strength 0.19. These and the preliminary value obtained earlier in this laboratory[12] are difficult to compare with the present result for several reasons. First, ionic strength was not varied. At the ionic strength used by McKenzie and Wake light scattering measurements reveal unpredictable aggregation. Also, a charge correction was not attempted for ultracentrifuge data. Correction would increase sedimentation molecular weights to an extent which is at present difficult to estimate.[16,17] Finally, partial specific volume, which has been found to be pH dependent for several proteins,[15] was not determined for the specific conditions employed. It should be noted that using equilibrium centrifugation we have obtained at pH 12 and ionic strength 0.4 reduced molecular weights equivalent to those given by McKenzie and Wake.[10] It is of interest that McKenzie and Wake reported a molecular weight of 27,600 at pH 7.3 in the presence of 6 *M* urea, using sedimentation-diffusion.

The studies of Burk and Greenberg[24] were the first of several[25-27] determinations of the average molecular weight of whole casein. These authors have used osmotic pressure and light scattering measurements and a variety of dissociating solvents. Minimum molecular weights in the range of 24,500 to 33,600 were obtained. Although these molecular weights are interesting by virtue of their being close to the molecular weight of $\alpha_{s1,2}$-caseins, no serious comparison can be made in view of the complexity of whole casein.[9]

In the experiments at pH 12 reported here unexpected aggregation occurred at low ionic strength, with low protein dialysis concentrations, and with a rapid pH-ionic strength increase after the protein was in solution at pH 7. The fact that no particular relationship between size and time has been observed in any of the aggregating systems studied, suggests that first order denaturation followed by higher order aggregation is not part of the fundamental mechanism. The characteristics of casein polymers, including association-dissociation phenomena at extreme pH values, have been investigated for example, by Halwerda,[28] Svedberg and Petersen,[29] Halwer,[30] von Hippel and Waugh,[31] Sullivan, *et al.*[32] and McMeekin and Peterson.[33] The extent to which unpredicable, irreversible aggregation might have influenced the results obtained in some of these studies is of course not known. Aggregation phenomena should be examined critically in future work.

Acknowledgment.—We wish to thank Mr. D. W. McGowan for assisting us in programming the computer.

(25) V. Vilensky and T. Kastorskaya, *Kolloid Zhur.*, **2**, 193 (1936).

(26) D. Carpenter, *J. Am. Chem. Soc.*, **57**, 129 (1935).

(27) P. F. Dyachenko and I. N. Vlodavets, *Kolloid Zhur.*, **14**, 338 (1952).

(28) B. J. Halwerda, *Rec. trav. chim.*, **50**, 601 (1931).

(29) T. Svedberg and K. O. Petersen, "The Ultracentrifuge," The Clarendon Press, New York, N. Y., 1940.

(30) M. Halwer, *Arch. Biochem. Biophys.*, **51**, 79 (1954).

(31) P. H. von Hippel and D. F. Waugh, *J. Am. Chem. Soc.*, **77**, 4311 (1955).

(32) R. Sullivan, M. Fitzpatrick, E. Stanton, R. Annino, G. Kissel and F. Palermiti, *Arch. Biochem. Biophys.*, **55**, 455 (1955).

(33) T. L. McMeekin and R. F. Peterson, Abst. 128th Meeting Am. Chem. Soc., p. 2A (1955).

[CONTRIBUTION FROM THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH, NEW BRUNSWICK, N. J.]

8-L-Citrulline Vasopressin and 8-L-Citrulline Oxytocin[1,2]

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The protected nonapeptides S-benzyl-N-benzyloxycarbonyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide and S-benzyl-N-benzyloxycarbonyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-citrullyl-glycinamide were synthesized stepwise from their C-terminal amino acid by the nitrophenyl ester method. Removal of the protecting groups and conversion to the corresponding cyclic disulfides gave the 8-citrulline analogs of vasopressin and oxytocin. Biological activities of the two hormone analogs are reported.

This paper presents a part of our studies on peptides containing a citrulline moiety.[1] The objectives in undertaking this work were to find methods for the synthesis of citrulline peptides and to study the changes in biological activity that occur when an arginine moiety in a physiologically active peptide is replaced by citrulline. We are dealing here with the synthesis of 8-L-citrulline vasopressin (XII) and 8-L-citrulline oxytocin (XVI). Although oxytocin does not contain an arginine residue, the corresponding hormone of birds and reptiles, vasotocin, has an arginine or lysine in the 8 position; thus the oxytocin analog described here can be called 8-L-citrulline vasotocin as well, and it is of considerable interest to gain information on its biological properties.

The protected nonapeptide S-benzyl-N-benzyloxycarbonyl - L - cysteinyl - L - tyrosyl - L - phenylalanyl - L - glutaminyl - L - asparaginyl - S-benzyl - L - cysteinyl - L - prolyl - L - citrullylglycinamide (XI), the key intermediate in the synthesis of the vasopressin analog, was prepared by the stepwise nitrophenyl ester method[3,4]

(1) For a preliminary report of this work see M. Bodanszky, M. A. Ondetti, B. Rubin, J. Piala, J. Fried, J. T. Sheehan and C. A. Birkhimer, *Nature*, **194**, 485 (1962).

(2) In order to designate analogs of oxytocin and vasopressin the numbering system proposed by M. Bodanszky and V. du Vigneaud was used, *J. Am. Chem. Soc.*, **81**, 1258 (1959).

(3) M. Bodanszky and V. du Vigneaud, *Nature*, **183**, 1324 (1959); *J. Am. Chem. Soc.*, **81**, 5688 (1959); M. Bodanszky, J. Meienhofer and V. du Vigneaud, *ibid.*, **82**, 3195 (1960).

