18 were pooled, evaporated to dryness, and the residue was thoroughly washed with water and then methanol to remove unreacted heptapeptide base. The protected heptadecapeptide VIII was obtained in 48.6% yield (0.160 g.).

Seryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginine (IX).—The above protected heptadecapeptide (0.638 g., 0.194 μmole) was dissolved in 200 cc. of liquid ammonia. Small pieces of metallic sodium (weighing approximately 3 mg.) were added at the temperature of boiling liquid ammonia until the blue color persisted for 30–40 min. The ammonia was then evaporated; all traces of excess ammonia were removed *in vacuo* over concentrated sulfuric acid. The residue was then dissolved in 0.1 *N* acetic acid (20 cc.) and the solution was passed through an Amberlite-cation exchanger, IRC-50 (XE-64), for desalting.[21] The column (3 × 10 cm.) was washed with 0.1 *N* acetic acid (100 cc.) and water (200 cc.) and the peptide was eluted with a pyridine–acetic acid–water solution (30:4:66, by volume). This was lyophylized to yield a slightly brownish powder; yield of crude heptadecapeptide, 0.451 g. (>100%). The crude heptadecapeptide was then divided into three portions and submitted to carboxymethylcellulose chromatography[22] using continuous gradient elution with ammonium acetate buffer on a 1 × 60 cm. column (see Fig. 4). Each of the major peaks was rechromatographed (Fig. 5) under the same conditions and the major peaks pooled and lyophilized three times to yield 0.253 g. (62%) of the purified heptadecapeptide (ultraviolet absorption studies indicated a peptide content of 87%). This product behaved as a homogeneous substance in paper electrophoresis at pH 3.7, 7.0, 11.0 and in polyacrylamide gel electrophoresis (Fig. 6). Samples were hydrolyzed and analyzed in the Spinco amino acid analyzer and by microbiological assay. See Table I for the results.

The electrophoretic mobility of IX relative to lysine on paper at pH 3.7 (400 v., 4 hr.) was found to be 0.82; in addition, $[\alpha]^{25}D$ −97.1° (*c* 0.5, 0.1 *M* acetic acid).

Enzymatic Digestion of the Heptadecapeptide.—One milligram of the heptadecapeptide was dissolved in 0.5 cc. of Tris buffer of pH 8.5 containing 0.01 *M* Mg^{++}. To this solution, 20 μl. of an enzyme solution, consisting of 1 mg. of trypsin (Armour lot no. 904CL3) and 1 mg. of chymotrypsin (Armour lot no. 381092) in 0.1 cc. of Tris buffer, was added and the digest incubated at 37° for 24 hr. (enzyme:substrate ratio, 1:50). The enzymes were inactivated by immersion in a 100° bath for 15 min. and slowly cooled to room temperature. Then 10 μl. of a solution of leucineaminopeptidase (Worthington lot no. 5917, 1 mg. in 0.2 cc. of Tris buffer) was added (enzyme:substrate ratio, 1:20) and the digest incubated at 37° for 24 hr. The digest was then analyzed directly in the Spinco amino acid analyzer.[24] The results are shown in Table I.