rides the activation of the later phase of the catalytic reaction. It will then appear that progressively lower concentration is required to produce inhibition as the pH is raised.

Thus, the mechanism we propose for the effects of ADP on the rapid and steady-state kinetics of glutamate dehydrogenase is sufficient to explain the variety of effects reported here and in the literature using only direct interactions between ADP and other ligands on the enzyme surface.

Acknowledgments

We express our appreciation for the excellent programming for data analysis and curve fitting provided by Mr. Joseph N. Simone.

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The Role of the Internal Cross-Link in Oxytocin. Preparation of Mercury Mercaptide Oxytocin Derivatives†

Ruth Sperling* and Marian Gorecki

ABSTRACT: The disulfide bond of oxytocin was reduced using an insoluble reducing reagent, dihydrolipoyl attached to polyacrylamide P-6. The sulfhydryl groups thus formed were bridged by bivalent mercury ion with the formation of an S–Hg–S bridge. The monomeric product obtained was shown to be homogeneous and contained one mercury atom per peptide molecule. Further addition of mercury ions led to the formation of a dimercurated open-chain derivative. The mercuration had a marked effect on the ultraviolet and circular dichroic spectral features of these derivatives in comparison with oxytocin. The tryosine's fluorescence intensity of mono- and dimercurated oxytocin derivatives was quenched in comparison with that of oxytocin and oxytoceine, respectively. The mercury–oxytocin derivatives were found to have negligible biological activity as muscular-contracting agents of isolated rat uteri. Elongation of the internal cross-link of oxytocin by 3 Å, thus dramatically affects the biological activity of the hormone, which is intimately connected with the native conformation of the peptide.

Internal disulfide cross-links play a decisive role in maintaining the three-dimensional conformation of proteins which possess such linkages (Anfinsen, 1965–1966). Yet not all the disulfide bonds are crucial for the stabilization of the biologically active conformation (Azari, 1966; Kress and Laskowski, 1967; Neumann *et al.*, 1967; Sperling *et al.*, 1969; Arnon and Shapira, 1969). It would be interesting, therefore, to know what demands there are on the length and the geometry of the internal cross-links which are essential for maintaining the biological active conformation of proteins.

Introduction of a mercury atom between the sulfur atoms of a disulfide cross-link results in the formation of an S–Hg–S bridge known to be linear (Grdenic, 1965). This insertion of mercury lengthened the disulfide bridge by about 3 Å (Yakel and Hughes, 1954; Pauling, 1960; Bradley and Kunchur, 1965). This approach was successfully applied to various proteins where a single disulfide bond was modified. Thus, insertion of mercury into the IV–V bond of RNase (Sperling *et al.*, 1969), 43–152 bond of papain (Arnon and Shapira, 1969), and A6–A11 bond of insulin (Sperling and Steinberg, 1974) did not affect the biological properties of these proteins. Elongation of all the disulfide bridges of RNase resulted in a marked decrease in the biological activity (Sperling and Steinberg, 1971).

In the small hormonal cyclic peptides, oxytocin and vaso-

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pressin, the role of the ring dimensions and geometry, in stabilizing the active conformation, was studied using a synthetic approach. A series of peptides, in which the 20-membered S–S ring was modified by amino acid and peptide analogs, was synthesized, and their biological as well as physical properties studied (Jarvis *et al.*, 1961; Jost and Sorm, 1971a,b; Rudinger, 1971; Prohazka *et al.*, 1972).

In the present study the ring of oxytocin was modified by insertion of a bivalent mercury ion into the disulfide bridge. This was achieved by reducing the disulfide bond by an insoluble dihydrolipoyl-containing polymer (Gorecki and Patchornik, 1973). The reduction product was oxytoceine, an unstable linear disulfhydryl derivative which was first characterized by Yamashiro *et al.* (1966). The sulfhydryl groups of oxytoceine were cross-linked by bivalent mercury according to the following reaction (Edelhoch *et al.*, 1953; Cecil, 1963; Webb, 1966)

$$2(\text{-SH}) + Hg^{2+} \rightleftharpoons \text{S-Hg-S} + 2H^{+}$$

This resulted in the formation of [1,6-mercuri-*S*,*S*′-dicysteine]-oxytocin–[oxytocin·1Hg]. It is shown below that elongation of the disulfide bridge of oxytocin by mercury had a dramatic effect on its biological activity. Further details concerning the properties of [oxytocin·1Hg] are presented. Biological and physical properties of [oxytocin·2Hg], an open-chain derivative are also discussed.

