

becomes stronger with an increase in temperature. Thus, if this argument is valid, an increase in temperature (within the range studied here) should result in a tightening of the internal structure of  $\beta$ -lactoglobulin, a greater restriction of motion of the groups within the native molecule, and a greater negative rotation. A denatured protein, on the other hand, behaves in solution essentially like a random coil; an increase in temperature should increase the freedom of orientation of its groups through increased internal rotation<sup>53</sup> and Brownian motion and lead to a decrease in the optical rotation. In connection with this, it is of interest to note that ribonuclease, in which Tanford has found that the stabilization due to the hydrophobic effect is not sufficient to counteract the structure disruptive force of the configurational entropy, has a complicated temperature dependence of  $[\alpha]_D$  in the native state, while this change is in the expected direction for the oxidized and denatured species.<sup>54</sup> An examination of Schellman's rotation data on some other proteins reveals that ovalbumin<sup>55</sup> and chymotrypsin<sup>54</sup> have a temperature dependence similar to that of  $\beta$ -lactoglobulin, although the native chymotrypsin case is complicated by a temperature-dependent transition. Ovalbumin has few if any internal disulfide bonds and therefore may owe most of its stabilization to noncovalent factors. In all cases, Schellman finds a positive temperature coefficient with denatured proteins. In some cases, such as insulin<sup>44</sup> and serum albumin,<sup>55</sup> the picture becomes complicated by aggregations and large conformational changes. It seems significant that nowhere can contradictions of the correlation with hydrophobic effect be found.

**Comparison of the Three Genetic Variants.**—The present optical rotation experiments have failed to show any vast differences between the gross native structures of the three genetic variants. The values of  $b_0$  are identical within experimental error, and indi-

(53) See, for example, M. V. Volkenstein, "Configurational Statistics of Polymeric Chains," Interscience Publishing Corp., New York, N. Y., 1963.

(54) J. A. Schellman, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **30**, 439, 450 (1958).

(55) J. A. Schellman, *ibid.*, **30**, 429 (1958).

cate little "helix" content in any of the three. Changes of  $a_0$  follow quantitatively known association effects. In the pH region between 4.5 and 5.5 all three undergo a small transition in  $a_0$ , which corresponds to Tiselius electrophoretic behavior, at least with  $\beta$ -B.<sup>36</sup> In some experiments it was found that  $\beta$ -C is more susceptible to structural changes than either of the other two variants. At low pH, high salt concentrations lead to a continuous increase in  $a_0$  (Fig. 6). Thus, while optical rotatory dispersion suggests great similarity of the three native isoelectric structures, in some respects it also indicates that  $\beta$ -C is less stable than the other two. A detailed study, aimed at elucidating further the differences between  $\beta$ -C and the other two better known variants, as well as the various structural changes found with changes in media, is presently under way in our laboratory.

### Conclusions

The quantitative agreement between the rotatory dispersion parameters and the associations of the well characterized  $\beta$ -lactoglobulin system suggests the use of this technique in the study of other protein-protein interactions (*e.g.*, enzyme-substrate complex formation, antigen-antibody reaction, and the functional association of proteins made up of subunits). It should be possible to use the  $a_0$  parameter in conjunction with other criteria of association which by themselves are insufficient to give quantitative answers. For example, while in the present case light-scattering equilibrium constants were available, the entire analysis could have been carried out by a combination of the changes in  $a_0$  with Gilbert analysis<sup>10,56</sup> of sedimentation data when the system is in a state of rapid re-equilibration. Moreover, as in the case of the association of  $\beta$ -lactoglobulin, the magnitude and sign of the change in  $a_0$  can lead to useful information about the geometry of the association.

**Acknowledgment.**—The authors wish to thank Miss Linda Mescanti for her very able performance of the spectropolarimetry measurements.

(56) G. A. Gilbert, *Proc. Roy. Soc. (London)*, **A250**, 377 (1959).

[CONTRIBUTION FROM THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH, NEW BRUNSWICK, NEW JERSEY]

## Synthesis of Arginine-Containing Peptides through Their Ornithine Analogs. Synthesis of Arginine Vasopressin, Arginine Vasotocin, and L-Histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine<sup>1</sup>

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A method for the preparation of arginine-containing peptides by selective guanylation of their partially protected ornithine analogs is demonstrated in the synthesis of arginine vasopressin, arginine vasotocin, and L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine. The ornithine analogs and their biological activities are also described.

The possibility of synthesizing arginine-containing peptides through guanylation of their ornithine analogs was proposed by Fruton as early as 1949.<sup>2</sup> Since then arginine-containing peptides have indeed been prepared

by such an approach<sup>3,4</sup>; however, to our best knowledge no syntheses of ornithine-containing peptide chains have been reported where the choice of protecting groups would allow selective guanylation of the  $\delta$ -amino

(1) These studies were presented, in preliminary form, at the Sixth European Peptide Symposium in Athens, Sept. 16, 1963.

(2) J. S. Fruton, *Advan. Protein Chem.*, **5**, 64 (1949); *cf.* also H. N. Christensen, *J. Biol. Chem.*, **160**, 75 (1945).

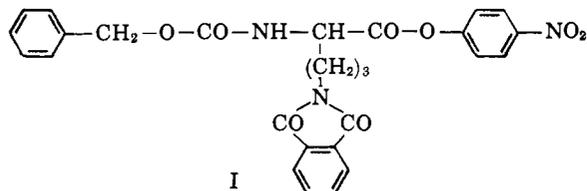
(3) E. Katchalski and P. Spitnik, *Nature*, **164**, 1092 (1949); *J. Am. Chem. Soc.*, **73**, 3992 (1951).

(4) B. C. Barrass and D. T. Elmore, *J. Chem. Soc.*, 3134 (1957).

group of ornithine at an appropriate stage of the synthetic procedure.

The fulfillment of several conditions seems to be necessary to permit the selective guanylation mentioned above. The protecting group on the  $\delta$ -amino group of the ornithine moiety should be stable under conditions which are commonly used for the removal of  $\alpha$ -amino protecting groups, *e.g.*, should resist hydrogenolysis, treatment with hydrobromic acid, etc. On the other hand, the same  $\delta$ -amino protection has to be selectively removable. This means that, at the point when guanylation becomes desirable, the removal of the  $\delta$ -amino protection should expose only the amino group or groups which are to be guanylated and leave other amino groups still protected. Finally, it has to be remembered that some derivatives of ornithine which can act as acylating agents in peptide synthesis show a definite tendency to cyclize to substituted piperidones.<sup>5,6</sup>

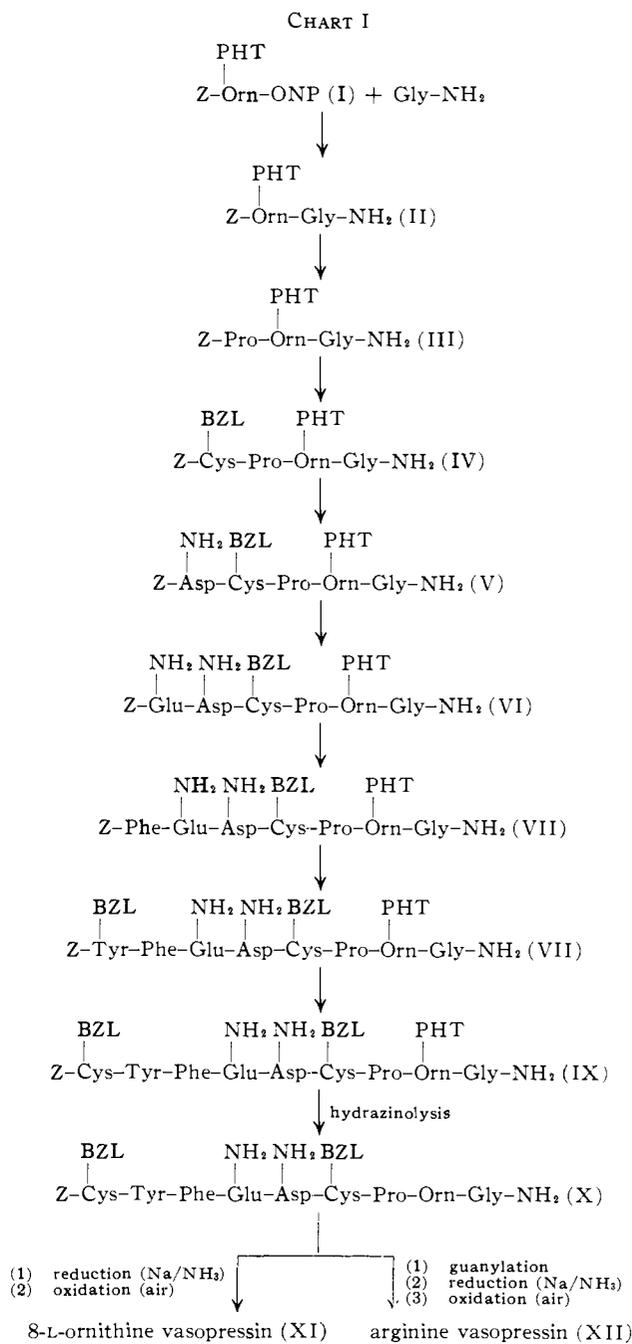
The phthalyl group fulfills these requirements. It resists hydrogenolysis and treatment with hydrobromic acid in acetic acid, and its removal by hydrazinolysis does not affect protecting groups such as benzoxycarbonyl or *t*-butoxycarbonyl groups. Moreover, if ornithine is applied in the form of a  $\delta$ -diacyl derivative, no lactam formation can occur. For these reasons,  $\alpha$ -benzloxycarbonyl- $N^{\delta}$ -phthalyl-L-ornithine *p*-nitrophenyl ester (I) was prepared and used as a key intermediate for the synthesis of arginine-containing peptides.



