THE SEQUENCE OF AMINO ACIDS IN OXYTOCIN, WITH A PROPOSAL FOR THE STRUCTURE OF OXYTOCIN*

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Previous communications from this laboratory have shown that oxytocin hydrolysates contain leucine, isoleucine, tyrosine, proline, glutamic acid, aspartic acid, glycine, and cystine in equimolar ratios to each other and ammonia in a molar ratio to any one amino acid of 3:1 (1, 2). The sequence of these amino acids in oxytocin has now been investigated through partial hydrolysis studies of oxytocin, of desulfurized oxytocin, and of the large fragment resulting from treatment of performic acid-oxidized oxytocin (3) with bromine water (4, 5). The resulting peptides were separated into acidic and neutral components by use of ion exchange resins and were then further separated by paper chromatography. The composition of the various peptides was determined by elution, hydrolysis, and analysis for amino acids by paper chromatography. On the basis of these data along with other information such as that derived from utilization of the Edman degradation (6) and from the cleavage with bromine water (5), together with certain assumptions which will be mentioned, a tentative structure for oxytocin is proposed.

EXPERIMENTAL

Partial Hydrolysis of Oxytocin and Desulfurized Oxytocin—Oxytocin and desulfurized oxytocin (7) were hydrolyzed essentially as described by Consden, Gordon, Martin, and Synge for the partial hydrolysis of gramicidin S (8). The techniques utilized in the partial hydrolysis of wool (9) were then applied for the separation of the resulting mixture. Approximately 10 mg. of oxytocin or desulfurized oxytocin were dissolved in 1 ml. of a mixture of equal volumes of concentrated HCl and glacial acetic acid and the solution was kept at 37° under an atmosphere of nitrogen for 6 days. The hydrolysate was freed of acid by evaporation under reduced pressure and by repeated evaporation with water under the same conditions. The residue was then placed on a column of Amberlite IR-4B

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(11 × 0.7 cm.), previously brought to pH 3 by treatment with 1 N HCl followed by washing overnight with water, and the neutral fraction of the hydrolysate was eluted with 20 ml. of water. Elution with 20 ml. of 1 N HCl then gave the acidic fraction of the hydrolysate.

In the case of the oxytocin hydrolysate, the eluate containing the neutral fraction, after evaporation, was taken up in 1 ml. of water and treated at 0° for one-half hour with an excess of bromine water. The acidic peptides so formed, in which cystine had been oxidized to cysteic acid, were separated from the residual neutral peptides by fractionation on an Amberlite IR-4B column at pH 3 as already described. Aliquots, usually one-seventh, of the three fractions so obtained, neutral, acidic, and cysteic acid-

<table>
<thead>
<tr>
<th>Peptide area No.</th>
<th>Amino acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenol</td>
</tr>
<tr>
<td>1*</td>
<td>0.72</td>
</tr>
<tr>
<td>2†</td>
<td>0.33</td>
</tr>
<tr>
<td>3†</td>
<td>0.17</td>
</tr>
<tr>
<td>4†</td>
<td>0.16</td>
</tr>
<tr>
<td>5‡</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* Peptide 1 was obtained from the neutral fraction of the hydrolysate.
† Peptides 2, 3, and 4 were obtained from the acidic fraction.
‡ Peptide 5 was obtained after treatment of the acidic fraction with bromine water.

containing, were then subjected to two-dimensional ascending paper chromatography on Whatman No. 4 paper with the solvent systems of 5:1:5 n-butyl alcohol-acetic acid-water and 75 per cent aqueous phenol. In some cases, n-butyl alcohol-water was used as the first solvent. After development of the ninhydrin color, the chromatogram was used as a guide to elute the peptide areas from similar undeveloped chromatograms. The eluates, after evaporation, were then hydrolyzed in 6 N HCl at 120° in an atmosphere of nitrogen for 16 hours and the constituent amino acids of the peptides were determined by two-dimensional paper chromatography. Leucine and isoleucine were differentiated in some cases according to the method of Work (10) with tert-amyl alcohol as the solvent. The results are given in Table I.

In the case of the desulfurized oxytocin, the hydrolysis was continued for 8 days under the conditions already described and the products were
separated into neutral and acidic fractions. The results are summarized in Table II.

