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Oxytoceine and Deamino-oxytoceine*

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ABSTRACT: Oxytoceine and deamino-oxytoceine were prepared from *N*-carbobenzoxy-*S*,*S*'-dibenzyl-oxytoceine and *S*,*S*'-dibenzyl-deamino-oxytoceine, respectively, by treatment with sodium in liquid ammonia. The disulfhydryl compounds so obtained were isolated by means of partition chromatography on Sephadex G-25 in acidic solvent systems containing EDTA. Elemental analysis for sulfur and examination of sulfhydryl content in these compounds showed that all the sulfur was in the sulfhydryl state within experimental error. Oxytoceine and oxytocin could be separated from each other by gel filtration on Sephadex G-25. Deamino-oxytoceine and deamino-oxytocin could also be separated from each other by the same technique.

Treatment of oxytoceine in liquid ammonia with benzyl chloride followed by bioassay indicated that the maximum amount of oxytocin that could have been present in the oxytoceine preparation was less than 1%. Solutions of both disulfhydryl compounds in 0.9% NaCl containing 0.25% acetic acid and 10^{-4} M EDTA were assayed for avian vasodepressor activity against the USP posterior pituitary reference standard. Oxytoceine and deamino-oxytoceine were found to possess approximately 70 units/mg and 60 units/mg of avian vasodepressor activity, respectively. Oxytoceine could also be obtained by the reaction of cysteine with oxytocin at pH 8.

The existence of a reduced form of the posterior pituitary hormone, oxytocin, in which a disulfide link had been cleaved by treatment with excess cysteine was first demonstrated by Sealock and du Vigneaud (1935). The fact that complete inactivation of the partially purified hormone preparation resulted when the reaction mixture containing cysteine was treated with benzyl chloride afforded evidence that a reduction had occurred since the hormone in the absence of cysteine was not inactivated by treatment with benzyl chloride under the same conditions. The reduced form of the hormone (Figure 1), for which the name *oxytoceine* was suggested by du Vigneaud *et al.* (1960a), was a key intermediate in the synthesis of oxytocin (du Vigneaud *et al.*, 1953, 1954) although it was not isolated as such. The hormone is readily obtained from oxytoceine by oxidation of the latter to the cyclic disulfide in neutral aqueous solution. Some earlier attempts to isolate oxytoceine indicated that it was very difficult to prevent oxidation to the cyclic disulfide. It was suggested to us by Dr. John Ferraro, then of this laboratory, that this linear disulfhydryl compound might be stable at low pH in the presence of Versene. A preliminary experiment on a crude preparation indeed indicated that the compound was highly resistant to oxidation over a period of 24 hr in 0.2 N acetic acid containing 0.01% Fe(III)-specific Versene (Bersworth Chemical Co., Framingham, Mass.) according to the qualitative nitroprusside test for thiols. Since oxytocin can be efficiently purified and isolated by partition chromatography on Sephadex by the method of Yamashiro (1964), it occurred to us that by using this technique it might be possible to isolate the oxytoceine in highly purified form if a suitable acidic solvent system containing a metal-complexing agent could be devised. The first encouraging results were obtained with the solvent system 1-butanol–ethanol–0.2 N aqueous acetic acid containing 0.01%

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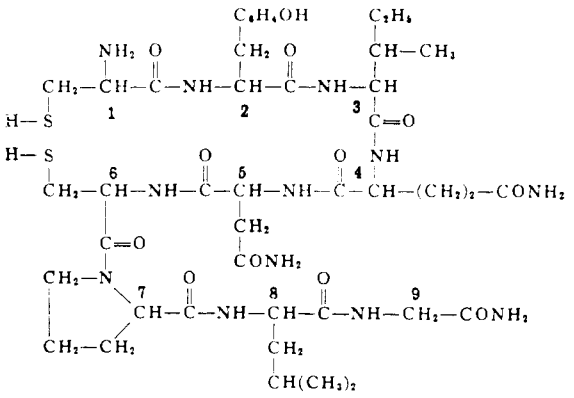

FIGURE 1: Structure of oxytoceine with numbers indicating the position of the individual amino acid residues. Oxytocin is the corresponding cyclic disulfide containing a cystine residue in place of the two cysteine residues in oxytoceine.

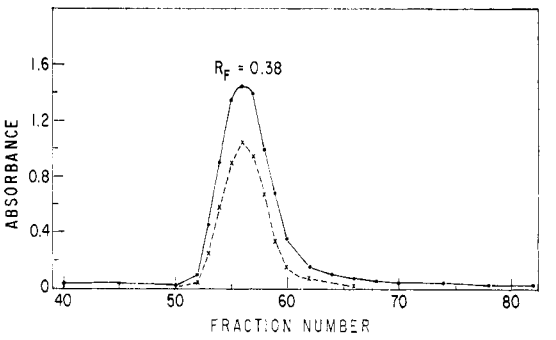

FIGURE 2: Partition chromatography of oxytoceine on Sephadex G-25 in 1-butanol–ethanol–benzene–0.5 N aqueous acetic acid containing 2×10^{-4} M EDTA (32:8:1:40) at 0°. ●——●, Folin–Lowry peptide determination; ×----×, Ellman sulfhydryl determination.

Fe(III)-specific Versene (4:1:5). In the course of time various modifications of this solvent system were tried, and one of the most useful was found to be 1-butanol–ethanol–benzene–0.5 N aqueous acetic acid containing 2×10^{-4} M EDTA (32:8:1:40). By use of this system the isolation of oxytoceine has been accomplished and is presented in this communication along with the isolation of deamino-oxytoceine. The latter analog is the reduced form of the highly potent deamino-oxytocin (du Vigneaud *et al.*, 1960b; Hope *et al.*, 1962) in which the free amino group of oxytocin has been replaced with hydrogen.

For the preparations of oxytoceine and deamino-oxytoceine the protected intermediates prepared by the stepwise *p*-nitrophenyl ester method, *N*-carbobenzoxy-*S*,*S*′-dibenzyl-oxytoceine (Bodanszky and du Vigneaud, 1959) and *S*,*S*′-dibenzyl-deamino-oxytoceine (du Vigneaud *et al.*, 1960b; Hope *et al.*, 1962), served as starting materials. These protected polypeptide derivatives were treated with sodium in liquid ammonia according to the method of Sifferd and du Vigneaud (1935) as used in the synthesis of oxytocin (du Vigneaud *et al.*, 1953, 1954) for removal of the *N*-carbobenzoxy and *S*-benzyl protecting groups. The reductions were carried out in such a manner that the blue color that spread throughout the solution at the end point due to sodium persisted for about 30 sec and then disappeared. The liquid ammonia solutions were evaporated to low volumes and then lyophilized.