Experimental Section

Materials

Oxytocin was a gift from Dr. Donald Yamashiro. 5,5′-Dithiobis(2-nitrobenzoic acid) (Nbs_2)[1] was purchased from Aldrich. Mercuric chloride labeled with ^{203}Hg was purchased from the Israel Atomic Energy Commission, Yavne, Israel. Aanlytical grade mercuric chloride was obtained from B.D.H. Urea was recrystallized from 95% aqueous ethanol. Lipoylated polyacrylamide was synthesized according to the method of Gorecki and Patchornik (1973) (0.38 mmol of lipoyl/g of dry polymer).

All buffer solutions were prepared from double-distilled water, boiled, and cooled under nitrogen.

Methods

Preparation of the Reducing Column. A column of dihydrolipoic acid bound to polyacrylamide P-6 was used for reducing the disulfide bond of oxytocin. The reagent, stored as lipoyl polyacrylamide, was converted to the thiol form by $NaBH_4$ treatment, just prior to its use for the reduction of the hormone, in the following way.

Dry polymer (500 mg) was swollen in 10 ml of distilled water; 10 ml of 1.0 M sodium borohydride was added to the stirred suspension. After 30 min the polymer was filtered off, and washed successively with water, 0.2 M acetic acid, water, and finally with 0.05 M Tris-Cl (pH 8.0). The swollen reduced polymer was packed in a glass column (4.4 × 50 mm), washed with 10 ml of 0.05 M Tris-Cl buffer (pH 8.0), and used immediately for the reduction of oxytocin.

Reduction of Oxytocin. Oxytocin (5 μmol, 5.04 mg) was dissolved in 50 μl of 0.1 M hydrochloric acid and brought to pH 8.0. This solution was applied to a 4.4 × 50 mm column packed with the reducing polymer and allowed to stand in the closed column, at 23°, for the appropriate reduction time. Oxytoceine was eluted from the column with 0.05 M Tris-Cl buffer (pH 8.0) into a volumetric flask, to which 5 μl of 4 M HCl/1 ml of effluent was added, sufficient to ensure a lowering of the pH of the effluent to pH 2.0.

The peptide concentration was determined by amino acid analysis of a hydrolyzed sample, and the concentration of sulfhydryl groups was estimated by the modified method of Ellman (1959). Samples (0.1 ml) of oxytoceine from the acidic solution were mixed with 0.5 ml of Nbs_2 (4 mg/ml in 0.1 M phosphate buffer, pH 7.3) and 4 ml of 0.2 M phosphate buffer (pH 7.3). The resulting color values were measured at 412 nm. A molar extinction coefficient value of 13,600 was used in the sulfhydryl content calculations.

Amino acid analyses were carried out with a Beckman-Spinco Model 120C automatic amino acid analyzer. Samples were hydrolyzed in constant-boiling HCl in evacuated sealed tubes for 22 hr at 110° (Spackman *et al.*, 1958).

Paper electrophoresis at 60 V/cm, at pH 3.5 (0.05 M pyridine–acetic acid buffer), was performed for 45 min using Whatman No. 1 paper. The peptides were stained with ninhydrin.

Potentiometric titrations of oxytoceine with mercuric chloride were conducted under nitrogen in a 5-ml titration vessel coupled to a pH-meter (Radiometer, Copenhagen) with a gold amalgam-saturated calomel electrode pair (Cecil, 1955).

Spectrophotometric measurements were performed using a Zeiss Model PMQ II spectrophotometer. Quartz cells with a 1-cm light path were used.

Circular dichroism was measured with a Cary Model 6001 spectropolarimeter at room temperature. The circular dichroism data are presented as $\Delta\epsilon$ difference between the molar absorption coefficients of left- and right-handed circularly polarized light. Signal to noise ratio at band maxima were 20:1. A value of 1008 was taken for the molecular weight to oxytocin.

Fluorescence measurements were performed with a Turner 21 spectrophotofluorimeter. All the various oxytocin derivatives tested had an optical density below 0.1.