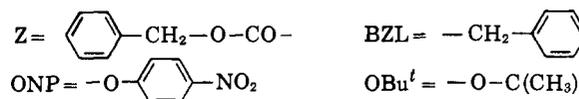
Three such peptides, all biologically active, were chosen as examples for these studies: arginine vasopressin,<sup>7</sup> arginine vasotocin,<sup>8</sup> and a pentapeptide sequence common to the melanophore-stimulating and the adrenocorticotrophic hormones, namely, L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine.<sup>9</sup> The corresponding three ornithine analogs were prepared and guanylated while still in partially protected form.

The protected nonapeptide intermediate in the synthesis of 8-L-ornithine vasopressin<sup>10</sup> was built up in a stepwise manner<sup>11</sup> starting from the C-terminal amino acid, glycine. The nitrophenyl ester method<sup>12</sup> was used for all coupling steps. Therefore, the present synthesis of ornithine vasopressin is quite similar to the

stepwise synthesis of lysine vasopressin<sup>13</sup> except that while in the case of lysine vasopressin the C-terminal amide was introduced by ammonolysis of the (protected) C-terminal tetrapeptide ester, in the present study the building of the chain was started with glycine-amide. This change was introduced in order to avoid the ammonolytic opening of the phthalimide ring on the  $\delta$ -amino group of the ornithine moiety. The synthesis proceeded uneventfully through intermediates which were obtained in good yields and in most cases in crystalline form, and it is summarized in Chart I.



The following abbreviations are used



(5) J. Rudinger, *Collection Czech. Chem. Commun.*, **24**, 95 (1959).

(6) M. Bodanszky and C. A. Birkhimer, *Chimia*, **14**, 368 (1960).

(7) V. du Vigneaud, H. C. Lawler, and E. A. Popenoe, *J. Am. Chem. Soc.*, **75**, 4880 (1953).

(8) P. G. Katsoyannis and V. du Vigneaud, *J. Biol. Chem.*, **233**, 1352 (1958).

(9) K. Hofmann, M. E. Woolner, G. Spuhler, and E. T. Schwartz, *J. Am. Chem. Soc.*, **80**, 1486 (1958); K. Hofmann, M. E. Woolner, H. Yajima, G. Spuhler, T. A. Thompson, and E. T. Schwartz, *ibid.*, **80**, 6458 (1958); R. Schwyzler and C. H. Li, *Nature*, **182**, 1669 (1958).

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

(11) M. Bodanszky and V. du Vigneaud, *Nature*, **183**, 1324 (1959); *J. Am. Chem. Soc.*, **81**, 5688 (1959).

(12) M. Bodanszky, *Nature*, **175**, 685 (1955); *cf.* also M. Bodanszky, *Ann. N. Y. Acad. Sci.*, **88**, 655 (1960).

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

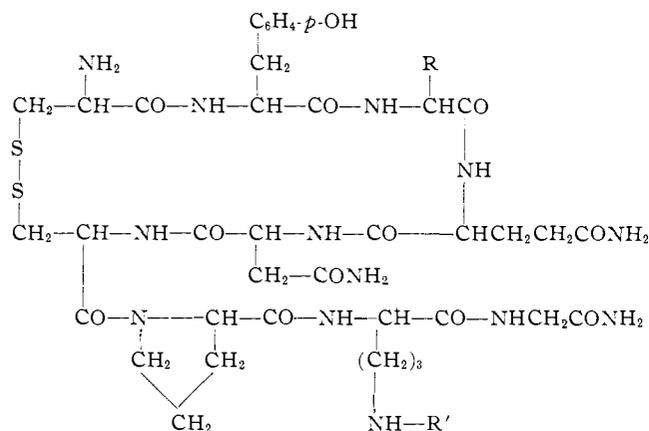


Fig. 1.—8-L-Ornithine vasopressin: R = CH<sub>2</sub>C<sub>6</sub>H<sub>5</sub>, R' = H; arginine vasopressin: R = CH<sub>2</sub>C<sub>6</sub>H<sub>5</sub>, R' = C(=NH)NH<sub>2</sub>; 8-L-ornithine vasotocin: R = CH(CH<sub>3</sub>)CH<sub>2</sub>CH<sub>3</sub>, R' = H; arginine vasotocin: R = CH(CH<sub>3</sub>)CH<sub>2</sub>CH<sub>3</sub>, R' = C(=NH)NH<sub>2</sub>.

The fully protected nonapeptide N-benzoxycarbonyl-S-benzyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N<sup>δ</sup>-phthalyl-L-ornithylglycinamide (IX) was treated with hydrazine (3 moles) in dimethylformamide at room temperature. A sample of the dephthalylated nonapeptide was reduced with sodium in liquid ammonia to give, after oxidation to the disulfide, 8-L-ornithine vasopressin. Another sample was treated with 1-guanyl-3,5-dimethylpyrazole<sup>14</sup> to guanylate the ornithine moiety. The arginine-containing protected nonapeptide was then reduced with sodium in liquid ammonia, and the reduction product was oxidized to the cyclic disulfide, arginine vasopressin (Chart I). A sample of the hexapeptide intermediate, benzoxycarbonyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N<sup>δ</sup>-phthalyl-L-ornithylglycinamide (VI), was treated with hydrobromic acid in acetic acid to remove the benzoxycarbonyl group, and then the hexapeptide amide was lengthened stepwise by addition of isoleucine, tyrosine, and S-benzylcysteine. In this way the protected nonapeptide XV, which corresponds to 8-L-ornithine vasotocin, was obtained. From this protected peptide both 8-L-ornithine vasotocin and arginine vasotocin were prepared in the way described above for arginine vasopressin and its ornithine analog (Chart II). The hormones and hormone analogs<sup>15</sup> were purified by conventional methods. Their biological activities are described in the experimental part.

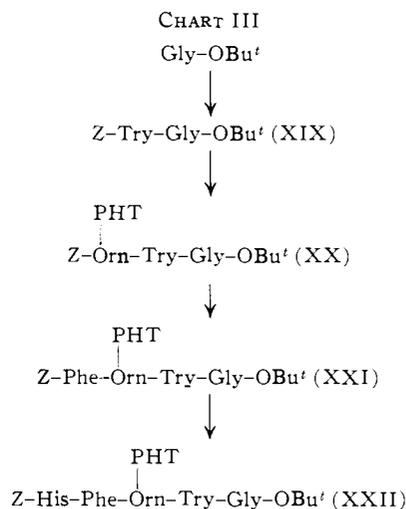
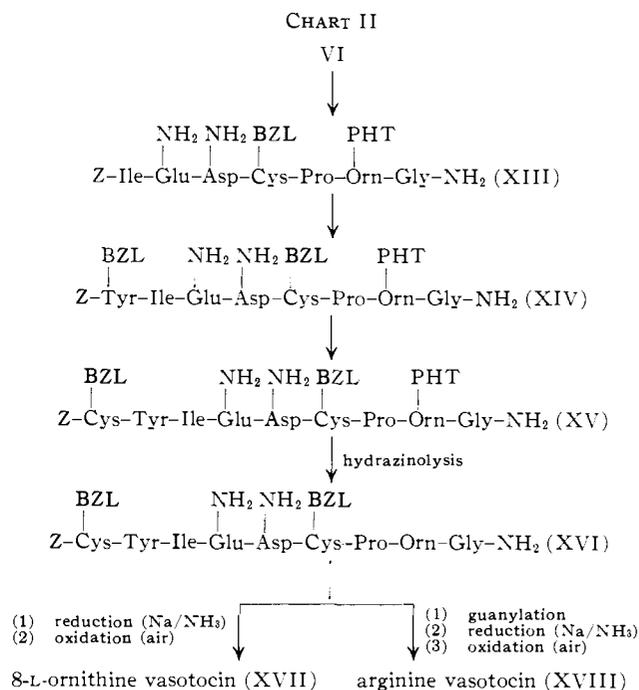
The synthesis of the protected pentapeptide benzoxycarbonyl-L-histidyl-L-phenylalanyl-N<sup>δ</sup>-phthalyl-L-ornithyl-L-tryptophylglycine *t*-butyl ester (XXII) was also achieved by the stepwise approach starting from the C-terminal (Chart III). In the first two steps the nitrophenyl ester<sup>12</sup> and the dicyclohexylcarbodiimide<sup>16</sup> methods lead to identical products with practically equal yields. The phenylalanine moiety was introduced by the nitrophenyl ester method and the histidine moiety by the azide method.<sup>17</sup>

(14) A. F. S. A. Habeeb, *Can. J. Biochem. Physiol.*, **38**, 493 (1960).

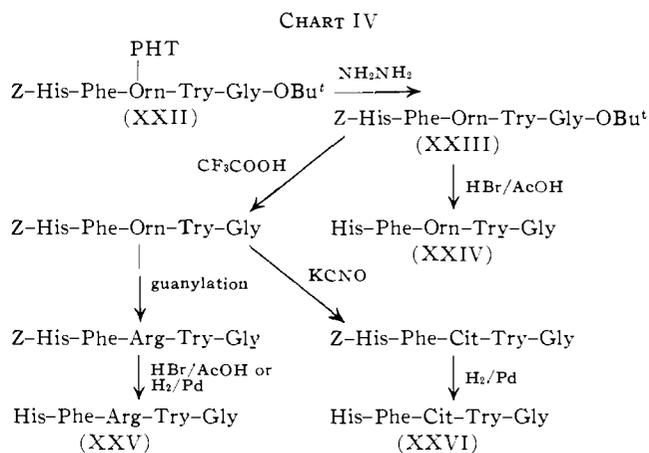
(15) The hormone analogs 8-L-ornithine vasopressin and 8-L-ornithine vasotocin were prepared independently also by R. L. Huguenin and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1669 (1963).