Partial Hydrolysis of Dinitrophenyl (DNP) Derivative of Large Degradation Fragment—The large degradation fragment was obtained from performic acid-oxidized oxytocin by treatment with bromine water and separated by counter-current distribution as described previously (5). 10 mg. of this material were converted to the N-DNP derivative by treatment with dinitrofluorobenzene (DNFB) and the product was separated from salts by adsorption on talc (11). The colored band was eluted with an 80:20 dioxane-water mixture. The eluate was lyophilized and heated under a reflux for a total of 15 hours with 2.5 ml. of 0.1 N HCl. After each 3 hour period, hydrolysis was interrupted and the solution was extracted with ethyl acetate. The DNP derivative of the large fragment is essentially insoluble in ethyl acetate; however, most of the yellow color was extractable by ethyl acetate after the first two periods. DNP-isoleucine and a high proportion of DNP-isoleucyl peptides were thus removed and from the aqueous layer the smaller peptides derived from the end furthest from the isoleucine residue were obtained. This study of the DNP heptapeptide fragment was particularly helpful since only one of the 2 molecules of cysteic acid originally present in the performic acid-oxidized oxytocin was present and the leucine-isoleucine spot could be readily identified as leucine, the isoleucine having been combined as the DNP derivative. The hydrolysate was then evaporated to dryness and fractionated on a column of Amberlite IR-4B into neutral and acidic components at pH 3.3. The acidic fraction was further fractionated at pH 2.6 into weakly acidic and cysteic acid peptides (12). The $R_F$ values and amino acid composition of peptides in the cysteic acid fraction are given in Table III.

Edman Degradation—8 mg. of performic acid-oxidized oxytocin (3) were allowed to react with 4.8 µl. of phenyl isothiocyanate at a pH of approximately 8.5. The procedure of Edman (6) was followed with the following

<table>
<thead>
<tr>
<th>Peptide area No.</th>
<th>$R_F$ value</th>
<th>Amino acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>$n$-Butyl alcohol-acetic acid-water</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.55</td>
<td>0.52</td>
</tr>
<tr>
<td>7</td>
<td>0.20</td>
<td>0.25</td>
</tr>
<tr>
<td>8</td>
<td>0.46</td>
<td>0.26</td>
</tr>
<tr>
<td>9</td>
<td>0.63</td>
<td>0.63</td>
</tr>
</tbody>
</table>
modifications. The white residue left after treatment with nitromethane-
HCl solution was well dried over KOH and was dissolved in 1 ml. of water. An aliquot corresponding to 1.5 to 2.5 μm was removed, concentrated in vacuo to dryness, and the residue was hydrolyzed in 1 ml. of 6 N HCl for 12 to 16 hours in a sealed tube under nitrogen. The acid was then removed in vacuo and the sample was analyzed on the starch column (13) for residual amino acids. The pattern in the second column of Table IV

**Table III**

*Peptides from Cysteic Acid Fraction from Hydrolysis of DNP Derivative of Large Degradation Fragment*

<table>
<thead>
<tr>
<th>Peptide area No.</th>
<th>RF value</th>
<th>Amino acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenol</td>
<td>α-Butyl alcohol-acetic acid-water</td>
</tr>
<tr>
<td>10</td>
<td>0.15</td>
<td>0.17</td>
</tr>
<tr>
<td>11</td>
<td>0.27</td>
<td>0.18</td>
</tr>
<tr>
<td>12</td>
<td>0.61</td>
<td>0.44</td>
</tr>
<tr>
<td>13</td>
<td>0.65</td>
<td>0.53</td>
</tr>
</tbody>
</table>

**Table IV**

*Edman Degradation of Performic Acid-Oxidized Oxytocin (Molar Ratios)*

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Untreated</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.00</td>
<td>1.00</td>
<td>0.74</td>
<td>0.34</td>
<td>0.24</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.93</td>
<td>0.69</td>
<td>0.17</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>1.13</td>
<td>1.08</td>
<td>1.05</td>
<td>1.00</td>
<td>1.10</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.03</td>
<td>1.03</td>
<td>0.97</td>
<td>0.90</td>
<td>0.55</td>
</tr>
<tr>
<td>Aspartic</td>
<td>1.08</td>
<td>0.95</td>
<td>1.02</td>
<td>0.97</td>
<td>0.99</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.08</td>
<td>1.16</td>
<td>0.95</td>
<td>0.95</td>
<td>0.95</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>3.02</td>
<td>4.13</td>
<td>2.72</td>
<td>3.09</td>
<td>3.82</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td>1.99</td>
<td>1.12</td>
<td>1.13</td>
<td>1.06</td>
<td>0.95</td>
</tr>
</tbody>
</table>

shows that approximately 1 mole of cysteic acid had disappeared from performic acid-oxidized oxytocin in Step 1. The remainder of the aqueous solution was lyophilized and again treated with phenyl isothiocyanate. The entire procedure with the removal of successive aliquots was repeated in this way three times. The results are given in Table IV. The nature of the phenylthiohydantoins that had been successively removed was established by hydrolysis in dilute barium hydroxide and identification of the resulting amino acids by paper chromatography. The results agree qualitatively with the quantitative analyses obtained with the use of the starch column on the hydrolysates of the residues, although the yields were low.
DISCUSSION