Biological oxytocic activity was measured on isolated uteri from rats according to the method of Holton as modified by Munsick (1960), with the use of magnesium-free Van Dyke–Hastings solution as the bathing fluid.

Results

Reduction of Oxytocin. A column of dihydrolipoyl attached to polyacrylamide P-6 was used as the reducing agent. The concentration of sulfhydryl groups on the polymer was ~120 μmol/ml of carrier. Quantitative reduction was obtained using 30 μmol of SH groups of polymer/μmol of oxytocin.

The reduction could be performed in the pH range 7.5–8.5 with either Tris-Cl or phosphate buffers. We selected 0.05 M Tris-Cl (pH 8.0) as the standard conditions.

The peptide was applied to the column and kept there for the appropriate reduction time. It was then eluted with the same buffer used for the application. The elution products were immediately acidified (see Methods) and could be kept for a few hours without any detectable oxidation. The peptide emerged just after the void volume of the column and was quantitatively eluted with 12 ml of eluent (80% can be collected in the first 4 ml from a 4.4 × 50 mm column. Even when oxytocin which was not completely dissolved was applied to the column, the recovery of oxytoceine was quantitative.)

Figure 1 demonstrates the time course of the reduction. It is seen that within 3 hr complete reduction is obtained,

[1] Abbreviation used is: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

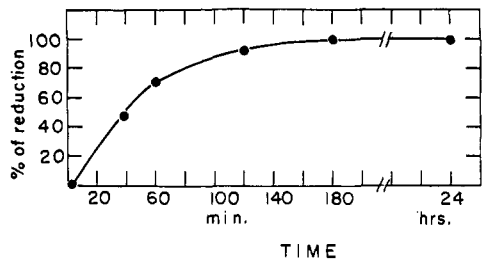


FIGURE 1: Reduction of oxytocin on dihydrolipoyl polyacrylamide P-6 column. Oxytocin (5 μmol) in 0.5 ml of Tris-Cl buffer (pH 8.0) was applied onto the reducing column (0.5 × 5 cm) and the flow was halted. After a given time the peptide was eluted and extent of reduction was measured.

as indicated by the comparison of the amino acid content of the eluted peptide with its sulfhydryl content.

Preparation of Mercurated Derivatives of Oxytocin. Mercurated oxytocin was prepared by potentiometric titration of oxytoceine with mercuric chloride using a gold amalgam electrode, which is reversible to mercuric ions; 5 ml of a 0.3 mM solution of oxytoceine in 0.05 M Tris-Cl buffer, brought to pH 2.5 with 25 μl of 4 M HCl, were flushed with nitrogen gas and titrated slowly with 5 mM $HgCl_2$; a gold amalgam-saturated calomel electrode pair serving as indicator (Cecil, 1955), Figure 2 illustrates such a potentiometric titration. As can be seen, there is a very sharp end point in the titration curve, with the concomitant jump in electrode potential of about 200 mV. The end point corresponds to the addition of one mercury ion per two sulfhydryl groups in the titrated solution (Steinberg and Sperling, 1967), and results in the formation of an oxytocin derivative in which one mercury atom is introduced between two sulfur atoms of cystine forming an S–Hg–S bridge

$$2(-SH) + Hg^{2+} \rightleftharpoons -S-Hg-S- + 2H^+$$

Further slow addition of a second equivalent of $HgCl_2$ resulted in the formation of an open oxytocin derivative, having two S–Hg^+ bonds (Edelhoch *et al.*, 1953; Cecil, 1963; Webb, 1966)

$$S-Hg-S + Hg^{2+} \rightleftharpoons 2(-SHg^+)$$

Properties of the Oxytocin Mercury Derivatives. HOMOGENEITY. Both mercurated derivatives are homogeneous, as checked by paper electrophoresis at pH 3.5, and do not contain polymeric by products. The migration of monomercurated oxytocin is slightly slower than that of native oxytocin while the bimercurated oxytocin migrated almost twice as fast.