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

(17) (a) T. Curtius, *Ber.*, **35**, 3226 (1902); (b) R. W. Holley and E. Sondheimer, *J. Am. Chem. Soc.*, **76**, 1326 (1954).



The sequence of reactions leading to the free ornithine and arginine pentapeptides is shown in Chart IV. The



guanylation step was performed after removal of the *t*-butyl ester because in this way a product of better purity was obtained, and the yield was also higher.

All the reactions involving acids were carried out under an atmosphere of nitrogen to minimize the destruction of the tryptophan moiety.

The ornithine-containing pentapeptide was converted also into the corresponding citrulline derivative with potassium cyanate (Chart IV), albeit in low yield. The product was shown to be identical with the pentapeptide,<sup>18</sup> which was prepared by stepwise synthesis, using benzoxycarbonyl-L-citrulline *p*-nitrophenyl ester to incorporate the citrulline moiety. Purification, properties, and biological activities of the free pentapeptides are described in the Experimental part.

### Experimental

Melting points are uncorrected.

**Copper Complex of  $\delta$ -Phthalylornithine.**—Ornithine monohydrochloride (16.85 g.) was dissolved in water (175 ml.) containing sodium hydroxide (8.0 g.). Cupric sulfate pentahydrate (12.5 g.) in water (175 ml.) was mixed with the above solution producing a deep blue color. Sodium bicarbonate (10 g.) and carbethoxyphthalimide<sup>19</sup> (25 g.) were added and the reaction mixture was stirred for 0.5 hr. The blue precipitate which appeared was washed with water, ethanol, chloroform, and ether, in that sequence. The product weighed 28 g. (94%).

**$\delta$ -Phthalyl-L-Ornithine Hydrochloride.**—The copper salt of phthalylornithine (5.0 g.) was powdered and stirred with 6 *N* hydrochloric acid (25 ml.) for 1 hr. It was filtered on a fritted glass filter and washed with 6 *N* hydrochloric acid until the filtrate (50 ml.) was nearly colorless. The crude hydrochloride was sucked "dry" on the filter. A sample (0.5 g.) was dissolved in methanol (7.5 ml.). The solution was diluted with ethyl acetate; the crystalline precipitate was washed with ethyl acetate and dried; it weighed 0.42 g., m.p. 223–224° dec. (sinters at 220°).

*Anal.* Calcd. for C<sub>13</sub>H<sub>13</sub>ClN<sub>2</sub>O<sub>4</sub>: C, 52.3; H, 5.1; Cl, 11.9; N, 9.4. Found: C, 52.4; H, 5.2; Cl, 11.0; N, 9.3.

**$\delta$ -Phthalyl-L-ornithine.**—To a solution of phthalyl-L-ornithine hydrochloride (0.50 g.) in water (10 ml.) a 0.5 *N* solution of potassium bicarbonate (4.5 ml.) was added. The pH was adjusted to about neutral by the addition of a drop of acetic acid. The long needles which separated from the solution were filtered and washed with water. The product (0.32 g.) sinters at 215° and melts with decomposition at 217–218°. Recrystallization from water–ethanol gave 0.13 g. of a product which shrinks at 215° and melts with decomposition at 220–221°. In butanol–acetic acid–water (4:1:5)  $\delta$ -phthalyl-L-ornithine shows an *R<sub>f</sub>* value of 0.5. The material is a monohydrate; loss of weight on drying 6.9% (calculated 6.4%).

*Anal.* Calcd. for C<sub>13</sub>H<sub>14</sub>N<sub>2</sub>O<sub>5</sub>: C, 59.5; H, 5.4; N, 10.7. Found: C, 59.6; H, 5.5; N, 10.5.

**N $\alpha$ -Benzoxycarbonyl-N $\delta$ -phthalyl-L-ornithine.**—The crude hydrochloride (from 5.0 g. of copper complex) was dissolved in water (250 ml.) and a solution of potassium bicarbonate (0.5 *N*) was added until the solution was neutral and then an additional 100 ml. was added. Carbobenzoxy chloride (2.9 ml.) was added and the mixture was stirred vigorously for 3 hr. Sodium bicarbonate (1.5 g.) was added and the stirring was continued for another 4 hr. The reaction mixture was extracted with ethyl acetate (500 ml.) and the ethyl acetate was in turn extracted with 0.5 *N* potassium bicarbonate. The two aqueous phases were combined and acidified with 6 *N* hydrochloric acid. An oil appeared and soon crystallized. The product was disintegrated, filtered, and washed well with water. The N $\alpha$ -benzoxycarbonyl-N $\delta$ -phthalyl-L-ornithine weighed 4.47 g. (66%), m.p. 129–131° with sintering at 125.5°. Recrystallization from ethyl acetate–ether–hexane did not change the melting point.

*Anal.* Calcd. for C<sub>21</sub>H<sub>30</sub>O<sub>6</sub>N<sub>2</sub>: C, 63.6; H, 5.1; N, 7.1. Found: C, 63.7; H, 5.1; N, 7.3.

**N $\alpha$ -Benzoxycarbonyl-N $\delta$ -phthalyl-L-ornithine *p*-Nitrophenyl Ester (I).**—N $\alpha$ -Benzoxycarbonyl-N $\delta$ -phthalyl-L-ornithine (12 g.) was dissolved with heating in a solution of *p*-nitrophenol (4.8 g.) in ethyl acetate (120 ml.). The solution was cooled in an ice bath and dicyclohexylcarbodiimide (6.18 g.) was added. After 0.5 hr. it was allowed to come to room temperature. Acetic

acid (0.6 ml.) was added 3.5 hr. later; after 10 min. the dicyclohexylurea was filtered off and washed with ethyl acetate (60 ml.). The precipitate was extracted with ethyl acetate (four 50-ml. portions) to separate any of the product which might have precipitated with it. The mother liquor and the extracts were combined and evaporated *in vacuo*. The residue was suspended in 95% ethanol, transferred to a filter, and washed with 95% ethanol. The dry product weighed 13.66 g. (88%), m.p. 133–135°. A recrystallized sample melted at 135–137°, [ $\alpha$ ]<sub>D</sub><sup>20</sup> –34° (*c* 2, dimethylformamide + 1% acetic acid).

*Anal.* Calcd. for C<sub>27</sub>H<sub>23</sub>N<sub>3</sub>O<sub>8</sub>: C, 62.7; H, 4.5; N, 8.1. Found: C, 62.7; H, 4.5; N, 7.9.

**Benzoxycarbonylglycinamide.**—To a solution of *p*-nitrophenyl benzoxycarbonylglycinate<sup>20</sup> (28.05 g.) in chloroform (100 ml.) a saturated solution of ammonia in methanol (30 ml.) was added. After 2 hr. at room temperature the precipitate which formed was filtered and washed with chloroform. The crude amide was recrystallized from ethyl acetate (500 ml.) to give 13.7 g. of purified material, m.p. 136–138°. Evaporation of the chloroform mother liquor gave a second crop, which was recrystallized from ethyl acetate; 0.5 g., m.p. 136–137.5°. The mother liquor from the recrystallization of the main crop yielded an additional 0.8 g. with similar melting point. The three crops were combined (15 g.) and recrystallized from 95% ethanol (65 ml.). The crystals were washed with 95% ethanol and with ethyl acetate. The pure material (14.1 g., 80%) melted at 137–138.5°.

*Anal.* Calcd. for C<sub>10</sub>H<sub>12</sub>N<sub>2</sub>O<sub>3</sub>: C, 57.7; H, 5.8; N, 13.5. Found: C, 57.9; H, 5.9; N, 13.9.

**Glycinamide Hydrobromide.**—To a solution of benzoxycarbonylglycinamide (13.3 g.) in acetic acid (150 ml.) a 4 *N* solution of hydrogen bromide in acetic acid (150 ml.) was added. After 1 hr. at room temperature ether (600 ml.) was added; the hydrobromide was collected, washed with ether, and dried in a desiccator over sodium hydroxide pellets overnight. Recrystallization from methanol–ether gave 10 g. of purified material. An analytical sample was recrystallized a second time, m.p. 199–203°.

*Anal.* Calcd. for C<sub>2</sub>H<sub>7</sub>BrN<sub>2</sub>O: Br, 51.6; N, 18.1. Found: Br, 51.4; N, 17.9.

**N $\alpha$ -Benzoxycarbonyl-N $\delta$ -phthalyl-L-ornithylglycinamide (II).**—Glycinamide hydrobromide (7.6 g.) was dissolved in dimethylformamide (120 ml.). The solution was neutralized with triethylamine and more triethylamine (6 ml.) was added, followed by N $\alpha$ -benzoxycarbonyl-N $\delta$ -phthalyl-L-ornithine *p*-nitrophenyl ester (I, 20.7 g.). The next day acetic acid was added until the reaction mixture was neutral and then it was poured onto ice and diluted with water (about 300 ml.). The precipitate was collected and washed well with water. The dry product weighed 18.16 g., m.p. 186–192° with sintering at 180°. It was recrystallized from 95% ethanol (400 ml.). The crystals were washed with 95% ethanol and with ethyl acetate. The weight of this product was 13.59 g.; it sinters at 186°, melts at 192.5–195°. A second crop obtained by concentration of the recrystallization mother liquor weighed 2.23 g., m.p. (sinters at 186°) 190–193.5°, [ $\alpha$ ]<sub>D</sub><sup>21</sup> +7° (*c* 2, dimethylformamide + 1% acetic acid). The total yield was 87%.