For purification of oxytoceine a Sephadex G-25 column (2.8 × 49 cm) was equilibrated at 0° to both phases of the solvent system 1-butanol–ethanol–benzene–0.5 N aqueous acetic acid containing 2×10^{-4} M EDTA (32:8:1:40), in the same manner as described previously for the preparation of Sephadex partition columns (Yamashiro, 1964; Yamashiro *et al.*, 1966). The pH of the aqueous phase of this system was 2.97 when measured at 18°. For chromatography the product obtained by reduction of 200 mg of *N*-carbobenzoxy-*S*,*S*′-dibenzyl-oxytoceine was dissolved in a small volume (12 ml) of the organic phase to which trifluoroacetic acid and an additional amount of EDTA had been added. It was essential to add a sufficient amount of strong acid to combine with the sodium that had been used in the reduction. When acetic acid was used for this purpose, unsatisfactory chromatograms were obtained. The eluates obtained during chromatography were collected in tubes which had been pretreated with a dilute aqueous solution of EDTA. Analysis of aliquots from some of the tubes by the Folin–Lowry procedure (Lowry *et al.*, 1951) gave the curve shown in Figure 2. In a similar manner sulfhydryl groups were detected by the method of Ellman (1959) and the values obtained are also plotted in Figure 2. Both curves coincided in a major peak with an R_F of 0.38. In a separate run oxytocin under the same conditions appeared with an R_F of 0.11. For isolation of the oxytoceine the materials represented by the central portion of the peak were pooled and mixed with cold dilute acetic acid containing a slight trace of EDTA, in addition to HCl in excess of the molar amount of peptide as calculated from the Ellman color values. The cold mixture was evaporated *in vacuo* to low volume and then lyophilized.

The purification of deamino-oxytoceine was carried out on a Sephadex G-25 column at 25° with the use of the solvent system 1-butanol–benzene–10% aqueous acetic acid containing 2×10^{-4} M EDTA (4:1:4). Chromatography of this analog presented particular difficulties due to its low solubility in the solvent systems tested and its higher solubility in the organic phase relative to the aqueous phase. Although an R_F value between 0.5 and 0.2 is desirable for optimum purification (Dixon, 1962), we were greatly restricted in the amount of benzene that could be used to achieve this end. For the same reason a relatively large column (4.3 × 63 cm) was used for the purification of product obtained from the reduction of 120 mg of *S*,*S*′-di-

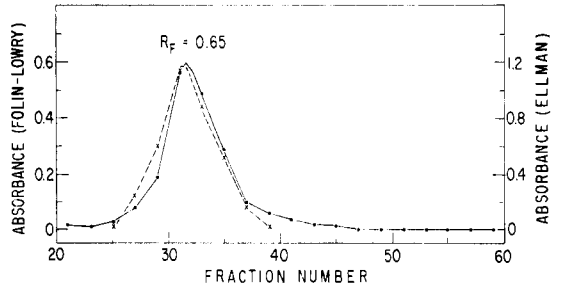

FIGURE 3: Partition chromatography of deamino-oxytoceine on Sephadex G-25 in 1-butanol–benzene–10% aqueous acetic acid containing 2 × 10^{-4} M EDTA (4:1:4) at 25°. ●——●, Folin–Lowry peptide determination; ×----×, Ellman sulfhydryl determination.

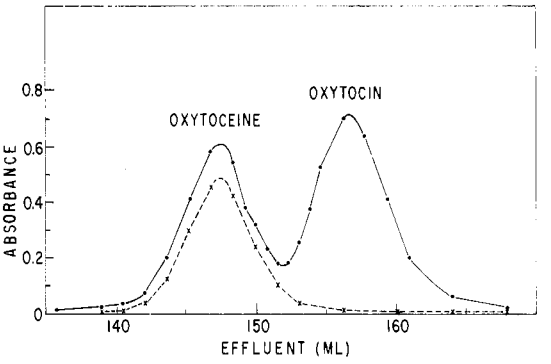

FIGURE 4: Gel filtration of a mixture of oxytoceine (2.24 mg) and oxytocin (2.20 mg) on Sephadex G-25 in 0.5 N acetic acid containing 2 × 10^{-4} M EDTA at 25°. ●——●, Folin–Lowry peptide determination; ×----×, Ellman sulfhydryl determination.

benzyl-deamino-oxytoceine. The deamino-oxytoceine appeared with coinciding Folin–Lowry and Ellman peaks with an R_F of about 0.65 as shown in Figure 3. Under the same conditions deamino-oxytocin emerged with almost the same R_F. It was therefore essential to obtain independent evidence for the absence of this cyclic disulfide, and this evidence will be presented subsequently. For isolation of the deamino-oxytoceine, the materials represented by the central portion of the major peak were pooled, mixed with water, evaporated to low volume, and lyophilized. No EDTA was added to the water at this point as was done in the case of oxytoceine since deamino-oxytoceine is more resistant to oxidation than oxytoceine. It may be recalled that in the synthesis of deamino-oxytocin the intermediate disulfhydryl compound was more resistant to oxidation by aeration than was the corresponding intermediate in the synthesis of oxytocin (Hope *et al.*, 1962).

In the case of deamino-oxytoceine its separation from deamino-oxytocin was demonstrated on thin layer chromatography on silica gel G with the use of the solvent system 1-butanol–benzene–acetic acid–water (3:1:1:5). The deamino-oxytoceine itself traveled as a single spot, and no deamino-oxytocin, which travels slower than deamino-oxytoceine, was detected on the chromatogram.

The oxytoceine and deamino-oxytoceine were analyzed for elemental sulfur and examined for sulfhydryl content by a number of reagents: DTNB[1] according to Ellman (1959), PCMB according to Boyer (1954), potassium ferricyanide, and iodine. The values obtained were expressed as moles of sulfhydryl per gram-atom of sulfur or more conveniently in practice as micromoles of sulfhydryl per milligram of undried lyophilized powder. The results of these analyses shown in Table I demonstrate that within the errors of the analyses all the sulfur in our preparations of oxytoceine and deamino-oxytoceine was in the sulfhydryl state.

[1] Abbreviations used: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PCMB, *p*-chloromercuribenzoic acid.