(4) In the stepwise synthesis a technique somewhat different from those previously used[3] was applied. In a personal communication Dr. J. E. Stouffer and Professor V. du Vigneaud suggested the use of pyridine both as a solvent and as the base necessary to liberate the

and also by coupling the protected N-terminal pentapeptide S-benzyl-N-benzyloxycarbonyl-L-cysteinyl - L - tyrosyl - L - phenylalanyl - L - glutaminyl-L-asparagine[5] to the C-terminal tetrapeptide part of the molecule, S-benzyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide (from VI). In the synthesis of 8-L-citrulline oxytocin the corresponding protected nonapeptide S-benzyl-N-benzyloxycarbonyl-L - cysteinyl - L - tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl-L - prolyl - L - citrullylglycinamide (XV) was obtained by the stepwise synthesis[3,4] mentioned earlier. The two stepwise syntheses follow the same route up to and employ the same hexapeptide derivative VIII. After removal of the protective groups from XI and XV with sodium in liquid ammonia, the free peptides were converted to the corresponding cyclic disulfides by aeration[6] and the hormone analogs thus obtained were purified by counter-current distribution.

Due to the presence of the citrulline moiety most of the intermediates had unfavorable solubility properties and were difficult to purify. Yields and purity of intermediates were generally less satisfactory in the present syntheses of 8-L-citrulline oxytocin and 8-L-citrulline vasopressin than in the similar syntheses of oxytocin and vasopressin.[3]

The pressor[7] and anti-diuretic[8] potency of the vasopressin analog was found to be one-eighth to one-tenth that of the natural hormone. In the isolated rat uterus test[9] 8-L-citrulline vasopressin is at least as potent as the natural vasopressins, 8-L-Citrulline oxytocin shows only slight pressor activity; its potency in the isolated rat uterus test[9] is about equal to that of oxytocin, and it is about half as active as oxytocin in the avian depressor test.[10] In the amphibian bladder assay 8-L-citrulline oxytocin is only slightly more active than oxytocin itself and much less active than arginine vasotocin.[11]

amines from their salts. Excellent results were obtained when we applied this technique for the preparation of III but with larger peptides—probably due to the presence of more than one HBr molecule in the intermediate hydrobromides-pyridine alone was not sufficient to liberate the amines from their salts. Therefore, the hydrobromides were dissolved in methanol and treated with the anion exchange resin Amberlite IRA 400 in the acetate cycle until the solution was free of bromide ions. Removal of the solvent left the acetic acid salt of the amino component as a residue and the latter was allowed to react with the appropriate nitrophenyl ester in pyridine. Using this technique the protected peptide intermediates up to the hexapeptide derivative were obtained in very satisfactory yield and purity. The protected hepta-, octa- and nonapeptide, however, separated in impure form. The protected heptapeptide and nonapeptide were purified by counter-current distribution in a system of butanol-acetic acid-water (4:1:5). Paper chromatography in the same solvent system revealed that after isolation of the desired compounds several by-products were left. Further separation of these by-products by partition chromatography, hydrolysis and chromatography of the hydrolysates has failed so far to give pertinent information regarding the nature of the by-products. Using our earlier technique, that is, the reaction between the active ester and the hydrobromide of the amino component plus triethylamine in dimethylformamide, formation of significant amounts of by-products was not observed. This version was used, therefore, in the preparation of the intermediates of 8-L-citrulline oxytocin.

(5) P. G. Katsoyannis and V. du Vigneaud, *J. Am. Chem. Soc.*, **78**, 4482 (1956).

(6) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, *ibid.*, **76**, 3115 (1954).

(7) J. Dekanski, *Brit. J. Pharmacol.*, **7**, 567 (1952).

(8) In unanesthesized dogs.

(9) J. H. Burn, D. J. Finney, L. G. Goodwin, "Biological Standardization," Oxford University Press, New York, N. Y., 1950.

(10) J. M. Coon, *Arch. Intern. Pharmacodynamic*, **62**, 79 (1939).

Experimental

N-α-Benzyloxycarbonyl-L-citrulline (I).—A solution of L-citrulline (7.0 g.) in *N* NaOH (40 ml.) was treated with stirring and cooling in an ice bath with benzyl chloroformate (8 ml.) and *N* NaOH (64 ml.) added simultaneously in five portions. After further stirring for 0.5 hr. at room temperature, the reaction mixture was extracted with ether (200 ml. in five portions) and the aqueous layer acidified with 5 *N* HCl (10 ml.). The resulting crystalline product was filtered, washed with water, and dried at 40° over P_2O_5 *in vacuo*. The crude product (10.3 g., 83%) melts at 115–117°. Recrystallization from ethanol did not change the melting point.

Anal. Calcd. for $C_{14}H_{19}O_5N_3$: C, 54.36; H, 6.19; N, 13.59. Found: C, 54.55; H, 6.18; N, 13.63.

***p*-Nitrophenyl N-α-Benzyloxycarbonyl-L-citrullinate** (II).—Dicyclohexylcarbodiimide[12] (14.4 g.) was added to a solution of N-α-benzyloxycarbonyl-L-citrulline (I) (21.7 g.) and *p*-nitrophenol (11.7 g.) in dimethylformamide (210 ml.) with stirring and cooling in an ice bath. After the reaction mixture was stirred for 0.5 hour at 0° and 4 hours at room temperature, acetic acid (0.7 ml.) was added and 5 minutes later the N,N'-dicyclohexylurea was filtered off and was washed with dimethylformamide (60 ml.). Water (1300 ml.) was added to the combined filtrates and washings at 0°. The crude ester separated as an oil, forming a milky suspension, but soon turned into a crystalline solid. This was collected on a filter, washed with water (700 ml.), and dried in the air. The crude product (30.0 g., m.p. 149–152°) was recrystallized from hot ethanol (500 ml.) to which acetic acid (5 ml.) had been added. The purified ester (20 g., 67%) melts at 163–165°. Further recrystallization did not change its m.p.; $[\alpha]^{20}D$ −22° (*c*, 2, dimethylformamide).