*Anal.* Calcd. for C<sub>23</sub>H<sub>24</sub>N<sub>4</sub>O<sub>6</sub>: C, 61.1; H, 5.4; N, 12.4. Found: C, 61.3; H, 5.5; N, 12.4.

**Benzoxycarbonyl-L-prolyl-N $\delta$ -phthalyl-L-ornithylglycinamide (III).**—N $\alpha$ -Benzoxycarbonyl-N $\delta$ -phthalyl-L-ornithylglycinamide (II, 10.4 g.) was dissolved in warm acetic acid (57 ml.) and a 40% solution of hydrogen bromide in acetic acid (57 ml.) was added. After 1 hr. at room temperature the salt was precipitated by the addition of ether (1700 ml.). The precipitate was filtered off, washed with ether, and dried briefly *in vacuo* before dissolution in dimethylformamide (57 ml.). Triethylamine (8.5 ml.) was added, followed by N-benzoxycarbonyl-L-proline *p*-nitrophenyl ester<sup>11</sup> (9.3 g.). The reaction mixture was left at room temperature overnight. The mixture was neutralized with acetic acid, poured onto ice, and diluted with water to 1 l. An oil appeared, a small aliquot of which was crystallized from ethyl acetate and used to seed the rest. The crystals were collected, washed with water, and dried *in vacuo* over phosphorus pentoxide. A suspension of the crystals in ethyl acetate (450 ml.) was boiled, cooled, and filtered, to yield 10.8 g. (86%), m.p. 195–198°. Recrystallization of a sample from 95% ethanol raised the m.p. to 198–199°, [ $\alpha$ ]<sub>D</sub><sup>21</sup> –29° (*c* 2, dimethylformamide + 1% acetic acid).

(18) M. Bodanszky, M. A. Ondetti, B. Rubin, J. J. Piala, J. Fried, J. T. Sheehan, and C. A. Birkhimer, *Nature*, **194**, 485 (1962).

(19) G. H. L. Nefkens, G. I. Tesser, and R. J. F. Nivard, *Rec. Trav. Chim.*, **79**, 688 (1960).

(20) M. Bodanszky and V. du Vigneaud, *Biochem. Prepn.*, **9**, 110 (1962).

*Anal.* Calcd. for  $C_{28}H_{41}N_5O_7$ : C, 61.2; H, 5.7; N, 12.7. Found: C, 61.2; H, 5.7; N, 12.5.

**S-Benzyl-N-benzoxycarbonyl-L-cysteinyl-L-prolyl-N<sup>δ</sup>-phthalyl-L-ornithylglycinamide (IV).**—Benzoxycarbonyl-L-prolyl-N<sup>δ</sup>-phthalyl-L-ornithylglycinamide (III, 27.5 g.) was dissolved in warm acetic acid (125 ml.), cooled, and hydrogen bromide in acetic acid (125 ml.) was added. One hour later the hydrobromide was precipitated and washed with ether. It was dissolved in dimethylformamide (125 ml.) and neutralized with triethylamine (10 ml.). Three milliliters of the base was added in excess before addition of S-benzyl-N-benzoxycarbonyl-L-cysteine *p*-nitrophenyl ester<sup>11</sup> (29.2 g.). After 2.75 hr. the reaction mixture was acidified with acetic acid (5 ml.) and poured onto ice. It was diluted with water to 2 l. and seeded. The product was collected, washed with water (about 1 l.), and dried (42 g.). It was then boiled with ethyl acetate (1 l.), and when cool, it was collected and washed with ethyl acetate. It weighed 31.5 g. (85%), m.p. (sinters at 170°) 175–178°. Recrystallization from 95% ethanol (2 l.) yielded 29.5 g. of crystals (79%), m.p. 177–180°. Further recrystallization did not substantially change the melting point;  $[\alpha]^{20}_D -39^\circ$  (*c* 2, dimethylformamide + 1% acetic acid).

*Anal.* Calcd. for  $C_{38}H_{42}N_6O_8S$ : C, 61.4; H, 5.7; N, 11.3; S, 4.3. Found: C, 61.7; H, 5.7; N, 11.6; S, 4.6.

**Benzoxycarbonyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N<sup>δ</sup>-phthalyl-L-ornithylglycinamide (V).**—S-Benzyl-N-benzoxycarbonyl-L-cysteinyl-L-prolyl-N<sup>δ</sup>-phthalyl-L-ornithylglycinamide (IV, 2.88 g.) was dissolved in warm acetic acid (20 ml.). Hydrogen bromide in acetic acid (20 ml.) was added and the reactants were left at room temperature for 1 hr. before the hydrobromide was precipitated with 20 volumes of ether. The salt was filtered on a fritted glass filter, washed with ether, and then dissolved on the filter with dimethylformamide (10 ml.). The solution was neutralized with triethylamine (2 ml.) and 0.6 ml. was added in excess, followed by benzoxycarbonyl-L-asparagine *p*-nitrophenyl ester<sup>11</sup> 3.10 g., 100% excess. Within an hour the reaction mixture had solidified. After 2 hr. it was acidified with acetic acid (6 ml.) and the product was disintegrated in 95% ethanol (100 ml.), filtered off, boiled with ethyl acetate (100 ml.), and washed on the filter with acetone (75 ml.) and hot acetone (25 ml.). The product weighed 3.0 g. (88%), m.p. (sinters at 210°) 213–215°. A sample recrystallized from dimethylformamide (containing 1% acetic acid) and ethyl acetate melted at 213–216.5°,  $[\alpha]^{20}_D -48^\circ$  (*c* 2, acetic acid).

*Anal.* Calcd. for  $C_{42}H_{48}N_{10}O_{10}S$ : C, 58.9; H, 5.7; N, 13.1; S, 3.7. Found: C, 58.5; H, 5.8; N, 13.2; S, 3.9.

**Benzoxycarbonyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N<sup>δ</sup>-phthalyl-L-ornithylglycinamide (VI).**—The protected pentapeptide V (5.87 g.) was dissolved in warm acetic acid (35 ml.), cooled, and hydrogen bromide in acetic acid (35 ml.) was added. One hour later, the hydrobromide was precipitated by addition of ether (600 ml.) and the precipitate was washed with ether and dissolved in dimethylformamide (35 ml.). The solution was neutralized with triethylamine (3.5 ml.), and an excess (0.9 ml.) of the base was added, followed by benzoxycarbonyl-L-glutamine *p*-nitrophenyl ester<sup>11</sup> (3.43 g., 25% excess). The reaction mixture solidified in about 1 hr. After 3 hr., it was acidified with acetic acid (3.5 ml.) and disintegrated under 95% ethanol (175 ml.), filtered off, and washed with 100 ml. each of 95% ethanol, hot acetone, ethyl acetate, and hot ethyl acetate. The product weighed 6.13 g. (91%), m.p. 203–213°. A sample crystallized from 95% ethanol melted at 204–210°,  $[\alpha]^{20}_D -60^\circ$  (*c* 2, acetic acid). It is a monohydrate.

*Anal.* Calcd. for  $C_{47}H_{58}N_{10}O_{13}S$ : C, 56.3; H, 5.8; N, 14.0; S, 3.2, loss of wt., 1.8. Found: C, 56.2; H, 6.2; N, 13.8; S, 3.3, loss of wt. on drying at 130°, 2.6.

**Benzoxycarbonyl-L-phenylalanyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N<sup>δ</sup>-phthalyl-L-ornithylglycinamide (VII).**—The protected hexapeptide VI (4.0 g.) was treated with hydrogen bromide in acetic acid in the usual manner to remove the benzoxycarbonyl group. The hydrobromide was dissolved in dimethylformamide (24 ml.), the solution was neutralized with triethylamine (2 ml.) and an excess of 0.6 ml. was added. Benzoxycarbonyl-L-phenylalanine *p*-nitrophenyl ester<sup>13</sup> (1.05 g.) was added. In about 1 hr. the reaction mixture had solidified. After 3.5 hr. it was acidified with acetic acid (2 ml.) and triturated with 95% ethanol (100 ml.). The precipitate was collected and washed with 60 ml. each of 95% ethanol, acetone, ethyl acetate, and hot ethyl acetate. The product weighed 4.57 g. (quantitative yield), m.p. (sinters at 207°) 214–220°. Recrystallization

from 80% ethanol yielded a crystalline product melting at 219–225°,  $[\alpha]^{20}_D -47^\circ$  (*c* 2, acetic acid).

*Anal.* Calcd. for  $C_{56}H_{68}N_{11}O_{13}S$ : C, 59.4; H, 5.8; N, 13.6; S, 2.8. Found: C, 59.3; H, 5.9; N, 13.8; S, 2.9.

**O-Benzyl-N-benzoxycarbonyl-L-tyrosyl-L-phenylalanyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N<sup>δ</sup>-phthalyl-L-ornithylglycinamide (VIII).**—The protected heptapeptide VII (2.26 g.) was treated with acetic acid (10 ml.) and hydrogen bromide in acetic acid (10 ml.) at room temperature for 1 hr. Ether (about 400 ml.) was added. The precipitate was collected on a fritted glass filter, washed with ether, and dissolved on the filter with dimethylformamide (10 ml.). After neutralization of the solution with triethylamine (1.0 ml.), an excess of the base (0.3 ml.) was added, followed by O-benzyl-N-benzoxycarbonyl-L-tyrosine *p*-nitrophenyl ester<sup>11</sup> (1.32 g.). Four hours later, the now semisolid reaction mixture was acidified with acetic acid (1 ml.) and disintegrated in 95% ethanol (50 ml.). It was filtered and washed with 30 ml. each of 95% ethanol, acetone, ethyl acetate, and hot ethyl acetate. The crystalline product weighed 2.6 g. (94%), m.p. 232–236°. Recrystallization from 80% ethanol did not change the melting point;  $[\alpha]^{20}_D -39^\circ$  (*c* 2, acetic acid).