TABLE I: Sulfhydryl Content of Oxytoceine and Deamino-oxytoceine.

	Oxytoceine		Deamino-oxytoceine	
Method	μmoles of Sulfhydryl/mg[a]	μmoles of Sulfhydryl/g-atom of Sulfur	μmoles of Sulfhydryl/mg[a]	μmoles of Sulfhydryl/g-atom of Sulfur
Ellman	1.74	1.01	1.81	0.97
PCMB	1.69	0.98	1.84	0.98
Potassium ferricyanide	1.69	0.98	—	—
Iodine	1.71	0.99	—	—

[a] Based on lyophilized powders.

Furthermore, the data from the four methods employed were in agreement with each other.

From the results of sulfhydryl and sulfur analyses of oxytoceine and deamino-oxytoceine the presence of the corresponding cyclic disulfides could be excluded, but an independent procedure was sought for their detection. Since the oxytoceine and deamino-oxytoceine are resistant to oxidation in dilute acetic acid containing EDTA, the preparations were subjected to gel filtration on Sephadex G-25 in this solvent. A long column (1.24 × 159 cm) packed with the G-25 block polymerisate (200–270 mesh) was equilibrated with 0.5 N acetic acid containing 2 × 10^{-4} M EDTA. A mixture of oxytoceine and oxytocin was then subjected to gel filtration. Two peaks were detected by the Folin–Lowry reagent; the faster traveling one coincided with the Ellman curve while the slower moving one contained no sulfhydryl material, as shown in Figure

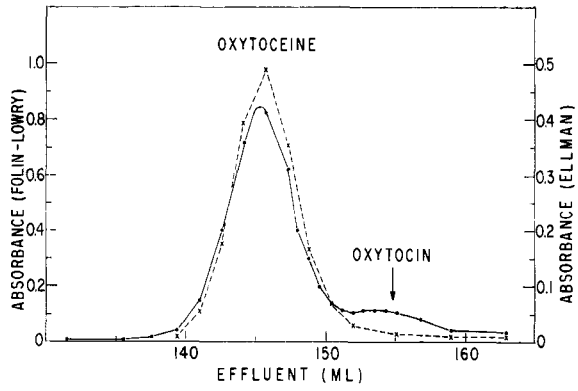

FIGURE 5: Gel filtration of a mixture of oxytoceine (2.21 mg) and oxytocin (0.095 mg) on Sephadex G-25 in 0.5 N acetic acid containing 2×10^{-4} M EDTA at 25°. ●——●, Folin–Lowry peptide determination; ×----×, Ellman sulfhydryl determination.

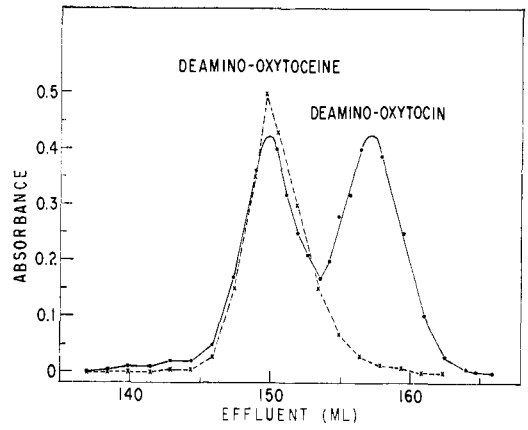

FIGURE 6: Gel filtration of a mixture of deamino-oxytoceine (1.58 mg) and deamino-oxytocin (1.46 mg) on Sephadex G-25 in 0.5 N acetic acid containing 2×10^{-4} M EDTA at 25°. ●——●, Folin–Lowry peptide determination; ×----×, Ellman sulfhydryl determination.

4. No additional peaks were detected. Gel filtration of either oxytocin or oxytoceine alone in the same solvent gave only one symmetrical peak for each compound at the respective positions shown in Figure 4. When oxytoceine and oxytocin were mixed in a weight ratio of 23:1 and analyzed under the same conditions, the added oxytocin was readily detected as a shoulder in the Folin–Lowry curve with no corresponding shoulder in the Ellman curve (Figure 5). No trace of oxytocin was detected in the oxytoceine chromatogram by these criteria. In a similar manner deamino-oxytoceine and deamino-oxytocin were separated from one another by this technique, as shown in Figure 6. Deamino-oxytoceine alone gave a single symmetrical peak at the position shown in the figure. A mixture of deamino-oxytoceine and deamino-oxytocin in a ratio of 20:1 gave a Folin–Lowry curve with a shoulder corresponding to the latter compound and no shoulder in the Ellman curve (Figure 7). No trace of deamino-oxytocin could be detected in our preparation of deamino-oxytoceine by these criteria. Thus, the data obtained from these gel filtration experiments are in accord with those obtained by sulfhydryl determination.

Elemental analyses of both oxytoceine and deamino-oxytoceine were in agreement with calculated values. For amino acid analyses samples of each compound were hydrolyzed in 6 N HCl at 110° for 22 hr, and the hydrolysates were treated with potassium ferricyanide near neutral pH before analyses for the amino acids and ammonia were carried out by the procedure of Spackman *et al.* (1958). The expected constituents were found in the same molar ratios as for oxytocin and deamino-oxytocin, respectively.

It is of interest that the optical rotation of oxytoceine differed markedly from that of oxytocin whereas the difference between the values for deamino-oxytoceine and deamino-oxytocin were less marked. The specific rotations of oxytoceine and oxytocin at 18° were −70 and −4°, respectively, at a concentration of 0.5% in 50% aqueous acetic acid containing 10^{-4} M EDTA. The values for deamino-oxytoceine and deamino-oxytocin at 18° were −63 and −52°, respectively, at a concentration of 0.5% in 98% aqueous acetic acid.

Previous work has shown that when oxytocin is reduced with sodium in liquid ammonia and the resulting disulfhydryl intermediate is treated with benzyl chloride, the biologically inactive compound *S,S'*-dibenzyl-oxytoceine is obtained, whereas treatment of oxytocin with benzyl chloride alone in this solvent caused no inactivation of the hormone (Gordon and du Vigneaud, 1953). Since it has also been shown that cysteine hydrochloride can be benzylated directly with benzyl chloride in liquid ammonia (Lutz *et al.*, 1959), we decided to attempt benzylation of our preparation of oxytoceine in this manner to convert it to the *S,S'*-dibenzyl-oxytoceine without the use of sodium. If complete benzylation occurred, the biological activity remaining after such treatment could be a measure of any traces of oxytocin present in the oxytoceine preparation. A sample of oxytoceine was therefore treated with excess benzyl chloride. After removal of the liquid ammonia it was found that only a trace of thiols remained in the product, thus indicating that the benzylation had been practically complete. The avian vasodepressor activity remaining in this product indicated that the maximum amount of oxytocin that could have been present in the oxytoceine preparation was less than 1%. Other experiments showed that no inactivation of oxytocin could be detected when the hormone was treated with benzyl chloride under exactly the same conditions and, furthermore, *S,S'*-dibenzyl-oxytoceine did not significantly inhibit the biological activity of oxytocin.