Anal. Calcd. for $C_{20}H_{22}O_7N_4$: C, 55.81; H, 5.15; N, 13.02. Found: C, 55.60; H, 5.61; N, 13.05.

Ethyl N-α-Benzyloxycarbonyl-L-citrullylglycinate (III).—Ethyl glycinate hydrochloride (11.1 g.) was added to pyridine (160 ml.), but was only partially dissolved. *p*-Nitrophenyl-N-α-benzyloxycarbonyl-L-citrullinate (II) (17.2 g.) was added; with occasional shaking, the ethyl glycinate hydrochloride went slowly into solution. The reaction was left to proceed at room temperature for 24 hours. The solvent was then removed *in vacuo* and the residue was triturated with water (200 ml.). The crystalline product was collected on a filter and washed with water, ethanol and ethyl acetate (100 ml. each). The dried product weighed 13.8 g. (88%), m.p. 166.5–168°; $[\alpha]^{20}D$ −2° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{18}H_{26}O_6N_4$: C, 54.82; H, 6.65; N, 14.2. Found: C, 54.62; H, 6.82; N, 14.1.

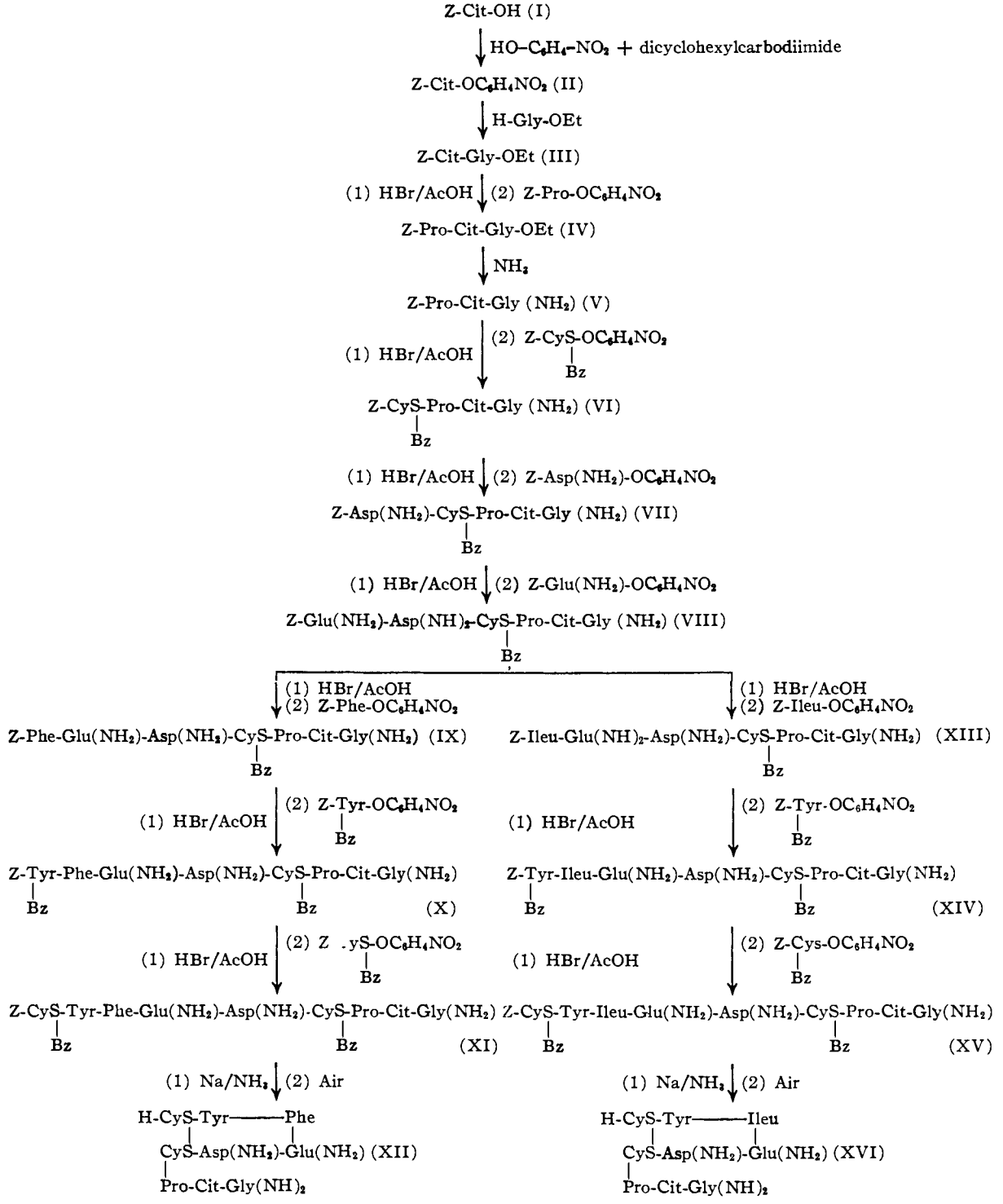
Ethyl Benzyloxycarbonyl-L-prolyl-L-citrullylglycinate (IV).—To a solution of ethyl N-α-benzyloxycarbonyl-L-citrullylglycinate (III) (7.0 g.) in acetic acid (25 ml.), a solution of HBr in acetic acid (*ca.* 36%, 25 ml.) was added. After the reaction proceeded 1 hour at room temperature, ether (*ca.* 900 ml.) was added. The semi-solid HBr salt which separated was washed with ether by decantation and was dried briefly *in vacuo* over NaOH. It then was dissolved in dimethylformamide (35 ml.) and triethylamine (7 ml.) was added to the solution, followed by *p*-nitrophenyl benzyloxycarbonyl-L-prolinate[3] (7.4 g.). Upon standing at room temperature the reaction mixture turned into a semi-solid mass. After 3 days it was disintegrated, triturated with ethyl acetate, washed on a filter with ethyl acetate, and air dried. It then was triturated with water (200 ml.), washed with water on a filter and dried *in vacuo* over P_2O_5. The product (5.71 g., 65%) melts at 157.5–162° (but completely only at 198–199°).