*Anal.* Calcd. for  $C_{72}H_{80}N_{12}O_{16}S$ : C, 62.4; H, 5.8; N, 12.1; S, 2.3. Found: C, 63.1; H, 6.2; N, 12.2; S, 2.4.

**S-Benzyl-N-benzoxycarbonyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N<sup>δ</sup>-phthalyl-L-ornithylglycinamide (IX).**—The benzoxycarbonyl group was removed from the protected octapeptide VIII (2.77 g.) in the usual manner. The hydrobromide was dissolved in dimethylformamide (10 ml.) and neutralized with triethylamine (1.2 ml.), with an excess (0.4 ml.) also being added. S-Benzyl-N-benzoxycarbonyl-L-cysteine *p*-nitrophenyl ester<sup>11</sup> (1.17 g.) was added and the mixture was left at room temperature for about 4 hr. Acetic acid (1.2 ml.) was added to the solidified reaction mixture before disintegrating it with 95% ethanol (50 ml.). The product was filtered off and was washed as the protected octapeptide VIII had been. It weighed 2.86 g. (96%), m.p. 222–234°. A sample recrystallized from 80% ethanol melted 230–235°,  $[\alpha]^{20}_D -44^\circ$  (*c* 2, acetic acid).

*Anal.* Calcd. for  $C_{73}H_{83}N_{13}O_{16}S_2$ : C, 60.5; H, 5.8; N, 12.2; S, 4.3. Found: C, 60.5; H, 5.8; N, 12.0; S, 4.6.

**S-Benzyl-N-benzoxycarbonyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N<sup>δ</sup>-phthalyl-L-ornithylglycinamide (X) and 8-L-Ornithine Vasopressin (XI).**—Benzoxycarbonyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N<sup>δ</sup>-phthalyl-L-ornithylglycinamide (IX, 1.5 g.) was dissolved with heating in dimethylformamide (10 ml.). A molar solution of hydrazine (3 ml.) in dimethylformamide was added. After 3 hr. the mixture was acidified with acetic acid (1.5 ml.) and the solvent was removed *in vacuo*. Water (10 ml.) was added to the residue. The next day the precipitate was collected and washed with water. It was dried at 35° over phosphorus pentoxide to a constant weight of 1.57 g. A solution of the partially protected nonapeptide X (0.25 g.) in liquid ammonia (250 ml.) was reduced with sodium until the blue color persisted for about 5 min. Ammonium chloride (0.4 g.) was added; the ammonia was allowed to evaporate. After the removal of traces of ammonia *in vacuo* the residue was dissolved in water (250 ml.) and aerated at pH 6.5 for about 2 hr. A second batch was prepared similarly, except the amounts were doubled. The two aqueous solutions were combined and the peptide was adsorbed on a column (1.9 × 20 cm.) of IRC 50 (XE64) resin. The column was washed with dilute acetic acid (0.25%, 400 ml.) and water (25 ml.). The crude hormone analog was eluted with a mixture of pyridine, acetic acid, and water (30:4:66). The eluate (35 ml.) was evaporated *in vacuo*. A solution of the residue in 0.05 M ammonium acetate buffer (2 ml.) was placed on a carboxymethylcellulose column (3.8 × 28 cm.) with 0.05 M ammonium acetate buffer. The column was eluted using a solution of ammonium acetate of pH 6.5. The elution was started with 0.05 M solution and the concentration of the eluent was gradually increased (in an approximately linear way) to 0.5 M. Fractions of 10 ml. were collected and were scanned by ultraviolet absorption at 280 m $\mu$ . The desired material was found in tubes 90–115. The contents of these tubes were pooled and lyophilized several times until a solid (371 mg.) was obtained. A portion (100 mg.) of this solid was dissolved in a small amount of dilute acetic acid (0.25%) and placed on a column (3 × 30 cm.) of Sephadex G-25. The column was eluted with dilute acetic acid (0.25%), collecting 10-ml. fractions. Ultraviolet absorption at

280 m $\mu$  showed the hormone analog to be in tubes 14–17. These were pooled and lyophilized. On paper chromatograms the product reveals a single spot with  $R_f$  0.1 (butanol-acetic acid-water, 4:1:5);  $R_f$  0.44 (phenol-butanol-acetic acid-water, 20:20:8:40, on paper buffered with a solution of 0.67 M KCl (5 ml) + 0.67 M HCl (10.66 ml.)). Paper electrophoresis shows: triethanolammonium citrate, pH 4.0,  $E_{arg}$  0.63; triethanolammonium acetate, pH 6.3,  $E_{arg}$  0.56;  $[\alpha]^{20}_D - 28^\circ$  ( $c$  2, 1 N acetic acid). Ratio of amino acids shows: Asp, 1.1; Glu, 1.0; Gly, 1.0; Pro, 1.0; Cys, 0.9; Tyr, 0.85; Phe, 1.0; Orn, 1.1.

*Anal.* Calcd., for  $C_{45}H_{63}N_{13}O_{12}S_2 \cdot C_2H_4O_2$ : C, 51.2; H, 6.1; N, 16.5; S, 5.8. Found: C, 51.4; H, 6.2; N, 16.8; S, 5.8.

In the oxytocic assay on the isolated rat uterus<sup>21</sup> 8-L-ornithine vasopressin showed a potency of 14 units per mg. of the base. The pressor activity<sup>22</sup> is 360 units per mg.

**S-Benzyl-N-benzoxycarbonyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide and Arginine Vasopressin (XII).**—The partially protected nonapeptide (X, 1.57 g.) was dissolved in dimethylformamide (10 ml.). A solution of 1-guanyl-3,5-dimethylpyrazole nitrate, (0.60 g.) in dimethylformamide (10 ml.), adjusted to pH 8–9 with triethylamine (0.4 ml.), was added and the pH of the reaction mixture was brought to 8–9 with more triethylamine (0.8 ml.). After 4 days the solvent was removed *in vacuo*, the residue was suspended in water (10 ml.), and the reaction of the suspension was adjusted to pH 5 with acetic acid. The solid was filtered off, washed with water, and dried (1.35 g.). The protecting groups were removed from the partially protected nonapeptide (1.2 g.) by reduction with sodium in liquid ammonia as described for XI. Cyclization to the disulfide and purification of the crude product were also similar. The purified material (0.35 g.) was hygroscopic and was, therefore, dissolved in 0.25% acetic acid, and the solution was kept in the frozen state;  $R_f$  0.16 (butanol-acetic acid-water, 4:1:5);  $R_f$  0.59 (phenol-butanol-acetic acid-water, 20:20:8:40, on paper buffered with a solution of 0.67 M KCl (5 ml.) + 0.67 M HCl (10.66 ml.)). Paper electrophoresis shows: triethanolammonium citrate, pH 4.0,  $E_{arg}$  0.60; triethanolammonium acetate, pH 6.3,  $E_{arg}$  0.49;  $[\alpha]^{20}_D - 21^\circ$  ( $c$  2.2, 1 N acetic acid). Ratio of amino acids shows: Asp, 1.05; Glu, 1.0; Pro, 1.2; Gly, 1.1; Cys, 1.0; Tyr, 0.9; Phe, 1.05; Arg, 1.0.

*Anal.* Calcd. for  $C_{46}H_{65}N_{13}O_{12}S_2 \cdot C_2H_4O_2$ : C, 50.4; H, 6.1; N, 18.4; S, 5.4. Found: C, 50.4; H, 5.4; N, 18.1; S, 5.4.

The synthetic hormone preparation showed 500 units of pressor activity<sup>22</sup> and 17 units of oxytocic activity<sup>21</sup> per mg. of the base.

**Benzoxycarbonyl-L-isoleucyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N<sup>6</sup>-phthalyl-L-ornithylglycinamide (XIII).**—The synthesis of this compound was performed in the same manner as described for compound VII. Starting from VI it was completed with an 83% yield, m.p. 223–230°. Recrystallization from 80% ethanol produced a material melting at 230–232°,  $[\alpha]^{25}_D - 56^\circ$  ( $c$  2, acetic acid).

*Anal.* Calcd. for  $C_{53}H_{67}N_{11}O_{13}S$ : C, 58.0; H, 6.2; N, 14.0; S, 2.9. Found: C, 58.1; H, 6.2; N, 14.1; S, 2.9.

**O-Benzyl-N-benzoxycarbonyl-L-tyrosyl-L-isoleucyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N<sup>6</sup>-phthalyl-L-ornithylglycinamide (XIV).**—The protected octapeptide XIV was obtained through the same methods described above. The yield was 78%, m.p. 234–240°. Two recrystallizations from acetic acid-ethanol produced a purified peptide which melted at 240–245°, after sintering at 236°,  $[\alpha]^{25}_D - 42^\circ$  ( $c$  2, acetic acid).

*Anal.* Calcd. for  $C_{65}H_{82}N_{12}O_{16}S$ : C, 61.3; H, 6.1; N, 12.4; S, 2.4. Found: C, 61.4; H, 6.2; N, 12.3; S, 2.5.