Since the official method of the United States Phar-

macopeia (1965) for the assay of oxytocin is based on avian vasodepressor activity, our preparations of oxytoceine and deamino-oxytoceine were subjected to bioassay for this activity according to the procedure of Munsick *et al.* (1960). The assays were carried out against the USP posterior pituitary reference standard.

For bioassay it is evident that special precautions would have to be taken to avoid oxidation of the disulfhydryl compounds in the very dilute solutions required for intravenous injection into the chickens to establish the blood pressure lowering response to oxytoceine and deamino-oxytoceine. It was finally found that the thiol content (Ellman method) of a solution of oxytoceine in 0.9% NaCl containing 0.25% acetic acid and 10^{-4} M EDTA (pH 2.97) remained unchanged over a period of 24 hr at a peptide concentration of 2 μg/ml. When such solutions of oxytoceine as well as of deamino-oxytoceine were injected into the chicken, protracted vasodepressor responses were observed in contrast to the transient responses characteristic of oxytocin. Neither the potency nor the pattern of response to oxytocin was noticeably affected when solutions of oxytocin in the same saline–acetic acid–EDTA solvent were injected into the chicken. After the initial precipitous drop in blood pressure following the injection of oxytoceine or deamino-oxytoceine, the blood pressure gradually returned to the base line in approximately 5 min, and sometimes longer depending on the dosage, whereas after injections of oxytocin the blood pressure returns to the base line in 20–60 sec, also depending on the dose. If only the maximum drop in blood pressure is taken as index of response, the potency of oxytoceine is about 70 units/mg and that of deamino-oxytoceine is about 60 units/mg. On this basis of comparison the avian vasodepressor potencies of the disulfhydryl compounds were substantially lower than those of the corresponding cyclic disulfides which for highly purified oxytocin are about 500 units/mg (Chan and du Vigneaud, 1962) and for crystalline deamino-oxytocin about 975 units/mg (Ferrier *et al.*, 1965). It may well be that oxytoceine and deamino-oxytoceine are gradually converted to the cyclic disulfides *in vivo* to produce the protracted biological response observed.

Since the avian vasodepressor activity of our oxytoceine samples was substantially lower than that indicated by the earlier work (Sealock and du Vigneaud, 1935) in which the partially purified preparations of oxytocin available at that time were treated with cysteine in a ratio of 40:1 of cysteine to the hormone preparation, the effect of cysteine on oxytocin was investigated with the chemical methods developed in this work. L-Cysteine (224 mg) was added to a solution of 5.85 mg of highly purified oxytocin (500 units/mg) in sodium borate–HCl buffer, pH 8. The hormone concentration was 0.5 mg/ml. The reaction mixture was kept under nitrogen, and 4- and 24-hr aliquots were removed and acidified to a pH of approximately 1.5 with HCl containing EDTA. The reaction products in these samples were analyzed by employing the gel filtration technique devised for oxytoceine. It was fortunate that the cysteine traveled at a slower rate than both the oxytoceine and the oxytocin, thus simplifying the identification of these constituents in the reaction mixture. It was also established in a separate experiment that L-cystine traveled at a rate close to that of oxytocin. The chromatograms of the 4- and 24-hr reaction aliquots were virtually identical and that of the latter is shown in Figure 8. In order of emergence from the column, the peaks corresponded to oxytoceine, L-cystine, and L-cysteine, respectively. Since oxytocin emerges close to the position of cystine, the contents of the cystine peak were subjected to bioassay and only a trace of avian vasodepressor activity was detectable.

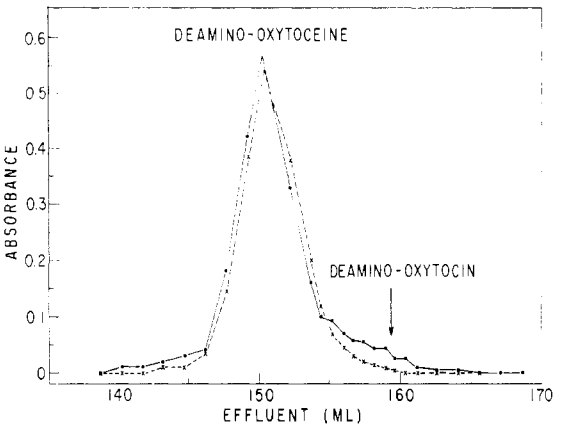

FIGURE 7: Gel filtration of a mixture of deamino-oxytoceine (1.71 mg) and deamino-oxytocin (0.075 mg) on Sephadex G-25 in 0.5 N acetic acid containing 2 × 10^{-4} M EDTA at 25°. ●——●, Folin–Lowry peptide determination; ×----×, Ellman sulfhydryl determination.

The avian vasodepressor activity of the reaction mixture was then determined. An aliquot of the mixture was acidified to approximately pH 2 with HCl containing EDTA, and a 500-fold dilution was made with 0.9% NaCl containing 0.25% acetic acid and 10^{-4} M EDTA. This solution was then subjected to bioassay. The potency obtained corresponded to a total activity of approximately 580 units in the entire reaction mixture. This indicated that some oxytocin was still present in the reaction mixture, for if the potency of oxytoceine is taken as 70 units/mg as determined for highly purified oxytoceine and all of the oxytocin had been converted to oxytoceine by the action of cysteine, then a total of 410 units should have been obtained. When an aliquot of the reaction mixture, neither acidified nor treated with EDTA, was diluted with a 0.9% solution of NaCl, which had been deaerated and flushed with nitrogen, the activity found on bioassay was twice as great as that found when acidification and addition of EDTA had been employed. These results demonstrated that, even in the presence of excess cysteine, oxytoceine is highly susceptible to