In a parallel experiment using pyridine (70 ml.) instead of the dimethylformamide, the yield was similar (5.82 g., 66%),

(11) The authors wish to thank Dr. Howard Rasmussen, University of Wisconsin, for the comparison of 8-L-citrulline oxytocin with oxytocin and arginine vasotocin in his toad bladder assay.

(12) D. F. Elliott and D. W. Russel, *Biochem. J.*, **66**, 49P (1957); M. Rothe and F. W. Kunitz, *Ann.*, **609**, 88 (1957); M. Bodanszky and V. du Vigneaud, "Biochemical Preparations," Vol. IX, John Wiley and Sons, Inc., New York, N. Y., 1962, p. 110.

SYNTHESIS OF 8-L-CITRULLINE VASOPRESSIN AND 8-L-CITRULLINE OXYTOCIN



as was the m.p.; $[\alpha]^{20}D$ −30° (*c*, 1, dimethylformamide). This compound was amorphous and difficult to purify. It was used directly in the preparation of the corresponding amide, which was more tractable.

Anal. Calcd. for $C_{32}H_{38}O_7N_6$: C, 56.20; H, 6.77; N, 14.25; OC_2H_5, 9.18. Found: C, 57.02; H, 6.83; N, 14.02; OC_2H_5, 10.5.

Benzyloxycarbonyl-L-prolyl-L-citrullylglycinamide (V).—Ethyl benzyloxycarbonyl-L-prolyl-L-citrullylglycinate (IV) (5.8 g.) was dissolved in methanol (300 ml.) with heating. The solution was cooled in an ice-water bath and was saturated with NH_3. The flask was stoppered and the reaction mixture stood at room temperature for two days. It then was evaporated to dryness and the residue was recrystallized from hot methanol (300 ml.). The crystals were filtered off and washed with methanol (100 ml.). The evaporation of the mother liquor and washings to approximately 50 ml. produced a second crop. The total amount of amide obtained weighed 5.1 g. (93%), m.p. 217–221°; $[\alpha]^{20}D$ −31° (*c*, 2, dimethylformamide).

Anal. Calcd. for $C_{21}H_{30}O_6N_6$: C, 54.54; H, 6.54; N, 18.17. Found: C, 54.76; H, 6.69; N, 18.14.

S-Benzyl-N-benzyloxycarbonyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide (VI).—Benzyloxycarbonyl-L-prolyl-L-citrullylglycinamide (V) (1.85 g.) in acetic acid (10 ml.) was treated with a solution of HBr in acetic acid (*ca.* 36%, 10 ml.). After one hour at room temperature, the hydrobromide was precipitated with ether, washed with ether and dissolved in methanol (50 ml.). Amberlite IRA-400 in acetate cycle was added until the solution gave no reaction with $AgNO_3$. The resin was filtered off and washed with methanol. The methanol was evaporated *in vacuo* and the residue was dissolved in pyridine (12 ml.) and dimethylformamide (6 ml.). S-benzyl-N-benzyloxycarbonyl-L-cysteine *p*-nitrophenyl ester[13] (2.1 g.) was added to the solution. After 24 hours at room temperature, the solvents were removed, *in vacuo*. The residue was triturated with ethyl acetate, filtered, washed with ethyl acetate and dried. The product (1.35 g.) melts at 181–184°.

In another experiment starting from 3.54 g. of benzyloxycarbonyl-L-prolyl-L-citrullylglycinamide (V), 3.82 g. (76%) of crude S-benzyl-N-benzyloxycarbonyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide (VI) was obtained. From this 2.80 g. was recrystallized from hot methanol (30 ml.); the crystals (2.50 g., 68%) melted at 185–187°; $[\alpha]^{20}D$ −46° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{31}H_{41}O_7N_7S$: C, 56.78; H, 6.30; N, 14.95; S, 4.89. Found: C, 56.84; H, 6.33; N, 14.83; S, 4.92.

Benzyloxycarbonyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide (VII).—The benzyloxycarbonyl group was removed from S-benzyl-N-benzyloxycarbonyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide (VI) (3.28 g.) with HBr in acetic acid, as described in the previous paragraph, and the HBr was removed from the resulting salt with the aid of the anion exchange resin. The residue remaining after evaporation of the methanol *in vacuo* was dissolved in pyridine (10 ml.) and allowed to react with benzyloxycarbonyl-L-asparagine *p*-nitrophenyl ester (2.13 g.). The mixture soon turned into a semi-solid mass. After three days at room temperature, the mixture was triturated with ethyl acetate (150 ml.), filtered, washed with ethyl acetate (100 ml.), ethanol (100 ml.), ethyl acetate (100 ml.), respectively. The dried product weighed 3.30 g. (86%), m.p. 208–210°; decomposition at 225°; $[\alpha]^{20}D$ −41° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{35}H_{47}O_9N_9S$: C, 54.61; H, 6.15; N, 16.38; S, 4.17; Found: C, 54.59; H, 6.33; N, 16.18; S, 4.18.

Benzyloxycarbonyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide (VIII).—The benzyloxycarbonyl group was removed from benzyloxycarbonyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide (VII) (2.7 g.) as described in preceding paragraphs. After removal of the HBr with the aid of the anion exchange resin and evaporation of the methanol, the residue was dissolved in pyridine (10.5 ml.) and *p*-nitrophenyl benzyloxycarbonyl-L-glutaminate[3] (1.61 g.) was added to the mixture. The solution became a semi-solid mass in approximately one hour. The next day, ethyl acetate (120 ml.) was used to disintegrate the solid. The product was filtered and washed with ethyl acetate (80 ml.), ethanol (80 ml.) and ethyl acetate again (80 ml.). The dried protected hexapeptide weighed 2.72 g. (86%), m.p. 186–202° (dec.); $[\alpha]^{20}D$ −42° (*c*, 1, dimethylformamide).