**S-Benzyl-N-benzoxycarbonyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N<sup>6</sup>-phthalyl-L-ornithylglycinamide (XV).**—The techniques employed in the synthesis of the protected nonapeptide amide were identical to those used in the preparation of IX. The product was obtained in 93% yield, m.p. 242–251°. After recrystallization from acetic acid-ethanol, the melting point was 245–253°,  $[\alpha]^{25}_D - 49^\circ$  ( $c$  2, acetic acid). Further recrystallization of a sample from the same solvent raised the melting point to 257–261°.

*Anal.* Calcd. for  $C_{72}H_{87}N_{13}O_{16}S_2$ : C, 59.5; H, 6.0; N, 12.5; S, 4.4. Found: C, 59.5; H, 6.0; N, 12.5; S, 4.4.

(21) J. H. Burn, D. J. Finney, L. G. Goodwin, "Biological Standardization," Oxford University Press, New York, N. Y., 1950, p. 180.

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

**S-Benzyl-N-benzoxycarbonyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-ornithylglycinamide (XVI) and 8-L-Ornithine Vasotocin (XVII).**—The phthalyl group was removed by the same method used for compound X, and compound XVI was obtained in 88% yield. A sample of XVI (620 mg.) was reduced with sodium in liquid ammonia, as described for XI. After cyclization to the disulfide 8-L-ornithine vasotocin (XVII) the product was purified as described for XI. The pure hormone analog (170 mg.) shows  $R_f$  0.20 (butanol-acetic acid-water, 4:1:5);  $R_f$  0.39 (phenol-butanol-acetic acid-water, 20:20:8:40, on paper buffered with a solution of 0.67 M KCl (5 ml.) + 0.67 M HCl (10.66 ml.));  $[\alpha]^{20}_D - 7^\circ$  ( $c$  1.7, 1 N acetic acid). Paper electrophoresis shows: triethanolammonium citrate, pH 4.0,  $E_{arg}$  0.69; triethanolammonium acetate, pH 6.3,  $E_{arg}$  0.55. Ratio of amino acids shows: Asp, 1.0; Glu, 1.0; Pro, 1.1; Gly, 1.0; Cys, 0.9; Ileu, 0.9; Tyr, 0.9; Orn, 1.1. Its potency is 190 units per mg. in the pressor<sup>22</sup> and 90 units per mg. in the oxytocic<sup>21</sup> assay.

*Anal.* Calcd. for  $C_{42}H_{63}N_{13}O_{12}S_2 \cdot C_2H_4O_2$ : C, 49.5; H, 6.5; N, 17.0; S, 6.0. Found: C, 49.6; H, 6.5; N, 16.0; S, 5.8.

**S-Benzyl-N-benzoxycarbonyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide and Arginine Vasotocin (XVIII).**—Guanylation of XVI was performed as described in connection with XII. The guanylated product was produced in 90% yield, m.p. 225–232° (dec.). The protecting groups were removed from this material (0.60 g.) as described and the product of reduction was aerated to form the desired cyclic disulfide. After purification, similar to those described above, a product (0.21 g.) was obtained;  $R_f$  0.1 (butanol-acetic acid-water, 4:1:5);  $R_f$  0.56 (phenol-butanol-acetic acid-water, 20:20:8:40, on paper buffered with a solution of 0.67 M KCl (5 ml.) + 0.67 M HCl (10.66 ml.)). Paper electrophoresis shows: triethanolammonium citrate, pH 4.0,  $E_{arg}$  0.65; triethanolammonium acetate, pH 6.3,  $E_{arg}$  0.53;  $[\alpha]^{20}_D - 5^\circ$  ( $c$  2, 1 N acetic acid). Ratio of amino acids shows: Asp, 1.1; Glu, 1.1; Gly, 1.0; Cys, 0.9; Ileu 0.9; Tyr, 0.8; Arg, 0.9. It corresponded to the expected amino acid composition and had a potency of 120 units of pressor activity<sup>22</sup> and 160 units of oxytocic activity<sup>21</sup> per mg. of the base.

*Anal.* Calcd. for  $C_{43}H_{67}N_{15}O_{12}S_2 \cdot 2C_2H_4O_2$ : C, 48.2; H, 6.5; N, 17.9; S, 5.5. Found: C, 48.9; H, 6.6; N, 17.9; S, 5.3.

***t*-Butyl Benzoxycarbonyl-L-tryptophylglycinate (XIX).**—Benzoxycarbonyl-L-tryptophan *p*-nitrophenyl ester<sup>23</sup> (4.6 g.) and glycine *t*-butyl ester (1.5 g.) were dissolved in ethyl acetate (7.5 ml.) and the solution was kept overnight at room temperature. The reaction mixture was diluted with ethyl acetate (*ca.* 100 ml.) and washed ten times with *N* ammonium hydroxide, once with water, twice with *N* hydrochloric acid, and finally twice with water. The organic layer was dried over magnesium sulfate and the solvent was removed *in vacuo*. The oily residue was dissolved in benzene (10 ml.) and freeze-dried. The solid thus obtained (3.95 g., 88%) was very soluble in methanol, ethanol, benzene, chloroform, acetone, acetonitrile, and ether. It was insoluble in hexane or cyclohexane. From mixtures of ethyl acetate and hexane it separated as an oil. On thin layer chromatography (silica gel, butanol-acetic acid-water, 4:1:1) it showed a single spot which was ultraviolet absorbing and Ehrlich positive. The same product was obtained when benzoxycarbonyl-L-tryptophan was coupled to glycine *t*-butyl ester using dicyclohexylcarbodiimide as a coupling agent, as described by Li, *et al.*<sup>24</sup>;  $[\alpha]^{20}_D - 34^\circ$  ( $c$  2, dimethylformamide).

*Anal.* Calcd. for  $C_{25}H_{29}N_3O_5$ : C, 66.5; H, 6.5; N, 9.3. Found: C, 66.5; H, 6.5; N, 9.3.

***t*-Butyl L-Tryptophylglycinate Oxalate.**—*t*-Butyl benzoxycarbonyl-L-tryptophylglycinate (9 g.) and oxalic acid (0.9 g.) were dissolved in methanol (140 ml.) and hydrogenated for 3 hr. in the presence of a 10% palladium on charcoal catalyst (1 g.). The catalyst was removed by filtration and the filtrate was concentrated *in vacuo* to about 5 ml. Oxalic acid (1 g.) in methanol (10 ml.) was added and the solution was diluted with ether (*ca.* 300 ml.) to precipitate the crystalline oxalate which was filtered and washed with ether; yield, 6.7 g. (72%); m.p. 178–180°;  $[\alpha]^{20}_D + 3^\circ$  ( $c$  2, dimethylformamide).

*Anal.* Calcd. for  $C_{19}H_{25}N_3O_7$ : C, 56.0; H, 6.2; N, 10.4. Found: C, 56.1; H, 6.2; N, 10.6.

(23) Prepared according to the method described in ref. 20; *cf.* also M. Wilchek and A. Patchornik, *J. Org. Chem.*, **28**, 1874 (1963).

(24) C. H. Li, B. Gorup, D. Chung, and J. Ramachandran, *ibid.*, **28**, 178 (1963).

*t*-Butyl  $N^{\alpha}$ -Benzoxycarbonyl- $N^{\delta}$ -phthalyl-L-ornithyl-L-tryptophylglycinate (XX).—Tryptophylglycine *t*-butyl ester oxalate (4.5 g.) was dissolved in methanol (10 ml.) and the solution was passed through a column of the ion-exchange resin Amberlyst XN 1003 (Rohm and Haas Co., Philadelphia, Pa.). The free base was eluted with methanol, and the methanolic solution was concentrated to dryness *in vacuo*. The oily residue was dissolved, together with  $N^{\alpha}$ -benzoxycarbonyl- $N^{\delta}$ -phthalyl-L-ornithine (4 g.), in tetrahydrofuran (50 ml.). A small insoluble residue was removed by filtration and the filtrate was cooled in an ice-water bath. Dicyclohexylcarbodiimide (2.1 g.) was added and the mixture was stirred in the ice-water bath for 6 hr. and then overnight at room temperature. The dicyclohexylurea was filtered off and washed with ethyl acetate (ca. 200 ml.). The filtrate was washed with 1 *N* hydrochloric acid, water, 1 *N* ammonium hydroxide, and again with water. The organic layer was dried over magnesium sulfate. The solvent was removed *in vacuo*, the residue was disintegrated under ether, filtered, and crystallized from chloroform-hexane (6:1) to yield 5.2 g. (74%) of a material, m.p. 123–126°, sintering at 105°. A small sample, recrystallized from chloroform-hexane and dried overnight at room temperature, melted at 123–128° with sintering at 113–116°;  $[\alpha]^{20D} -7^{\circ}$  (*c* 2, dimethylformamide).

Anal. Calcd. for  $C_{38}H_{41}N_5O_8$ : C, 65.6; H, 5.9; N, 10.1. Found: C, 65.2; H, 6.2; N, 10.3.

The same product, in similar yield, was obtained by coupling  $N^{\alpha}$ -benzoxycarbonyl- $N^{\delta}$ -phthalyl-L-ornithine *p*-nitrophenyl ester with tryptophylglycine *t*-butyl ester oxalate in the presence of triethylamine.