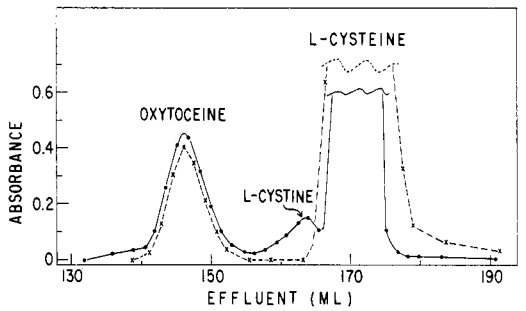

FIGURE 8: Gel filtration of a mixture of oxytocin and L-cysteine after 24-hr reaction at pH 8. Chromatography was performed on Sephadex G-25 in 0.5 N acetic acid containing 2×10^{-4} M EDTA at 25°. ●——●, Folin–Lowry peptide determination; ×----×, Ellman sulfhydryl determination.

oxidation in very dilute solution. Finally, an aliquot of the original reaction mixture was diluted 100-fold with 0.9% NaCl, exposed to oxygen by aeration for 10 min, and then diluted an additional tenfold with saline. Upon bioassay of this solution an amount of avian vasodepressor activity was found corresponding to the activity of the oxytocin (2900 units) used in the total reaction mixture, indicating complete conversion of oxytoceine to oxytocin had taken place by aeration.

Experimental Section

Materials and Methods. Sephadex G-25 block polymerisate (100–270 mesh) (Pharmacia Fine Chemicals, Inc., New Market, N. J.) was sieved on a No. 200 U. S. Standard sieve. The portion remaining on the screen (100–200 mesh) was used for the preparation of partition columns which were operated as described previously (Yamashiro, 1964; Yamashiro *et al.*, 1966). The portion going through the sieve (200–270 mesh) was washed in water by repeated decantation to remove the finer particles and then used for packing a 1.24 × 159 cm column for the gel filtration experiments.

Nitrogen was obtained from The Matheson Co., Inc., East Rutherford, N. J. (prepurified grade containing 8-ppm oxygen). Disodium ethylenediaminetetraacetate dihydrate reagent (J. T. Baker Chemical Co., Phillipsburg, N. J.) was used for the preparation of a 0.1 M stock solution. The concentrations of EDTA stated in this experimental section refer to the disodium salt. *p*-Chloromercuribenzoic acid (Mann Research Laboratories, Inc., New York, N. Y.) was used without further purification.

Peptides were detected by the Folin–Lowry procedure (Lowry *et al.*, 1951). A suitable aliquot of the sample solution was mixed with 3 ml of the alkaline copper solution and after 10 min the 1 N Folin–Ciocalteu phenol reagent (0.2 ml) was added. Color values were measured at 625 mμ.

The procedure of Ellman (1959) was used for the routine determinations of sulfhydryl groups. The starting material 5,5′-dithiobis(2-nitrobenzoic acid) (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was recrystallized from 60% aqueous acetic acid. A 10 mM stock solution of the DTNB was prepared by dissolving 396 mg of the compound in 100 ml of 0.1 M potassium phosphate buffer, pH 7, and stored at 0°. The reagent employed in this work was freshly prepared by diluting the stock solution of DTNB 50-fold with either 0.1 or 0.4 M potassium phosphate buffer, pH 8. A 3-ml aliquot of this 0.2 mM solution of DTNB was mixed with a suitable aliquot of the sample solution, with the appropriate buffer being used to obtain a pH in the range 7–8 for the color development. The resulting color values were measured at 412 mμ and compared with those obtained on a standard solution (0.1 mM) of L-cysteine tosylate (Zervas and Photaki, 1962) in 0.5 N acetic acid containing 10^{-4} M EDTA. The presence of 10^{-3} M EDTA in the 0.2 mM DTNB reagent did not affect these color values. Avian vasodepressor activity was measured according to the procedure of Munsick *et al.* (1960) against the USP posterior pituitary reference standard.

Oxytoceine. *N*-Carbobenzoxy-*S*-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (200 mg) prepared by the method of Bodanszky and du Vigneaud (1959) was dissolved in approximately 150 ml of liquid ammonia (distilled from sodium) in an apparatus protected from atmospheric moisture. The solution was brought to the boiling point and then treated with sodium which was delivered from a 2-ml graduated pipet until a blue color persisted throughout the solution for 30 sec. The approximate amount of sodium consumed (25 mg, 1.1 mequiv) was recorded. The solution was concentrated *in vacuo* to about 30 ml and then lyophilized.

A Sephadex partition column (2.85 × 49 cm) was prepared for operation at 0°. The required solvent system was obtained by mixing 1-butanol (960 ml), ethanol (240 ml), benzene (30 ml), water (1166 ml), glacial acetic acid (34 ml), and 0.1 M EDTA (2.4 ml). The test tubes used for the subsequent collection of eluates were allowed to soak for 6 hr at room temperature in a solution prepared by mixing 1 l. of water, 0.1 ml of 0.1 M EDTA, and 0.2 ml of 0.1 N HCl. The solvent was drained from these test tubes, and, without further rinsing, the tubes were dried in an oven.

The product obtained from the reduction of the protected nonapeptide was then cooled to 0° and dissolved in a 12-ml aliquot of a solution prepared by mixing 20 ml of the organic phase of the Sephadex solvent system, 0.04 ml of 0.1 M EDTA, and 0.25 ml (3.3 mequiv) of trifluoroacetic acid. The resulting solution was applied to the partition column, and elution with the organic phase was carried out at a flow rate of about 14 ml/hr. The eluates were collected in 149 fractions of 3.9 ml each. For detection of peptide materials in these fractions, aliquots (25 μl) of selected tubes were analyzed by the Folin–Lowry method.

For detection of thiols, aliquots (25 μl) were analyzed with the DTNB reagent, and the color values obtained were plotted along with those from the Folin–Lowry determination. Only one peak was detected with an R_F of 0.38, and a portion of the chromatogram near this peak is shown in Figure 2. When a sample (16.7 mg) of oxytocin was chromatographed under the same conditions, the hormone emerged as an unsymmetrical peak with an R_F of 0.11. The columns used in these experiments were regenerated with 50% aqueous acetic acid.