The homogeneity of the monomercurated derivative of oxytocin was further checked by gel filtration. Monomercurated oxytocin was prepared using [^{203}Hg]$HgCl_2$ and was concentrated by ultrafiltration through Diaflo UM-0.5 membrane. The concentrated solution (1 ml) was applied to a Sephadex G-10 column. Figure 3 illustrates the chromatographic pattern of monomercurated oxytocin (3a). For comparison the elution pattern of native oxytocin (3b) and a prepared mixture of 10% oxytocin and 90% monomercurated oxytocin (3c) are also included. The monomercurated derivative of oxytocin appears as a single symmetrical peak (3a) which is slightly retarded with respect to the native oxytocin (3b,3c). A tiny amount of polymeric products not exceeding 0.2% is eluted in the front of the column (3a). Therefore, it can be concluded that in the case of the monomercurated derivative, the bridging of the sulfhydryl groups of oxytocin with mercury resulted in the formation of monomeric material

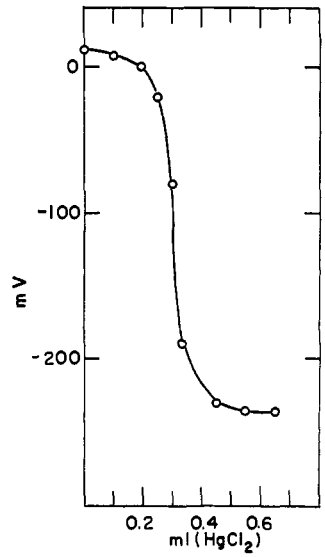


FIGURE 2: Potentiometric titration of oxytoceine (1.5 μmol) with 5 mM $HgCl_2$. All titrations were carried out with a gold amalgam-saturated calomel electrode pair at 25° in 0.05 M Tris-Cl brought to pH 2.5 with HCl.

having an intramolecular S–Hg–S bridge. This derivative, which has a 21-membered ring, will be designated [oxytocin·1Hg]. The bimercurated derivative will be designated [oxytocin·2Hg].

OXYTOCIC ACTIVITY. Measured as the muscular contraction of isolated rat uterus shows that the activity of [oxytocin·1Hg] is 1% and that of [oxytocin·2Hg] is much lower than 0.1% of that of the native oxytocin. It can thus be concluded that

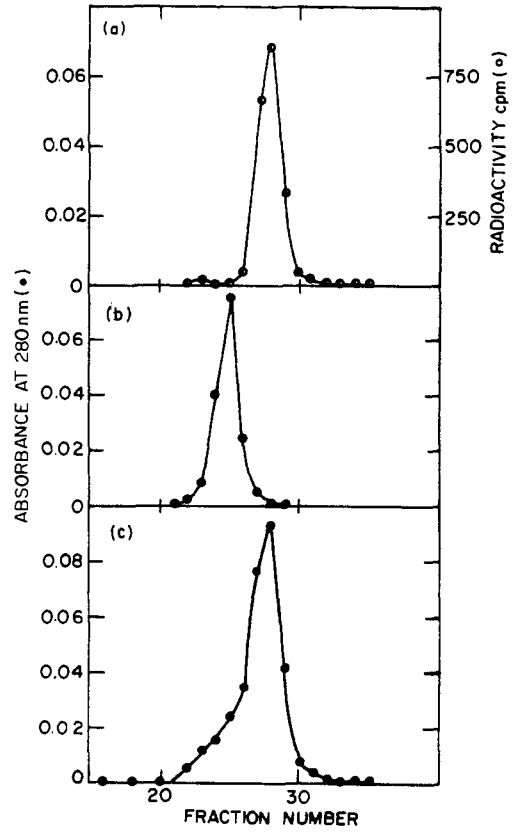


FIGURE 3: Gel filtration of [oxytocin·1Hg], labeled with [^{203}Hg]-$HgCl_2$ (a), oxytocin (b), and a mixture of [oxytocin·1Hg] (90%) and oxytocin (10%) (c), on Sephadex G-15 column (0.9 × 100 cm) in 0.001 M HCl.