*t*-Butyl Benzoxycarbonyl-L-phenylalanyl- $N^{\delta}$ -phthalyl-L-ornithyl-L-tryptophylglycinate (XXI).—*t*-Butyl  $N^{\alpha}$ -benzoxycarbonyl- $N^{\delta}$ -phthalyl-L-ornithyl-L-tryptophylglycinate (7.7 g.) was dissolved in a mixture of acetic acid (90 ml.) and methanol (90 ml.) and hydrogenated for 7 hr. in the presence of 10% palladium on charcoal (1.6 g. at the start and 1.6 g. more after 3 hr.). The catalyst was removed by filtration and the solvents were removed *in vacuo*, the oily residue was diluted with benzene and freeze-dried. The solid thus obtained and benzoxycarbonyl-L-phenylalanyl-*p*-nitrophenyl ester<sup>13</sup> (4.85 g.) were dissolved in pyridine (18 ml.). After 24 hr. at room temperature the reaction mixture was concentrated *in vacuo* to almost dryness, diluted with ethanol, and concentrated again. A crystalline residue was obtained which was suspended in ethyl acetate and re-evaporated. This process was repeated twice more; yield, 7.35 g. (79%); m.p. 167–169°;  $[\alpha]^{20D} -15^{\circ}$  (*c* 2, dimethylformamide).

Anal. Calcd. for  $C_{47}H_{50}N_6O_9$ : C, 67.0; H, 6.0; N, 10.0. Found: C, 67.1; H, 6.1; N, 10.2.

*t*-Butyl  $N^{\alpha}$ -Benzoxycarbonyl-L-histidyl-L-phenylalanyl- $N^{\delta}$ -phthalyl-L-ornithyl-L-tryptophylglycinate (XXII).—*t*-Butyl benzoxycarbonyl-L-phenylalanyl- $N^{\delta}$ -phthalyl-L-ornithyl-L-tryptophylglycinate (1.68 g.) was dissolved in a mixture of acetic acid (45 ml.) and methanol (45 ml.) and hydrogenated in the presence of 10% palladium on charcoal (320 mg. at the start and another 320 mg. after 3 hr.). The catalyst was removed by filtration and the filtrate was concentrated *in vacuo*. The residue, a heavy oil, was diluted with benzene and freeze-dried. The product was dissolved in pyridine (4 ml.) and added to a solution of  $N^{\alpha}$ -benzoxycarbonyl-L-histidine azide in ethyl acetate (prepared from 0.67 g. of  $N^{\alpha}$ -benzoxycarbonyl-L-histidine hydrazide according to Holley and Sondheimer<sup>17b</sup>).

After 24 hr. at 5° the solidified mixture was disintegrated under ether, filtered, and washed with ether. The crude product (1.78 g., m.p. 165–174° sintering at 155°) was purified by counter-current distribution in the system toluene-chloroform-methanol-water, 5:5:8:2. After 50 transfers the distribution train was scanned by weighing the residues of the evaporation of 10-ml. portions withdrawn from each third tube. Only one significant peak was observed (*K* = 0.5); the experimental and theoretical curves were in close agreement. The material isolated from this peak (1.38 g., 71%) melted at 169–174°, sintering from 160°,  $[\alpha]^{20D} -24^{\circ}$  (*c* 2, dimethylformamide).

Anal. Calcd. for  $C_{53}H_{57}N_7O_9$ : C, 64.9; H, 5.9; N, 12.9. Found: C, 64.9; H, 5.9; N, 12.7.

*t*-Butyl  $N^{\alpha}$ -Benzoxycarbonyl-L-histidyl-L-phenylalanyl-L-ornithyl-L-tryptophylglycinate (XXIII).—*t*-Butyl  $N^{\alpha}$ -benzoxycarbonyl-L-histidyl-L-phenylalanyl- $N^{\delta}$ -phthalyl-L-ornithyl-L-tryptophylglycinate (500 mg.) was dissolved in a mixture of methanol (2.5 ml.) and chloroform (1.0 ml.). A 2 *M* methanolic solution of hydrazine (1 ml.) was added and the solution was kept at room temperature for 3 hr. The mixture was acidified with a

4 *M* solution of acetic acid in methanol and cooled overnight. The tetrahydrophthalazine-1,4-dione was removed by filtration and the filtrate was concentrated to dryness *in vacuo*. The residue was extracted with five 10-ml. portions of 20% aqueous acetic acid. The combined extracts were filtered and the filtrate freeze-dried; yield, 430 mg., 99%. This material was used in the next step without further purification.

L-Histidyl-L-phenylalanyl-L-ornithyl-L-tryptophylglycine (XXIV).—*t*-Butyl  $N^{\alpha}$ -benzoxycarbonyl-L-histidyl-L-phenylalanyl-L-ornithyl-L-tryptophylglycinate (250 mg.) was dissolved in glacial acetic acid (5 ml.), and to the solution thus obtained hydrobromic acid in glacial acetic acid (5 ml.) was added. All these operations and the subsequent storage at room temperature for 1 hr. were performed in a nitrogen atmosphere. Ether (100 ml.) was added and the precipitate formed was filtered off and thoroughly washed with ether; yield, 228 mg.

This product (100 mg.) was adsorbed on a column of carboxymethylcellulose (4 g.) and eluted with a linear gradient of ammonium acetate. For this purpose an "Autograd" (Technicon Chromatography Corp., Chauncey, N. Y.) with seven chambers was used. The chambers were filled with the following solutions of ammonium acetate (250 ml. each): 0.01, 0.02, 0.03, 0.04, 0.06, 0.08, and 0.10 *M*. The elution was followed by reading the optical density of the fractions at 280 m $\mu$ . The tubes containing the desired product were pooled and the solution was freeze-dried four times to remove the ammonium acetate; yield, 49 mg.;  $[\alpha]^{20D} -13^{\circ}$  (*c* 1, water); lit.<sup>25</sup>  $-7.8^{\circ}$ . This product was shown to be homogeneous on paper chromatograms (1-butanol-acetic acid-water, 4:1:5, *R<sub>f</sub>* 0.44; isoamyl alcohol-pyridine-water, 7:7:6, *R<sub>f</sub>* 0.46) and on paper electropherograms (triethylammonium citrate pH 4, *E<sub>arg</sub>* 0.59; triethylammonium acetate pH 6.3, *E<sub>arg</sub>* 0.42).

A sample of the product was digested<sup>26</sup> with leucine aminopeptidase (Worthington). No unhydrolyzed peptide was found in the digest, which on quantitative amino acid analysis gave the following ratios of amino acids: Gly:Phe:His:Orn, 1.0:1.0:1.0:1.1.

Anal. Calcd. for  $C_{33}H_{41}N_5O_6 \cdot 0.5H_2O$ : C, 57.7; H, 6.5; N, 18.3. Calcd. for  $C_{33}H_{41}N_5O_6 \cdot C_2H_4O_2$ : C, 58.4; H, 6.3; N, 17.5. Found: C 57.6; H, 6.9; N, 18.6.

L-Histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine (XXV).—*t*-Butyl  $N^{\alpha}$ -benzoxycarbonyl-L-histidyl-L-phenylalanyl-L-ornithyl-L-tryptophylglycinate (240 mg.) was dissolved in trifluoroacetic acid (3 ml.) and the solution was kept at room temperature for 15 min. All these operations were performed in an atmosphere of nitrogen. Ether (50 ml.) was added and the precipitate was removed by filtration, washed thoroughly with ether, and dried *in vacuo* over potassium hydroxide. The  $N^{\alpha}$ -benzoxycarbonyl-L-histidyl-L-phenylalanyl-L-ornithyl-L-tryptophylglycine thus obtained (203 mg.) and 1-guanyl-3,5-dimethylpyrazole nitrate (120 mg.) were dissolved in a mixture of dimethylformamide (1.2 ml.) and triethylamine (0.24 ml.). The solution was kept for 4 hr. at 38–40° and for 60 hr. at room temperature and finally diluted with water (10 ml.). The precipitate was separated by centrifugation and dried *in vacuo* (178 mg.). The benzoxycarbonyl group was removed by dissolving this residue in a mixture of acetic acid (4 ml.) and hydrobromic acid in acetic acid (4 ml.), under nitrogen. After 1 hr. at room temperature the solution was diluted with ether and the precipitate was collected and washed thoroughly with ether.

The crude hydrobromide (190 mg.) was purified by chromatography on a column of carboxymethylcellulose (5 g.) in the way described for XXIV; yield, 95 mg.;  $[\alpha]^{20D} -11^{\circ}$  (*c* 1, hydrochloric acid); lit.<sup>27</sup>  $-11.8^{\circ}$ .

When compared on paper chromatograms (butanol-acetic acid-water, 4:1:5, *R<sub>f</sub>* 0.50; and isoamyl alcohol-pyridine-water, 7:7:6, *R<sub>f</sub>* 0.54) and paper electropherograms (triethylammonium acetate pH 6.3, *E<sub>arg</sub>* 0.37; triethylammonium citrate pH 4, *E<sub>arg</sub>* 0.53), this material was shown to be identical with a sample of L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine prepared in our laboratory by Dr. Saul Lande according to the procedure described in the literature.<sup>27</sup> A sample of this pentapeptide was digested with leucine aminopeptidase as described for XXIV. Paper chromatographic analysis of the digestion mixture

(25) C. H. Li, E. Schnabel, and D. Chung, *J. Am. Chem. Soc.*, **82**, 2062 (1960).

(26) K. Hofmann, H. Yajima, T. Liu, N. Yanaiharu, C. Yanaiharu, and J. Humes, *ibid.*, **84**, 4481 (1962).

(27) K. Hofmann and S. Lande, *ibid.*, **83**, 2286 (1961).

showed only the spots corresponding to histidine, phenylalanine, arginine, tryptophan, and glycine. Quantitative amino acid analysis showed the following ratios: Gly:Phe:His:Arg, 1.0:1.0:1.0:1.0.

