Preparation of S,S'-Dibenzyloxytocin and Its Reconversion to Oxytocin.* (20765)

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In previous communications evidence has been presented that oxytocin is a polypeptide consisting of cystine and 7 other amino acids plus 3 moles of ammonia(1,2). It was further shown that the disulfide linkage was unquestionably present in some form of cyclic structure since no evidence for the presence of more than one moiety was found after cleavage of the disulfide by means of oxidation with performic acid(3) or removal of the sulfur by Raney nickel(4). A study of the purified performic acid-oxidized oxytocin revealed that the oxidized material possessed 2 cysteic acid residues, one of the moles of cysteic acid occupying a terminal position with its amino group free(5). From these results it would be expected that reduction of the disulfide to a sulfhydryl form followed by benzylation should result in a benzylated oxytocin containing 2 *S*-benzylcysteine residues and having a structure analogous to the performic acid-oxidized material if no other reactions occurred in the formation of the respective derivatives. It may be recalled that in 1935 Sealock and du Vigneaud(6) had shown that treatment of partially purified oxytocin with cysteine in aqueous solution at pH 7.5 at room temperature did not result in inactivation. Treatment of the resulting solution with benzyl chloride inactivated the hormone, whereas treatment of the original oxytocin preparation under the same conditions did not result in inactivation. It was predicted from this behavior that oxytocin contained sulfur in the form of a disulfide, and that the reduced or sulfhydryl form of the hormone was also active.

Preliminary studies with partially purified oxytocin in these laboratories has also shown that oxytocin could be reduced in liquid ammonia with metallic sodium without appreciable loss of activity and further that addition of benzyl chloride to the liquid ammonia reaction mixture resulted in inactivation. With highly purified oxytocin available, it was felt that reduction and benzylation in liquid ammonia should be tried on such material in the hope that a benzylated oxytocin might be separated in reasonably pure form and that through quantitative amino acid analysis one would be able to ascertain whether the composition agreed with what might be expected.

229 mg of oxytocin, with a potency of approximately 500 units/mg and with a typical amino acid composition(7), were benzylated in batches of approximately 50-70 mg. The benzylation was carried out as follows: 50 mg of oxytocin were placed in the reaction vessel, dissolved in 15 ml of ammonia, and sodium was added until a lingering blue color was obtained. 0.1 ml of benzyl chloride was added and the solution was stirred for 20 minutes. Before removal of the ammonia, a few crystals of ammonium iodide were added. The dry residue was extracted with small volumes of anhydrous acetone, the residual acetone was removed *in vacuo*, the residue was extracted with small volumes of cold 2% acetic acid, and the product was dried *in vacuo* over P_2O_5 at room temperature. From 229 mg of oxytocin, 188.3 mg of benzylated oxytocin were obtained. A sample of this material gave a negative nitroprusside test, which remained negative on the addition of cyanide, whereas a sample of oxytocin gave a positive nitroprusside test

* A preliminary announcement of these results was made recently (du Vigneaud, V., Ressler, C., Swan, J. M., Roberts, C. W., Katsoyannis, P. G., and Gordon, S., *J. Am. Chem. Soc.*, 1953, v75, 4879.)

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after the addition of cyanide. A sample of the benzylated oxytocin was analyzed for its constituent amino acids by the starch column technic(8,9) and found to contain 2 moles of *S*-benzylcysteine, one mole each of leucine, isoleucine, tyrosine, proline, glutamic acid, aspartic acid, glycine, and a trace of cystine. The solvent system used by Stein and Moore (9) for the resolution of leucine and isoleucine was found when slightly modified to allow the determination of *S*-benzylcysteine in addition to leucine and isoleucine. When the solvent system 1:1:0.27 *n*-butyl alcohol-benzyl alcohol-water was used, an authentic sample of *S*-benzylcysteine was found to emerge at effluent ml 15 and to possess a color yield of 0.92. Assay of a portion of the benzylated oxytocin after solution in one drop of glacial acetic acid and dilution with water indicated it to be inactive.