The calculated sulfhydryl content in fractions 54–58 of the oxytoceine peak (see Figure 2) was 139 μmoles. For isolation of the oxytoceine a solution was prepared by mixing 300 ml of water, 15 μl of 0.1 M EDTA, 0.78 ml of 1 N HCl, and 0.85 ml of glacial acetic acid. This solution was cooled to 0°, and an aliquot (60 ml) was withdrawn which contained 154 μequiv of HCl, an amount slightly in excess of the micromolar sulfhydryl content in fractions 54–58. This aliquot was mixed with the material in these fractions, and the mixture was evaporated *in vacuo* in a 25° bath to a low volume. The resulting aqueous solution was lyophilized. The yield of product was 82 mg, $[\alpha]_D^{18}$ −70.2° (*c* 0.50, 50% acetic acid containing 10^{-4} M EDTA). No significant change in optical rotation was observed over a period of 47 hr. For analysis a sample was dried at 100° over phosphorus pentoxide *in vacuo* with a loss in weight of 5.9%.

Anal. Calcd for $C_{43}H_{65}N_{12}O_{12}S_2 \cdot HCl$: C, 49.4; H, 6.65; N, 16.1; S, 6.13. Found: C, 49.7; H, 6.74; N, 16.0; S, 5.88.

A sample (5.87 mg) was hydrolyzed in 6 N HCl (3 ml) *in vacuo* at 110° for 22 hr. The residue obtained after drying over NaOH was dissolved in 1.0 ml of water. Determination of the sulfhydryl content by the Ellman procedure showed the presence of 5 μmoles. The solution was mixed with 1.0 ml of 0.2 M sodium phosphate buffer, pH 6.4, and allowed to stand exposed to air at room temperature for 2.5 hr (Hirs *et al.*, 1954). Oxidation of the thiols remaining in the solution (2.4 μmoles) was completed by the addition of 0.25 ml of 0.01 N potassium ferricyanide. The pH of the solution was adjusted to 2.4 with 0.12 ml of 1 N HCl and then diluted to 10 ml with the citrate buffer (pH 2.2) used in the procedure of Spackman *et al.* (1958). An aliquot was analyzed by this procedure in the 50–50° system on a Beckman-Spinco amino acid analyzer. The following molar ratios of amino acids and ammonia were obtained, with the value of glycine taken as 1.0: half-cystine, 2.0; tyrosine, 1.0; isoleucine, 1.0; glutamic acid, 1.0; aspartic acid, 1.2; proline, 1.2; leucine, 1.0; glycine, 1.0; and ammonia, 3.1.

Deamino-oxytoceine. *S*-Benzyl-β-mercaptopropionyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (120 mg) prepared by the method of Hope *et al.* (1962) was treated with sodium (~20 mg, 0.85 mequiv) in liquid ammonia (~150 ml) as described for the preparation of oxytoceine. A Sephadex column (4.3 × 63 cm) was equilibrated at 25° for partition chromatography with a solvent system which was obtained by mixing 1-butanol (800 ml), benzene (200 ml), glacial acetic acid (80 ml), water (720 ml), and 0.1 M EDTA (1.8 ml). The product obtained from the reduction of the protected polypeptide amide was dissolved in a mixture of the organic phase of the Sephadex solvent system (50 ml), trifluoroacetic acid (0.12 ml, 1.6 mequiv), and 0.1 M EDTA (0.1 ml). The insoluble material was filtered off and the filtrate was applied to the partition column. Elution with the organic phase was carried out at a flow rate of about 40 ml/hr. The eluates were collected in 60 fractions of 10 ml each. The peptide and thiol content in these fractions was determined on 100-μl aliquots as described for oxytoceine, and only one peak was found, with an R_F of 0.65 as shown in Figure 3. When a mixture of deamino-oxytoceine (1.05 mg) and deamino-oxytocin (2.06 mg) was chromatographed on a 1.05 × 54 cm column in the same solvent system, the resulting Ellman and Folin–Lowry peaks were separated by an R_F difference of only 0.07, with the deamino-oxytocin traveling more slowly. The columns used in these experiments were regenerated by washing with pyridine–0.2 N aqueous acetic acid (4:5).

For isolation of the deamino-oxytoceine the contents of fractions 28–36 (see Figure 3) were combined, diluted with water (200 ml), evaporated *in vacuo* in a 20° bath to a low volume, and lyophilized. The yield of product was 72.0 mg, $[\alpha]_D^{17.5}$ −62.7° (*c* 0.5, 98% acetic acid). For analysis a sample was dried at 100° over phosphorus pentoxide *in vacuo* with a loss in weight of 3.8%.

Anal. Calcd for $C_{43}H_{67}N_{11}O_{12}S_2$: C, 52.0; H, 6.79; N, 15.5; S, 6.45. Found: C, 52.0; H, 6.81; N, 15.1; S, 6.24.

A sample was analyzed for amino acids and ammonia in the same manner as described for oxytoceine. The constituents and their molar ratios for the deamino-oxytoceine were the same as those for deamino-oxytocin.

Ascending thin layer chromatography of deamino-oxytoceine, deamino-oxytocin, and a mixture of the two was carried out on silica gel G. The chromatogram was obtained in stepwise fashion by alternate drying and development with the organic phase of the solvent system 1-butanol–benzene–glacial acetic acid–water (3:1:1:5). The R_F values of the spots detected by the chlorine method (Zahn and Rexroth, 1955) were 0.17 for deamino-oxytocin and 0.25 for deamino-oxytoceine.

Gel Filtration of Oxytoceine and Oxytocin. The 1.24 × 160 cm Sephadex column for gel filtration whose preparation was described above was equilibrated at 25° with a solvent prepared by mixing water (965 ml), glacial acetic acid (28.5 ml), 0.1 N HCl (4.0 ml), and 0.1 M EDTA (2.0 ml). A mixture of oxytoceine (2.24 mg) and oxytocin (2.20 mg) was dissolved in 50 μl of 50% aqueous acetic acid containing 10^{-4} M EDTA and then diluted with 0.95 ml of 0.05 N acetic acid containing 2 × 10^{-4} M EDTA and 4 × 10^{-4} N HCl. The solution was applied to the column, and

elution with the solvent used for equilibration of the column was carried out at a flow rate of 6 ml/hr. After a forerun (117 ml) was collected the eluates were then collected in 100 fractions of 0.78 ml each. Aliquots (0.25 ml) from selected tubes were analyzed by the Folin–Lowry and Ellman methods, and the color values obtained were plotted as shown in Figure 4. In the same manner oxytoceine (1.94 mg) and oxytocin (1.54 mg) were separately examined, and each gave a symmetrical peak at the position indicated in Figure 4. Finally, a mixture of oxytoceine (2.21 mg) and oxytocin (0.095 mg) was analyzed as shown in Figure 5 (Folin–Lowry determination on 0.45-ml aliquots; Ellman determination on 0.25-ml aliquots).