Anal. Calcd. for $C_{40}H_{55}O_{11}N_{11}S$: C, 53.50; H, 6.17; N, 17.16; S, 3.58. Found: C, 53.48; H, 6.16; N, 17.04; S, 3.79.

Benzyloxycarbonyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide (IX).—Starting with benzyloxycarbonyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide (VIII) (2.25 g.), the procedure for the removal of the benzyloxycarbonyl group and of HBr was repeated as described, *e.g.*, in the preparation of VI. The free amino component (as acetate) was treated with *p*-nitrophenyl benzyloxycarbonyl-L-phenylalaninate[14] (1.26 g.) and, after leaving the reaction mixture one day at room temperature, the product was isolated as above (*cf.* VIII). The crude product (2.23 g.), m.p. 184–204°; $[\alpha]^{20}D$ −47.7° (*c*, 1, dimethylformamide), on analysis proved to be grossly impure.

Anal. Calcd. for $C_{49}H_{64}O_{12}N_{12}S$: C, 56.31; H, 6.17; N, 16.08; S, 3.07. Found: C, 53.54; H, 6.08; N, 16.65; S, 3.44.

The experiment was repeated with very similar results. In a third experiment the HBr salt of the hexapeptide amide (from 180 mg. of VIII) was dissolved in dimethylformamide and was allowed to react with the active ester of protected phenylalanine (105 mg.). The product (181 mg.), m.p. 186–202°, dec. at 208°, is less impure than the one described above. A sample (100 mg.) reprecipitated from acetic acid–ethanol yielded 62 mg. (30%) of a product, m.p. 208–212° (dec.).

Anal. Found: C, 56.83; H, 6.68; N, 16.33; S, 3.14.

The impure benzyloxycarbonyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide (IX) (1.35 g.) was partially purified by extraction with hot methanol (100 ml. in several portions). An insoluble residue, A (0.48 g., m.p. 210–214°), a solid which separated from the methanol upon cooling and standing, B (0.33 g., m.p. *ca.* 210°), and a methanol soluble part, C (0.48 g., m.p. 165–173°) were obtained, $[\alpha]^{20}D$ 38, 38 and 52°, respectively. Fraction B was further purified by counter-current distribution in a system of 1-butanol–acetic acid–water (4:1:5). A train of seven Erlenmeyer flasks was used with 25 ml. of each phase. The lower phase was transferred and distribution was performed with complete withdrawal of both phases. The fractions were examined by paper chromatography in the same solvent system as for distribution. The spots were revealed by a modification of the procedure of Rydon, treatment with hypochlorite and spraying with a KI-starch solution.[15] Fractions 0, 1, 2 and 3 (upper phase only) contained the component fastest on chromatograms. This was isolated by evaporation of the solvent *in vacuo*. The residue was dissolved in acetic acid (2.5 ml.) and ethanol (20 ml. in portions) was added to the solution. The next day the precipitate was filtered, washed with ethanol and dried. The protected heptapeptide amide (146 mg.), thus purified, melts at 205–208° (dec.); $[\alpha]^{20}D$ −32° (*c*, 1, dimethylformamide).

Anal. Found: C, 55.83; H, 6.52; N, 15.80; S, 3.02.

O-Benzyl-N-benzyloxycarbonyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide (X).—Benzyloxycarbonyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide (IX) (105 mg.) in acetic acid (0.3 ml.) was treated with HBr in AcOH (36%, 0.3 ml.). After one hour at room temperature, ether (12 ml.) was added and the precipitate was washed with ether. The hydrobromide was dissolved in dimethylformamide (0.5 ml.) and triethylamine (0.25 ml.) was added, followed by *p*-nitrophenyl O-benzyl-N-benzyloxycarbonyl-tyrosinate[3] (63 mg.). All these operations were performed in a centrifuge tube. After the reaction mixture had been left three days at room temperature, acetic acid (0.05 ml.) and ethyl acetate (10 ml.) were added to the semi-solid mass. The precipitate was washed with ethyl acetate (5 ml.), ethanol (5 ml.), and ethyl acetate (5 ml.). After drying, the crude protected octapeptide weighed 116 mg., m.p. 189–221° (dec.). Part of this product (80 mg.) was dissolved in acetic acid (3 ml.) and ethanol was added in portions (a total of 21 ml.). The next day the precipitate was filtered and washed with ethanol (6 ml.) and ethyl acetate (6 ml.). The recovered product weighed 56 mg. (62%), m.p. 242–245° (dec.) (sintering at 220°); $[\alpha]^{20}D$ −36° (*c*, 2, dimethylformamide).

Anal. Calcd. for $C_{65}H_{70}O_{14}N_{13}S$: C, 60.12; H, 6.13; N, 14.02; S, 2.47. Found: C, 59.95; H, 6.01; N, 14.41; S, 2.57.

S-Benzyl-N-benzyloxycarbonyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-citrullylglycinamide (XI). A.—The benzyloxycarbonyl and O-benzyl groups were removed from O-benzyl-N-benzyloxy-carbonyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-

(13) M. Bodanszky, *Nature*, **175**, 685 (1955); M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 2504 (1959).

(14) M. Bodanszky and V. du Vigneaud, *ibid.*, **81**, 6072 (1959).

(15) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 922 (1952); S. C. Pan and J. D. Dutcher, *Anal. Chem.*, **28**, 836 (1956).