**Benzoxycarbonyl-L-tryptophylglycine Ethyl Ester (XXVII).**—To a solution of glycine ethyl ester hydrochloride (1.75 g.) in chloroform (20 ml.), triethylamine (1.8 ml.) was added followed by the addition of *p*-nitrophenyl benzoxycarbonyl-L-tryptophanate<sup>23</sup> (4.6 g.). After standing at room temperature for 3 days (probably a few hours are sufficient), the solvent was removed *in vacuo*, the residue was dissolved in a mixture of ethyl acetate (100 ml.) and water (50 ml.), and the organic layer was washed with 1 *N* ammonium hydroxide (twelve 25-ml. portions), water (50 ml.), 1 *N* hydrochloric acid (50 ml.), and water (50 ml.). The solution was dried over magnesium sulfate, the solvent was removed *in vacuo*, and the crystalline residue (4.2 g., m.p. 116–121°) was recrystallized from ethyl acetate–hexane. The purified material (3.45 g., 81%) melted at 119–120°. A second crop (0.50 g.) melted at 117° (softens at 115°),  $[\alpha]^{25}_D -20^\circ$  (*c* 2, ethanol).

*Anal.* Calcd. for  $C_{25}H_{25}N_3O_3$ : C, 65.2; H, 5.9; N, 9.9. Found: C, 65.4; H, 5.8; N, 10.1.

**Ethyl Benzoxycarbonyl-L-citrullyl-L-tryptophylglycinate (XXVIII).**—A solution of ethyl benzoxycarbonyl-L-tryptophylglycinate (8.9 g.) in a mixture of methanol (175 ml.) and acetic acid (5.25 ml.) was hydrogenated at normal pressure using 5% palladium on charcoal (900 mg.) as a catalyst. After 1.5 hr. the catalyst was removed and the solvent was evaporated *in vacuo*. The residue was dissolved in pyridine (40 ml.), benzoxycarbonyl-L-citrulline *p*-nitrophenyl ester<sup>23</sup> (9.03 g.) was added, and the solution was kept at room temperature for 2 days. The semisolid mass which formed was triturated with ethyl acetate (*ca.* 100 ml.), filtered, washed with ethyl acetate, and then with ethanol. The yield was 9.95 g. (81%), m.p. 214–215° (softens at 212°). A small portion was recrystallized for analysis, m.p. 216–217°,  $[\alpha]^{25}_D -15^\circ$  (*c* 2, dimethylformamide).

*Anal.* Calcd. for  $C_{29}H_{36}N_6O_7$ : C, 60.0; H, 6.3; N, 14.5. Found: C, 60.0; H, 6.2; N, 14.4.

**Ethyl Benzoxycarbonyl-L-phenylalanyl-L-citrullyl-L-tryptophylglycinate (XXIX).**—A solution of ethyl benzoxycarbonyl-L-citrullyl-L-tryptophylglycinate (8.7 g.) in methanol containing 5% acetic acid (125 ml.) was hydrogenated at normal pressure using 5% palladium on charcoal (1 g.) as catalyst. After 2 hr. the catalyst was removed, the solvent was evaporated *in vacuo*, and the residue was dissolved in pyridine (30 ml.). Benzoxycarbonyl-L-phenylalanine *p*-nitrophenyl ester<sup>13</sup> (6.5 g.) was added and the solution was kept at room temperature for 2 days. The solidified mixture was triturated with ethyl acetate, filtered, and washed with ethyl acetate and ethanol. The yield was 8.0 g. (73%), m.p. 216–219°,  $[\alpha]^{25}_D -19.5^\circ$  (*c* 2, dimethylformamide). A small sample recrystallized from 80% ethanol melted at 221–223°.

*Anal.* Calcd. for  $C_{38}H_{45}N_7O_8$ : C, 62.7; H, 6.2; N, 13.5. Found: C, 63.0; H, 6.2; N, 13.5.

**Ethyl Benzoxycarbonyl-L-histidyl-L-phenylalanyl-L-citrullyl-L-tryptophylglycinate (XXX).**—A suspension of ethyl benzoxycarbonyl-L-phenylalanyl-L-citrullyl-L-tryptophylglycinate (6.57 g.) in a mixture of acetic acid (30 ml.), methanol (60 ml.), and water (2 ml.) was hydrogenated at normal pressure using 5% palladium on charcoal (1 g.) as a catalyst. After 2.5 hr. the catalyst was removed, the solvent was evaporated *in vacuo*, and the residue was dissolved in pyridine. A solution of benzoxycarbonyl-L-histidine azide in 45 ml. of ethyl acetate (prepared from 2.7 g. of benzoxycarbonyl-L-histidine hydrazide<sup>17b</sup>) was added and the mixture was kept in the refrigerator for 2 days. The semisolid mass was worked out as described above and the product was twice recrystallized from 90% ethanol. The yield was 4.2 g. (56%), m.p. 205–208°,  $[\alpha]^{25}_D -26^\circ$  (*c* 2, dimethylformamide).

*Anal.* Calcd. for  $C_{44}H_{52}N_{10}O_9$ : C, 61.1; H, 6.1; N, 16.2. Found: C, 60.9; H, 6.2; N, 16.3.

**Benzoxycarbonyl-L-histidyl-L-phenylalanyl-L-citrullyl-L-tryptophylglycine (XXXI).**—The protected pentapeptide ester XXX (500 mg.) was dissolved in a mixture of methanol (50 ml.) and water (8 ml.) by gentle heating; the solution was rapidly cooled and made alkaline with normal sodium hydroxide (2.5 ml.).

After 1.5 hr. at room temperature it was diluted with water (12 ml.) and acidified with acetic acid (3.5 ml.). An amorphous solid separated which was collected and washed with methanol. It was dissolved in dimethylformamide and reprecipitated with water; yield, 350 mg. (72%); m.p. 228–229°;  $[\alpha]^{19}_D -21^\circ$  (*c* 2, dimethylformamide).

*Anal.* Calcd. for  $C_{42}H_{48}N_{10}O_9$ : C, 60.3; H, 5.8; N, 16.7. Found: C, 59.9; H, 6.5; N, 16.7.

**L-Histidyl-L-phenylalanyl-L-citrullyl-L-tryptophylglycine (XXVI).** A.—A suspension of the protected pentapeptide acid XXXI (500 mg.) in aqueous 10% acetic acid (35 ml.) was hydrogenated at normal pressure using 5% palladium on charcoal (60 mg.) as catalyst. After 24 hr. the catalyst was removed and the solvent was evaporated *in vacuo*. The pink residue was dissolved in water and freeze-dried to give 400 mg. of a pink solid. A portion of this crude material (100 mg.) was chromatographed on carboxymethylcellulose using stepwise elution with increasing concentrations of aqueous ammonium acetate (0.005, 0.01, 0.025, 0.05, and 0.1 *M*). The elution was followed by reading the optical density (270 m $\mu$ ) of the fractions. The contents of the tubes with the desired product were pooled. The solution was concentrated *in vacuo* and finally freeze-dried several times to eliminate ammonium acetate. Purified material (77 mg.) was recovered. A small sample was hydrolyzed with 6 *N* hydrochloric acid for 16 hr. and the hydrolysate was analyzed as described for XXIV. The ratio of amino acids was found to be the following: Gly:Phe:His:Cit, 0.9:1.0:1.1:0.9.

*Anal.* Calcd. for  $C_{34}H_{42}N_{10}O_7 \cdot C_2H_4O_2 \cdot H_2O$ : C, 56.8; H, 6.1; N, 18.4. Found: C, 56.8; H, 6.2; N, 17.9.

B.— $N^\alpha$ -Benzoxycarbonyl-L-histidyl-L-phenylalanyl-L-ornithyl-L-tryptophylglycine, obtained as described for XXV (177 mg.), and potassium cyanate (64 mg.) were dissolved in a mixture of water (0.1 ml.) and 1 *N* potassium hydroxide (0.4 ml.). Two more portions of potassium cyanate (64 and 32 mg. each) and water (0.2 ml. each) were added after 24 and 48 hr. The reaction mixture was periodically examined by paper chromatography. When the ninhydrin-positive spot had practically disappeared, the reaction mixture was acidified with 1 *N* hydrochloric acid and diluted with water (4 ml.). After standing overnight in the refrigerator the solid precipitate which formed was separated by centrifugation and dried *in vacuo* over potassium hydroxide. This residue (191 mg.) was treated with hydrobromic acid in glacial acetic acid as described for XXIV. The resulting hydrobromide was purified on a column of carboxymethylcellulose (4 g.) as described for XXIV; yield, 33 mg.;  $[\alpha]^{20}_D -9^\circ$  (*c* 1, 1 *N* acetic acid). The concentrations of the ammonium acetate solutions were somewhat different: 0.005, 0.01, 0.02, 0.03, 0.04, 0.06, and 0.08 *M*.

This product was found to be identical with the preparation obtained by the procedure described above when compared on paper chromatograms (1-butanol–acetic acid–water, 4:1:5,  $R_f$  0.57, and isoamyl alcohol–pyridine–water, 7:7:6,  $R_f$  0.37) and on paper electropherograms (triethanolammonium citrate pH 4.0,  $E_{arg}$  0.38; triethanolammonium acetate pH 6.3,  $E_{arg}$  0.10).

A sample of this pentapeptide was digested with leucine aminopeptidase as described for XXIV. Paper chromatographic analysis of the digest showed only the spots corresponding to histidine, phenylalanine, citrulline, tryptophan, and glycine. Quantitative amino acid analysis gave the following ratios: Gly:Phe:His:Cit, 1.0:1.1:1.1:0.9.

This compound lightens frog skin previously darkened with  $\alpha$ -melanocyte-stimulating hormone when the citrullyl peptide is present in the concentration of 0.01 mg. per ml. of Ringer's solution. The corresponding ornithine-containing pentapeptide XXIV also shows such a lightening activity, but two and one-half to five times as much material is required.

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