Gel Filtration of Deamino-oxytoceine and Deamino-oxytocin. The procedures just described for oxytoceine were employed for the gel filtration of a mixture of deamino-oxytoceine (1.58 mg) and deamino-oxytocin (1.46 mg). This mixture was dissolved in 50 μl of 50% aqueous acetic acid containing 10^{-4} M EDTA and then diluted with 1.0 ml of 0.05 N acetic acid containing 2×10^{-4} M EDTA and 4×10^{-4} N HCl. The solution was applied to the column, and elution was carried out in the same manner as described for oxytoceine. The results of Folin–Lowry (0.40-ml aliquots) and Ellman (0.25-ml aliquots) analyses of the eluates are shown in Figure 6. In the same manner deamino-oxytoceine (1.64 mg) was examined, and only one symmetrical peak was found, at the position indicated in Figure 6. Finally, a mixture of deamino-oxytoceine (1.71 mg) and deamino-oxytocin (0.075 mg) was analyzed as shown in Figure 7.

Determination of Sulfhydryl Groups in Oxytoceine and Deamino-oxytoceine. A. ELLMAN METHOD. Weighed samples (~1 mg) of oxytoceine were dissolved in 50% aqueous acetic acid containing 2×10^{-4} M EDTA (0.1 ml) and then diluted with 0.05 N acetic acid containing 2×10^{-4} M EDTA (2.2 ml). Aliquots (200 μl) of these solutions were withdrawn and analyzed with the aforementioned DTNB reagent. Weighed samples (~1 mg) of deamino-oxytoceine were dissolved in glacial acetic acid (0.1 ml) and then diluted with water (0.9 ml) prior to analyses of 100-μl aliquots.

B. PCMB METHOD. The method of Boyer (1954) was employed as described by Benesch and Benesch (1962). The titrations were carried out near pH 4 using minimal amounts of EDTA for analysis of oxytoceine and no EDTA in the case of deamino-oxytoceine. A stock solution of PCMB was prepared by dissolving a weighed sample (~9 mg) in 10 ml of 1 N Tris–HCl buffer, pH 7.5, containing 1 M sodium perchlorate (Coombs *et al.*, 1964). An approximately 0.08 mM solution of the reagent was prepared by adding dropwise an aliquot (0.96 ml) of the stock solution to 30 ml of 2.5 M sodium acetate buffer, pH 4.0, containing 0.9 M sodium perchlorate. This solution was standardized by titrating a 3-ml aliquot with 15-μl portions of a 3.61 mM solution of glutathione in 0.1 N acetic acid containing 2×10^{-5} M EDTA. The course of the titration was followed at 255 mμ with the sodium acetate–sodium perchlorate buffer in the reference cell. The standardized solution of PCMB was then titrated in the same manner with solutions of oxytoceine and deamino-oxytoceine. The solution of oxytoceine was prepared by dissolving a sample (1.115 mg) in 50% aqueous acetic acid containing 10^{-4} M EDTA (50 μl) and diluting with 0.1 N acetic acid containing 2×10^{-5} M EDTA (0.45 ml). The deamino-oxytoceine (1.110 mg) was dissolved in glacial acetic acid (50 μl) and diluted with water (0.55 ml). Linear titration curves were obtained in all cases.

C. POTASSIUM FERRICYANIDE. A stock solution of 0.01 N potassium ferricyanide was diluted 15-fold with 0.4 M potassium phosphate buffer, pH 8, to give close to a 0.67 mM solution of the reagent. The latter was standardized by titrating a 3-ml aliquot with 100-μl portions of a cold 4.0 mM solution of L-cysteine tosylate in 0.5 N acetic acid. The course of the titration was observed by measuring the disappearance of color at 412 mμ with the buffer as reference. Color values were recorded 2 min after each portion of the cysteine solution had been added. When these values were corrected for the dilution effect and then plotted against the volume of cysteine solution added, a linear titration curve was obtained. The standardized solution of potassium ferricyanide in the potassium phosphate buffer was then titrated in the same manner with a solution of oxytoceine. The latter was prepared by dissolving a sample (3.38 mg) of oxytoceine in 50% aqueous acetic acid containing 10^{-5} M EDTA (50 μl) and diluting with 0.5% acetic acid containing 10^{-6} M EDTA (1.41 ml). The oxytoceine gave a linear titration curve, as is the case with cysteine. The analysis of oxytoceine was also carried out in the same manner in the following buffers: 0.4 M potassium phosphate buffer, pH 6.8, 2.5 M sodium acetate buffer, pH 5.75, and 2.7 M sodium acetate buffer, pH 4.8. The results obtained with these buffers were in close agreement with those obtained with the pH 8 buffer.

D. IODINE. The procedure was patterned after that described by Ma (1963). A solution of potassium iodide was prepared by dissolving a sample (562 mg) in a mixture of water (9.6 ml), 0.1 M EDTA (0.1 ml), 0.1 N HCl (0.2 ml), and glacial acetic acid (0.1 ml). Potassium iodate (50 mg) was dissolved in a mixture of glacial acetic acid (0.5 ml), 0.1 N HCl (0.2 ml), and 0.1 M EDTA (0.1 ml) and then diluted to 50 ml with water. For standardization of the potassium iodate solution an aliquot (100 μl) of a 3.55 mM solution of L-cysteine tosylate in 50% aqueous acetic acid containing 10^{-4} M EDTA was mixed with 0.25 ml of the potassium iodide solution and then titrated with the potassium iodate solution until the yellow color of the end point appeared. A sample (2.43 mg) of oxytoceine was dissolved in 0.10 ml of 50% aqueous acetic acid containing 10^{-4} M EDTA and analyzed in the same manner.

Benzylation of Oxytoceine in Liquid Ammonia. A sample (9.74 mg) of oxytoceine containing 17.0 μmoles of thiol was dissolved in 50 ml of liquid ammonia (distilled from sodium) and treated with a 0.25-ml

aliquot of a mixture of benzyl chloride (2 ml) and anhydrous ether (23 ml). The solution was protected from atmospheric moisture with a trap containing NaOH and was kept at the boiling point for 70 min while the volume slowly decreased to about 10 ml. The solution was then lyophilized to give a dense powder which was dissolved in 2 ml of 50% aqueous acetic acid containing 0.02 N trifluoroacetic acid and 10^{-4} M EDTA. Samples of this solution were analyzed for sulfhydryl content by the Ellman method, which showed the presence of about 0.2 μmole of thiol in the benzylated material. Another sample of the solution was diluted with 0.9% NaCl and submitted for avian vasodepressor assay, which showed that the benzylated material contained approximately 30 units of this activity. This activity would represent less than 0.1 mg of oxytocin.