L-cysteinyl-L-prolyl-L-citrullylglycinamide (X) (1.7 g.). (This sample was X was obtained from a crude sample of benzyloxycarbonyl - L - phenylalanyl - L - glutaminyl - L-asparaginyl - S - benzyl - L - cysteinyl - L - prolyl - L - citrullylglycinamide (IX) and was not purified.) After the removal of HBr with resin and evaporation of the solvent, the residue was allowed to react with S-benzyl-N-benzyloxycarbonyl-L-cysteine *p*-nitrophenyl ester (0.75 g.). Ethyl acetate (100 ml.) was added the next day and the precipitate was washed with 50-ml. portions each of ethyl acetate, ethanol and ethyl acetate. The dried product, 1.46 g., m.p. 175–198° (dec.), $[\alpha]^{20}D$ − 51° (*c*, 1, dimethylformamide), was grossly impure, as demonstrated by analysis and paper chromatography. The crude peptide (1.4 g.) was extracted with hot methanol (100 ml.). A fraction (0.30 g.), m.p. 215–220° (dec.), remained undissolved; a second (0.58 g.), m.p. 210–218° (dec.), separated from the methanol on standing in the cold, and a third (0.44 g.), m.p. 100–110° (dec.), was obtained upon evaporation of the solvent. The first two fractions were combined and further purified by counter-current distribution as described for IX. In this purification, the protected peptide (750 mg.) was dissolved in 50 ml. of each of the two phases of the solvent system. A train of eight flasks was used, with complete withdrawal of both phases. Paper chromatography revealed that the protected nonapeptide—the fastest moving component—is present, without the contaminants, in flask 0 (only upper phase). Evaporation of the solvent left a residue (203 mg.), which was dissolved in acetic acid (4 ml.) and precipitated by the slow addition of ethanol (75 ml.). The purified protected nonapeptide was filtered on the next day, washed with ethanol and dried. One hundred and forty milligrams (8%) of a product, m.p. 201–210°; $[\alpha]^{21}D$ −42° (*c*, 1, dimethylformamide), was obtained.

Anal. Calcd. for $C_{68}H_{84}O_{15}N_{14}S_2$: C, 58.27; H, 6.04; N, 13.99; S, 4.57. Found: C, 58.39; H, 6.23; N, 13.96; S, 4.65.

B.—The same protected nonapeptide was prepared also by coupling S-benzyl-N-benzyloxycarbonyl-L-cysteinyl-L-tyrosyl - L - phenylalanyl - L - glutaminyl - L - asparagine[5] to the free tetrapeptide from S-benzyl-N-benzyloxycarbonyl-L - cysteinyl - L - prolyl - L - citrullylglycinamide (VI). Compound VI (1.0 g.) was treated with HBr in AcOH and the HBr was removed from the hydrobromide with Amberlite IRA-400 (OH cycle). Removal of the solvent left a foam (0.80 g.) which was dissolved in dimethylformamide (5 ml.). The protected pentapeptide (0.90 g.) and dicyclohexylcarbodiimide[16] (0.68 g.) were added to the solution and the mixture was left at room temperature for three days. Acetic acid (0.5 ml.) was added. The urea derivative was removed by filtration and was washed with dimethylformamide (5 ml.). The filtrate was diluted with ethyl acetate (100 ml.). The precipitate thus formed was filtered and washed with ethyl acetate (50 ml.). The crude product (1.25 g.) was extracted with methanol (50 ml.), then with warm methanol (30 ml.). The residue (0.51 g.), m.p. 215–220°, was not yet pure. A portion (200 mg.) was reprecipitated from acetic acid with ethanol. The recovered product weighed 149 mg. (27%), m.p. 210–220°; $[\alpha]^{20}D$ −40° (*c*, 1, dimethylformamide).

8-L-Citrulline Vasopressin (XII).—The protective groups were removed from XI (170 mg.) by treatment with sodium in liquid ammonia (250 ml.) until a permanent blue color was obtained.[5] Ammonium chloride was used to discharge the color. The ammonia was allowed to evaporate. The residue was dissolved in water (350 ml.) and aerated at pH 6.5 for 5 hours. The pH of the solution then was adjusted to about 3 with acetic acid and the water was removed *in vacuo* until the volume of the solution was about 5 ml. This concentrated solution was placed in the first three tubes of a counter-current distribution apparatus and distributed in a solvent system of 1-butanol–ethanol–0.05% acetic acid (4:1:5). After 800 transfers the contents of the tubes were scanned by measurement of the optical density at 270 mμ. The main part of the peptidic material was found in a peak corresponding to $K = 0.18$. The contents of the tubes no. 110 to 140 were pooled, the solvents removed *in vacuo* to a final volume of a few milliliters and the residual solution lyophilized. The 8-L-citrulline vasopressin thus purified weighed 60 mg. (In a solvent system of 1-butanol–acetic acid–water (4:1:5), freshly prepared, it migrates as a single spot with an R_f value of 0.20. Its specific rotation is $[\alpha]^{20}D$ − 33° (*c*, 1, *N* acetic acid). Amino acid analysis by a modification of the Stein–Moore procedure was performed after hydrolysis in constant boiling HCl in a sealed ampoule at 110° for 16 hours. Part of the citrulline is hydrolyzed during this time to give ornithine and ammonia. (About the same amount of decomposition was observed when citrulline itself was exposed to identical hydrolysis conditions.) The following molar ratio of amino acids (glutamic acid = 1.00) was found: aspartic acid 0.94, proline 1.00, glycine 1.04, cystine 1.02, tyrosine 0.85, phenylalanine 0.99, ammonia 2.6, citrulline 0.79 and ornithine 0.31. In the rat pressor assay XII exhibits a potency of about 40 u./mg., while in the anti-diuretic test in the dog only about 15 u./mg. was found. In the test on the isolated rat uterus slight potency (2–5 u./mg.) was demonstrated.