In the following derivation, peptides obtained from the partial hydrolysis of native oxytocin, desulfurized oxytocin, and the sulfonic acid heptapeptide obtained by degradation of performic acid-oxidized oxytocin with bromine water have been used. In the peptides derived from desulfurized oxytocin, an alanine residue obviously indicates the position of one-half the cystine residue in oxytocin. In the oxidized peptides a cysteic residue similarly corresponds to one-half of a cystine residue (CyS-), since the two cysteic acid residues are derived from the cystine residue in oxytocin.

In Peptide 10, Asp.Cysteic, which was isolated from the acidic fraction of the hydrolysate of the dinitrophenyl derivative of the large fragment, the presence of a free amino group on the aspartic acid was indicated by the results from the application of the Sanger technique for end-group analysis (16). The composition of this peptide was confirmed in part by the isolation of Peptide 6 [Asp,Ala] from the partial hydrolysis of desulfurized oxytocin. The peptide, Asp.Cysteic, together with the three other peptides isolated in the same acidic fraction from the heptapeptide, Peptide 11 [Cysteic,Pro], Peptide 12 [Cysteic,Pro,Leu], and Peptide 13 [Cysteic,Pro,Leu,Gly], readily allowed the following sequence to be derived:

\[
\text{Asp. Cysteic Pro. Leu. Gly}
\]  

and the corresponding sequence \((I')\) in oxytocin would then be

\[
\text{Asp. CyS-. Pro. Leu. Gly}
\]  

This sequence is consistent with the composition of Peptide 1 [Pro,Leu,Gly], which was obtained from the neutral fraction of the hydrolysates resulting from the treatment of oxytocin with 1:1 acetic acid-12 N HCl mixture for 6 days or with 0.25 M oxalic acid at 110° for 15 hours. The same tripeptide was isolated also from the neutral fraction resulting when the dinitrophenyl derivative of the large fragment was hydrolyzed in 0.1 N HCl for 15 hours. It traveled on paper in the solvents n-butyl alcohol-acetic acid-water and

1 The abbreviations used for the amino acids are those suggested by Brand and collaborators (14, 15). CyS- is used to indicate one-half of a cystine residue in a sequence. For the full cystine residue the representation CyS-SCy will be employed. To avoid confusion in peptides containing cysteic acid, the word itself instead of an abbreviation will be used. The convention is followed that, when peptides of known structure are referred to, the residues are joined by a period and that, in peptides of unknown sequence, the residues appear within brackets and are separated by commas. The amino acid appearing at the left in a known sequence is that bearing a free amino group. In discussing sequences in the performic acid-oxidized oxytocin, Roman numerals will be used. For the corresponding sequences arrived at for oxytocin, Roman numerals with a prime designation will be used.
phenol just behind free leucine and isoleucine and was separated from the latter two amino acids with tert-amyl alcohol as a solvent.

When Peptide 5 [Cysteic, Asp, Glu] is considered, sequence (I) becomes

\[ \text{Glu. Asp. Cysteic. Pro. Leu. Gly} \] (II)

Sequence (II) is also consistent with the composition of Peptide 7 [Ala, Asp, Glu] isolated from the hydrolysate of desulfurized oxytocin and with that of Peptide 4 [CyS-SCy, Asp, Glu] isolated from the hydrolysate of oxytocin. The corresponding sequence (II') in oxytocin would be

\[ \text{Glu. Asp. CyS. Pro. Leu. Gly} \]

Peptide 8 from the desulfurized oxytocin contained only isoleucine and glutamic acid and therefore the sequence (III') may be derived.

\[ \text{Ileu. Glu. Asp. CyS. Pro. Leu. Gly} \]

Peptide 3 [Tyr, CyS-SCy, Asp, Glu] obtained from oxytocin differed from Peptide 4 only by the additional presence of tyrosine and therefore the sequence (IV') follows:

\[ \text{Ileu. Glu. Asp. CyS. Pro. Leu. Gly} \]

In Peptides 2 and 9, which were isolated from the hydrolysates of oxytocin and desulfurized oxytocin, respectively, the differentiation between leucine and isoleucine was not made. However, with the assignment of isoleucine, Peptide 2 becomes [Tyr, CyS-SCy, Asp, Glu, Ileu] and Peptide 9 becomes [Ala, Asp, Glu, Ileu]. These could both be explained by sequence (IV').