A sample (9.65 mg) of oxytocin (500 units/mg) was treated with benzyl chloride in liquid ammonia in exactly the same manner as described for the benzylation of oxytoceine. When the material so treated was assayed against the starting hormone, no difference in potency was detected.

Avian Vasodepressor Assay of Highly Purified Oxytoceine and Deamino-oxytoceine. A sample (2.537 mg) of oxytoceine was dissolved in 50% aqueous acetic acid containing 10^{-4} M EDTA (0.288 ml) and diluted with 10^{-4} M EDTA (4.78 ml). An aliquot of the solution was diluted 150-fold with 0.9% NaCl containing 0.25% acetic acid, 10^{-4} M EDTA, and 2×10^{-4} M HCl. The resulting solution was submitted to avian vasodepressor assay without further dilution. Aliquots of this solution and of the standard were injected intravenously into the chicken and washed in with aliquots (1.0 ml) of 0.9% NaCl containing 0.1% acetic acid, 10^{-4} M EDTA, and 2×10^{-4} M HCl.

For assay of deamino-oxytoceine a sample (1.085 mg) was dissolved in 0.05 ml of glacial acetic acid and diluted with 4.95 ml of 0.9% NaCl containing 10^{-4} M EDTA. An aliquot of the solution was diluted and assayed as described for oxytoceine.

Reaction of Oxytocin with Cysteine. L-Cysteine (400 mg) was dissolved in 20 ml of 0.11 M sodium borate–HCl buffer, pH 8 (Clark, 1928), and the pH of the solution was adjusted to 7.95 with 5 N NaOH. A sample (5.85 mg) of oxytocin (500 units/mg) was dissolved in 0.5 ml of water and then mixed with an aliquot (11.2 ml) of the cysteine–borate solution that contained 224 mg of cysteine. Nitrogen was gently bubbled through the reaction mixture for 3 hr, and the mixture was then kept tightly covered.

Four and twenty-four hours after the treatment with nitrogen had been started, aliquots (2.0 ml) of the reaction mixture were withdrawn, and each was acidified to pH 1.5 by addition to a mixture of 0.1 M EDTA (2 μl) and concentrated HCl (60 μl). The two samples were subjected to gel filtration on Sephadex in the same manner as described for oxytoceine. Aliquots (0.50 ml) from the tubes containing the eluates were analyzed by the Folin–Lowry and Ellman methods, and the color values obtained for the 24-hr sample are shown in Figure 8. The material from the L-cystine peak contained approximately 9 units of avian vasodepressor activity.

For bioassay of the reaction mixture an aliquot (2.0 ml) corresponding to 18 hr of reaction was withdrawn and acidified with a mixture of concentrated HCl (60 μl) and 0.1 M EDTA (2 μl). This solution was diluted 500-fold with 0.9% NaCl containing 0.25% acetic acid, 10^{-4} M EDTA, and 2×10^{-4} M HCl and assayed for avian vasodepressor activity in the same manner employed for the assay of highly purified oxytoceine.

In preparation for bioassay of the reaction mixture without acidification and without the use of EDTA, a 0.9% NaCl solution was boiled for 10 min and then cooled in ice under a stream of nitrogen. This saline solution, protected from air, served as diluent in the following avian vasodepressor assays. An aliquot (50 μl) of the original reaction mixture, corresponding to 18 hr of reaction, was diluted 1000-fold with the saline (50 ml) out of contact with air. A portion of the resulting solution was injected into the chicken within 2 min and washed in with aliquots (1.0 ml) of the same saline solution. Further assays were performed in the same manner with fresh dilutions of the original reaction mixture.

Finally, an aliquot (100 μl) of the original reaction mixture, corresponding to an 18-hr reaction, was diluted with 0.9% NaCl (9.9 ml) and gently aerated for 10 min. The solution was then diluted tenfold with 0.9% NaCl and assayed for avian vasodepressor activity.

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8-Alanine-oxytocin, 8-Alanine-oxypressin, and Their Deamino Analogs. Their Synthesis and Some of Their Pharmacological Properties*

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ABSTRACT: 8-Alanine-oxytocin, 8-alanine-oxypressin, and their 1-deamino analogs have been synthesized and tested for some of the pharmacological activities characteristic of the neurohypophysial hormones. The analogs were prepared through the use of the stepwise *p*-nitrophenyl ester method as employed in the synthesis of oxytocin and deamino-oxytocin. L-Prolyl-L-alanylglycinamide served as the starting material for all four analogs. In each case the analog was isolated in highly purified form by countercurrent distribution. Upon bioassay the following mean potencies and standard errors were found for 8-alanine-oxytocin: 240 ± 25 units/mg of avian vasodepressor, 166 ± 6 units/mg of oxytocic, and 13 ± 1 units/mg of rat pressor activity. The corresponding values for 8-alanine-oxypressin were found to be 38 ± 2, 15 ± 0.6, and 21 ± 1 units/mg; for 1-deamino-8-alanine-oxytocin 453 ± 6, 314 ± 10, and 8 ± 0.4 units/mg; and for 1-deamino-8-alanine-oxypressin 47 ± 2, 25 ± 0.6, and 4.6 ± 0.1 units/mg.

In the course of extensive studies of the relationship between structure and biological activity of oxytocin (Figure 1) attention has been repeatedly focused on replacements of the leucine residue in the 8 position by other amino acid residues. Analogs in which a neutral amino acid residue, *e.g.*, isoleucine, valine (Jaquenoud and Boissonnas, 1961), or citrulline (Bodanszky and Birkhimer, 1962), was substituted for the leucine residue in oxytocin were all found to possess high oxytocic and avian vasodepressor activities. In view of the high potencies of these analogs it was decided to investigate the effect on these activities of a reduction in the length of the side chain of the amino acid residue in the 8 position of the oxytocin molecule. We therefore synthesized 8-alanine-oxytocin and 1-deamino-8-alanine-oxytocin, analogs of oxytocin and 1-deamino-oxytocin which contain an alanine residue in place of the leucine residue in the 8 position.

Another facet of this study was the evaluation of

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