From these studies it is obvious that the sulfhydryl form of oxytocin is biologically active and that when the sulfhydryl groups are covered by benzyl groups the resulting compound is inactive. Whether the sulfhydryl form *in vivo* is active *per se* or is converted to a disulfide form is an open question at the present time. On the other hand, it is possible that the hormone might have to be reduced in the tissues to exert its biological effect. Earlier attempts to regenerate active material from the benzylated oxytocin by debenzylation in our laboratories had been negative. It was obvious, however, that if debenzylation could be brought about to reactivate the material and if on oxidation the original oxytocin could be obtained with the disulfide in the cyclic structure, an approach to the synthesis of oxytocin was feasible once the sequence of the amino acids in the *S*-benzylated oxytocin or the performic acid-oxidized oxytocin had been established. Thus, an eventual synthesis of oxytocin might be possible through the application of the method of cystine peptide synthesis introduced by du Vigneaud and Miller(10), in which the sulfhydryl group of cysteine is protected by a benzyl group during a series of reactions and the benzyl group is removed at the end of the series by sodium in liquid ammonia. This method was based on the earlier findings that a sulfhydryl compound could be benzylated in liquid ammonia(11) and that the resulting benzyl thio ether could be cleaved by sodium in liquid ammonia(12). A study of the debenzylation of the inactivated benzylated oxytocin was therefore undertaken. This has led to the regeneration of what we believe to be oxytocin.

Approximately 20 mg of benzylated oxytocin were dissolved in 20 ml of ammonia and reduced with sodium. A few crystals of ammonium chloride were added to discharge the slight blue color and the ammonia was removed *in vacuo*. The dry residue was quickly dissolved in 100 ml of cold 0.005% acetic acid and the pH of the solution was adjusted to 7-7.5. The solution was aerated with CO_2-free air for one hour and then concentrated to a small volume in a rotary evaporator(13) at a temperature below 35° and lyophilized. Assays of the solution indicated the regeneration of activity. Several batches of the regenerated material were combined. From a total of 160 mg of benzylated oxytocin approximately 12,700 units of activity were obtained. This represented a yield of approximately 20% on the basis of the activity that might be theoretically derived from this amount of benzylated oxytocin. The regenerated material was subjected to countercurrent distribution(14) with the solvent system *sec*-butyl alcohol-0.05% acetic acid. One hundred transfers were applied and the distribution was analyzed by weight determination. From the results it was quite evident that complete separation of the salt peak and the activity peak had not taken place. Ultraviolet absorption at 275 mμ was also used to analyze the distribution. A maximum at approximately Tube 27 coincided with the peak containing the active principle found by the weight analysis. The partition coefficient was found to be approximately 0.37. The distribution was then continued for another 100 transfers. An assay of one of the peak tubes indicated an activity of 410 U.S.P. units per mg by the blood pressure method. The material had the expected activity on the isolated uterine strip. Tubes 43-60 inclusive were combined, concentrated, and lyophilized to yield 28.5 mg of regener-

ated, active material which was used to establish the nature of the regenerated material.

A sample of the regenerated active material was analyzed for constituent amino acids by the starch column method(8,9). It was found to have the same amino acids as the original oxytocin in essentially 1:1 ratio and *S*-benzylcysteine was found to be absent. Electrophoretic studies at pH 9.7 (glycine buffer) and pH 4.5 (acetate buffer) were carried out by the method described by Kunkel, Taylor, and du Vigneaud(15). Fifty μg of regenerated oxytocic material and the same amount of original oxytocin were applied to Whatman No. 3 MM filter paper and 300 volts were applied for 2 hours. The regenerated material behaved like the original oxytocin; however, in the case of the acetate buffer, a trace of a second component was noted. Crystallization of regenerated material as the flavianate was accomplished and the crystals were compared with the flavianate of the original oxytocin(7). No difference in melting point, solubility, or crystalline structure could be observed between the crystalline flavianate of the regenerated material and the crystalline flavianate of the original oxytocin. Optical rotations of both regenerated and original oxytocin were determined. For the regenerated oxytocin, the rotation was $[\alpha]_D^{22} = -27.9°$ (0.57% in water) and for the original oxytocin, the value was $[\alpha]_D^{22} = -26.2°$ (0.53% in water).

Although the activity of the regenerated material was not quite as high as that of the original oxytocin, we believe that the data obtained on the properties of the regenerated oxytocic material warrant the conclusion that oxytocin has been regenerated from the S,S'-dibenzyl derivative by debenzylation followed by oxidation.

Summary. The benzylation of oxytocin by reduction with metallic sodium in liquid ammonia and subsequent treatment with benzyl chloride has been found to result in the formation of an S,S'-dibenzyl derivative which is biologically inactive. The activity has been regenerated by reduction of the benzylated oxytocin with sodium in liquid ammonia and a comparison of the material after oxidation with oxytocin itself has led to the conclusion that the biologically active material so formed represents oxytocin.

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