Benzyloxycarbonyl-L-isoleucyl-L-glutaminyl-L-asparaginyl - S - benzyl - L - cysteinyl - L prolyl - L -citrullylglycinamide (XIII).—The benzyloxycarbonyl group was removed from the protected hexapeptide (VIII) (0.72 g.) with HBr in AcOH. The hydrobromide of the resulting free amine was dissolved in dimethylformamide (7 ml.); triethylamine (0.48 ml.) and *p*-nitrophenyl-benzyloxycarbonyl-L-isoleucinate[3] (0.39 g.) were added. Two days later acetic acid (0.3 ml.) and ethyl acetate (50 ml.) were added to the mixture. The product was filtered and washed well with ethyl acetate, ethanol and again with ethyl acetate. The protected heptapeptide amide (0.70 g.) sinters at 206°, m.p. 216–218°, dec. at 224°. A portion (100 mg.) was partially purified by dissolving it in acetic acid (5 ml.) and precipitating by the slow addition of ethanol (50 ml.). The precipitate was washed with ethanol. After drying, it weighed 74 mg. (64%), m.p. 229–232°, dec. at 235°; $[\alpha]^{20}D$ −32° (*c*, 1, dimethylformamide). Further attempts of purification failed; the material was used as such in the next step.

Anal. Calcd. for $C_{46}H_{65}O_{12}N_{12}S$: C, 54.64; H, 6.58; N, 16.62; S, 3.17. Found: C, 53.06; H, 6.77; N, 16.54; S, 3.18.

O-Benzyl-N-benzyloxycarbonyl-L-tyrosyl-L-isoleucyl-L-glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl- L - prolyl - L citrullylglycinamide (XIV).—The crude benzyloxycarbonyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S-benzyl - L - cysteinyl - L - prolyl - L - citrullylglycinamide (XIII) (606 mg.) was converted into the octapeptide derivative in the manner described in the preceding paragraph, using *p*-nitrophenyl O-benzyl-N-benzyloxycarbonyl-L-tyrosinate[3] (394 mg.) as the acylating agent. The product weighed 623 mg., m.p. 235–239° (dec.). A portion (125 mg.) was purified by reprecipitation from acetic acid with ethanol; the recovered material (101 mg., 67%), m.p. 232–243°, dec. at 244°, was still impure, as indicated by analysis; however, no solvent system was found for its purification.

Anal. Calcd. for $C_{62}H_{81}O_{14}N_{13}S$: C, 58.89; H, 6.46; N, 14.40; S, 2.54. Found: C, 57.08; H, 6.44; N, 14.59; S, 2.58.

S-Benzyl-N-benzyloxycarbonyl-L-cysteinyl-L-tyrosyl-L-isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L -cysteinyl - L - prolyl - L - citrullylglycinamide (XV).—The crude octapeptide derivative O-benzyl-N-benzyloxycarbonyl-L-tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S-benzyl - L - cysteinyl - L - prolyl - L - citrullylglycinamide (XIV) (505 mg.) was treated with HBr in AcOH. The resulting hydrobromide was dissolved in dimethylformamide (3 ml.). Triethylamine (0.35 ml.) and S-benzyl-N-benzyloxycarbonyl-L-cysteine *p*-nitrophenyl ester[13] (233 mg.) were added. Three days later the product was isolated from the reaction mixture as described previously. The crude protected nonapeptide (460 mg.), m.p. 236–240° (dec.), was purified by reprecipitation from acetic acid with ethanol. The recovered material (390 mg., 68%), m.p. 245–248°, was used in the preparation of the hormone analog.

Anal. Calcd. for $C_{65}H_{86}O_{15}N_{14}S_2$: C, 57.09; H, 6.34; N, 14.34; S, 4.69. Found: C, 56.49; H, 6.43; N, 14.24; S, 4.36.

8-L-Citrulline Oxytocin (XVI).—Removal of the protecting groups from XV (250 mg.) was performed as described at the conversion of XI into XII; cyclization to the disulfide and isolation of the purified free peptide (88 mg.) were also

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

similar. In the counter-current distribution (500 transfers) $K = 0.16$ was observed; the hormone analog has an R_f value of 0.15 in the solvent systems described by XII; $[\alpha]^{20}D$ −20° (*c*, 1, N acetic acid). Amino acid analysis gives the molar ratios (glutamic acid = 1.0): aspartic acid 1.09, proline 0.92, glycine 0.97, cystine 1.2, isoleucine 0.98, tyrosine 0.90, ammonia 3.8, citrulline 0.77, and ornithine 0.26.

In the assay on the isolated rat uterus the oxytocic potency of XVI is about 500 u./mg., while about 250 u./mg. was exhibited in the avain depressor test. The pressor activity in rat was found to be about 7 u./mg. In the amphibian bladder test XII is only slightly more active than oxytocin and much less active than arginine vasotocin.

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[CONTRIBUTION FROM THE RESEARCH DIVISION OF THE CLEVELAND CLINIC FOUNDATION, CLEVELAND 6, OHIO]

Synthesis of Alanyl⁴-isoleucyl⁵-angiotensin II[1]

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The octapeptide alanyl⁴-isoleucyl⁵-angiotensin II has been synthesized to study further the significance of the aromatic ring in position 4 of the natural peptide. This synthetic peptide possesses about 3% of the pressor activity of isoleucyl⁵-angiotensin II.

Recent studies on analogs and homologs of angiotensin II, the pressor octapeptide L-aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine, have shown the two amino acids with aromatic rings are important to biological activity.[3,4,5] Removal of the aromatic ring of phenylalanine greatly reduces pressor activity.[3] The peptide without the phenolic hydroxyl group of tyrosine, phenylalanyl⁴-angiotensin, has only 2 to 10%[4,6] of the activity of the parent octapeptide. The two aromatic side groups are positioned very close to each other in a conformation recently suggested for angiotensin II.[7] When the position of the two groups relative to each other is changed by the substitution of alanine for proline in position 7,[8] pressor activity is greatly reduced. To study further the importance of the aromatic ring of tyrosine for biological activity, we have synthesized alanyl⁴-isoleucyl⁵-angiotensin II, the octapeptide without this ring in position four.