In a previous study in this laboratory, by use of the dinitrophenyl technique for end-group analysis, it was found that only one of the two cysteic acid residues in performic acid-oxidized oxytocin carried a free amino group (5), and from these results it was deduced that in oxytocin one-half of the cystine moiety possesses a free amino group if it is assumed that no rearrangement had occurred in the performic acid oxidation. Evidence was also offered that this residue is linked through its carboxyl group to the amino group of tyrosine (5). On this basis the partial sequence (V') for oxytocin follows.

\[ \text{CyS. Tyr} \]

\[ \text{Ileu. Glu. Asp. CyS. Pro. Leu. Gly} \]
Although no peptide was separated from the hydrolysates of either oxytocin or desulfurized oxytocin which would indicate clearly a linkage between tyrosine and isoleucine, a linkage between these two amino acids has been considered likely on the basis of the results of a study of the treatment of performic acid-oxidized oxytocin with bromine water (5). It was shown that performic acid-oxidized oxytocin was degraded by the action of bromine water into two fragments. One fragment was identified as β-sulfoalanyldibromotyrosine; the other contained the seven remaining amino acids in a fragment in which isoleucine now possessed a free amino group. It appeared reasonable to assume that the cleavage by bromine water into these two fragments was the result of a cleavage of a tyrosine-isoleucine bond involving the amino group of isoleucine, and the sequence Cysteic.Tyr.Ileu was suggested. To make more certain that a Tyr.Ileu linkage exists in the molecule, the Edman degradation has been applied to performic acid-oxidized oxytocin. The results from the analysis on the starch column of the hydrolysates of the residues remaining after each of four applications of the Edman reaction are given in Table IV. It can be seen that approximately 1 mole of cysteic acid has been removed in the first application of the Edman reaction to performic acid-oxidized oxytocin. In Step 2 the disappearance of tyrosine is almost complete and a small amount of isoleucine has been removed. Step 3 of the Edman reaction results in the further disappearance of isoleucine, and in Step 4 the loss of considerable amounts of glutamic acid is apparent. Thus independent evidence for the sequence Cysteic.Tyr.Ileu.Glu for the amino end of performic acid-oxidized oxytocin is afforded.

With a linkage between tyrosine and isoleucine, the sequence (VI') of oxytocin then would follow:

\[
\]

(VI')

Thus oxytocin would be an octapeptide involving a cyclic pentapeptide containing cystine with one-half of the cystine moiety possessing a free amino group and with the carboxyl group adjacent to the latter joined to the amino group of tyrosine. The other half of the cystine residue would be connected through its amino group to aspartic acid and would be linked through its carboxyl group to the tripeptide prolylleucylglycine.

In order to derive a structure for oxytocin based on this sequence, one would then have to establish the source from which the 3 moles of ammonia, which are found in a hydrolysate of oxytocin, are derived. Also remaining to be established are whether it is the α- or γ-carboxyl group of glutamic acid and the α- or β-carboxyl group of aspartic acid which are involved in peptide linkage.
Tentatively, it would seem logical to assume that 2 moles of ammonia are derived from amide linkages involving the carboxyl groups of glutamic acid and aspartic acid. Some evidence has been derived that glycine in the terminal position may also carry an amide grouping accounting for the third mole of ammonia. It has been shown that oxytocin, when treated with 1 N HCl at 90–100° for 1 hour, loses approximately 1 mole of ammonia. Oxytocin so treated has therefore been subjected to the thiohydantoin carboxyl end-group determination (17, 18). By use of paper chromatography on the hydrolysate of the thiohydantoin obtained, glycine was obtained in good yield along with a trace of leucine. Oxytocin itself gave a negative result.

Moreover, it has been shown that oxytocin is not inactivated or cleaved by carboxypeptidase. It might also be pointed out that the isoelectric point of oxytocin has been found to agree with that to be expected of a compound possessing one free amino group and one free phenolic group (19, 20).

On the basis of the evidence so far obtained and with the assumptions for the positions of the amide groups, and with the further assumption that glutamine and asparagine moieties are present rather than their isomers, we would propose as a working hypothesis the accompanying structure for oxytocin.

\[
\text{SUMMARY}
\]

On the basis of partial hydrolysis studies on oxytocin, desulfurized oxytocin, and the large fragment obtained by treating oxytocin with bromine water, along with other data and certain assumptions, a tentative structure

2 Taylor, S. P., Jr., and du Vigneaud, V., unpublished data.
3 Evidence so far obtained in this laboratory would indicate that the amino acids present in oxytocin are of the L configuration.
has been proposed for oxytocin, the principal uterine contracting and milk-ejecting hormone of the posterior pituitary.

BIBLIOGRAPHY

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