Experimental[9]

Carbobenzoxy-L-valyl-L-alanine Methyl Ester (I).—To 25.1 g. (100 mmoles) of carbobenzoxy-L-valine in 53 ml. of tetrahydrofuran was added 23.8 ml. of tri-*n*-butylamine. The solution was cooled to −10° and then 9.45 ml. of ethyl chloroformate was added. After stirring for 20 minutes at −10°, 14.0 g. (100 mmoles) of alanine methyl ester hydrochloride and 24.0 ml. of tri-*n*-butylamine in 100 ml. of tetrahydrofuran were added. The mixture was stirred at room temperature for 2 hours and then 200 ml. of water and 200 ml. of ethyl acetate were added. The two liquid phases were separated and the aqueous phase was again extracted with 50 ml. of ethyl acetate. The combined extract then was washed twice with 50 ml. of *N* hydrochloric acid and once with 50 ml. of distilled water. Then 350 ml. of ethyl acetate and 50 ml. of methanol were added to redissolve a precipitate which formed in the organic phase. The organic phase was further washed once with 50 ml. of distilled water, twice with 50 ml. of saturated sodium bicarbonate, and twice with 50 ml. of distilled water. This entire extraction procedure was conducted at 4°. The organic solution was dried over sodium sulfate and the solvent removed *in vacuo*. The residue was dissolved in a minimum of ethyl acetate; then pet. ether (b.p. 30–60°) was added to the point of incipient turbidity. The peptide crystallized on standing to yield 23.3 g. of product, m.p. 162°. An additional 2 g. of product was obtained from the mother liquor (yield 75%). Upon recrystallization as above, a product melting at 162.5–163° was obtained; $[\alpha]^{22}D$ −49.45° (*c*, 1.65, in methanol). *Anal.* Calcd. for $C_{17}H_{24}N_2O_5$: C, 60.70; H, 7.19; N, 8.33. Found: C, 60.70; H, 7.13; N, 8.16.

Carbobenzoxy-L-valyl-L-alanine Hydrazide (II).—To 15 g. of carbobenzoxy-L-valyl-L-alanine methyl ester in 375 ml. of methanol was added 15 ml. of 85% hydrazine hydrate and the solution was heated under reflux for two hours. Upon cooling to room temperature, 1100 ml. of water was added and the solution was allowed to stand at 4° overnight. The crystalline product was collected by filtration and recrystallized from methanol by the addition of water to yield 13.9 g. (92.5%) of peptide melting at 219–222°; $[\alpha]^{22}D$ −57.67° (*c*, 0.86, in methanol). *Anal.* Calcd. for $C_{16}H_{24}N_4O_4$: C, 57.30; H, 7.19; N, 16.66. Found: C, 57.78; H, 7.25; N, 16.90.

Carbobenzoxy-L-valyl-L-alanyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine-*p*-nitrobenzyl Ester (III).—To 10.95 g. (14 mmoles) of carbobenzoxy-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-nitrobenzyl ester[10] in 35 ml. of anhydrous acetic acid was added 30 ml. of freshly prepared 4.2 *N* hydrogen bromide in anhydrous acetic acid. After 50 minutes at room temperature, 900 ml. of anhydrous ether was added to precipitate the peptide ester dihydrobromide. The precipitate was collected by filtration, washed with a large volume of anhydrous ether and dried over sodium hydroxide and phosphorus pentoxide. This material on paper chromatography showed two spots; R_{fBAW} 0.70 and 0.46 (both ninhydrin and Pauly positive), so it was converted to the free base as described below.

(1) This work was supported in part by the National Heart Institute U. S. Public Health Service, Grant H-96.

(2) Research Fellow of the Frank E. Bunts Educational Institute.

(3) I. H. Page and F. M. Bumpus, *Physiological Rev.*, **41**, 331 (1961).

(4) R. Schwyzer, *Helv. Chim. Acta*, **44**, 667 (1961).

(5) F. M. Bumpus, P. A. Khairallah, K. Arakawa, I. H. Page and R. R. Smeby, *Biochim. et Biophys. Acta*, **46**, 38 (1961).

(6) T. B. Paiva and A. C. M. Paiva, *Brit. J. Pharmacol.*, **15**, 557 (1960).

(7) R. R. Smeby, K. Arakawa, F. M. Bumpus and M. M. Marsh, *Biochim. et Biophys. Acta*, **58**, 550 (1962).

(8) J. H. Seu, R. R. Smeby and F. M. Bumpus, *J. Am. Chem. Soc.*, **84**, 3883 (1962).

(9) All melting points were taken on a Kofler hot-stage and are corrected. Microanalyses were done by Micro-Tech Laboratories, Skokie, Ill. Paper chromatograms were developed using butanol:acetic acid:water (4:1:5) abbreviated as R_{fBAW}; acetonitrile:methanol (1:1) abbreviated as R_{fAM}; acetonitrile:1-butanol (1:1) abbreviated as R_{fAB}; dimethylformamide:methanol (1:3) abbreviated as R_{fDM}. The conditions used for paper electrophoresis were: solvent, 95 ml. of acetic acid and 36 ml. of formic acid diluted to 2 liters with distilled water; *p*H 2.1; voltage, 14.8 volts per cm. of paper. Electrophoretic mobilities are reported as the ratio: distance the peptide moved/distance glutamic acid moved and abbreviated as E_G.

(10) K. Arakawa and F. M. Bumpus, *J. Am. Chem. Soc.*, **83**, 728 (1961).