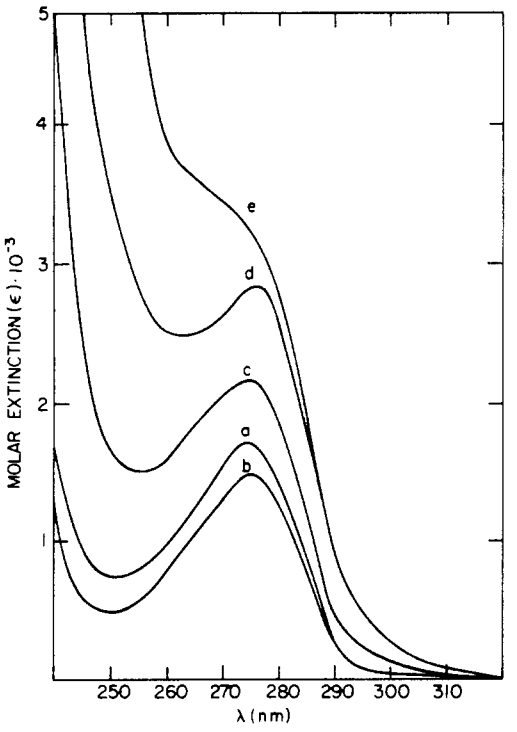


FIGURE 4: Ultraviolet absorption spectrum of oxytocin (curve a), oxytoceine (curve b), [oxytocin·1Hg] at pH 2.5 (curve c), [oxytocin·1Hg] at pH 9.0 (curve d), and [oxytocin·2Hg] at pH 2.5 (curve e). Measurements were performed in 0.05 M Tris-Cl.

the mercuration has greatly damaged the oxytocic activity of the mercury derivatives. However, the 1% activity of [oxytocin·1Hg] may be regarded as an upper limit as some oxidation of the reduced oxytocin before mercuration might not be excluded.

ULTRAVIOLET ABSORPTION SPECTRA. Figure 4 illustrates the ultraviolet absorption spectra of oxytocin, oxytoceine and its mercurated derivatives at pH 2.5. There is a distinct difference between oxytocin and its reduced form, which can be accounted for by the contribution of the disulfide bond. The ultraviolet (uv) spectrum of [oxytocin·1Hg] in the 300–250-nm region (Figure 4c) is greatly enhanced with respect to that of oxytocin (Figure 4a). This change is probably due to the difference in absorption between the S–Hg–S chromophore in the modified hormone compared with the S–S group it replaces. Indeed, the conversion of an S–S bond into an S–Hg–S bridge, in the case of cystine, resulted in increase in the absorption at the near-uv region with the formation of a shoulder in the absorption spectrum at 260 nm and a difference spectrum of $\Delta\epsilon \sim 400$ at 275 nm (Steinberg and Sperling, 1967). An increase in the absorption of RNase in the near-uv region was also obtained upon conversion of a disulfide bond (IV–V) into an S–Hg–S bridge (Sperling *et al.*, 1969). The uv absorption of the mercurated cystine derivative with an S–Hg–S bridge, was dependent on the pH of the solution and increased upon raising the pH (Steinberg and Sperling, 1967). As shown in Figure 4d, [oxytocin·1Hg] exhibits a marked increase in its molar absorption coefficient upon raising the pH from 2.5 to 9.0. On the other hand, the uv absorption of oxytocin suffered almost no change when the pH was increased similarly. The uv absorption of [oxytocin·2Hg] at pH 2.5 (Figure 4e) is much higher than all the other derivatives. The increase in absorption can be explained by the convertion of the disulfide bond of oxytocin into two

TABLE I: Relative Fluorescence Yield of Oxytocin Derivatives.

Compound	Rel Fluorescence Yield (%)[a]
Oxytoceine	100
[Oxytocin·2Hg]	45
Oxytocin	58.5
[Oxytocin·1Hg]	25

[a] The fluorescence intensity of oxytoceine was chosen as 100%.

S–Hg^+ bonds (Steinberg and Sperling, 1967; David *et al.*, 1974).

FLUORESCENT INTENSITY of oxytocin and oxytoceine was compared with that of [oxytocin·1Hg] and [oxytocin·2Hg] (Table I), in an excitation wavelength of 276 nm and an emission wavelength of 310 nm. High quenching of the fluorescent intensity was obtained in the transition from an open-chain form (oxytoceine and [oxytocin·2Hg]) to a closed-ring form (oxytocin and [oxytocin·1Hg]). Introducing mercury atom bound to sulfhydryl group, either in the S–Hg–S or SHg^+ forms, causes further quenching of the tyrosines' fluorescence intensity.

CD SPECTRA IN THE NEAR-ULTRAVIOLET REGION. Figure 5 illustrates the CD spectra in the near-uv region of oxytocin and [oxytocin·1Hg], both of which have a closed-ring structure. The spectrum of oxytocin (5a) shown here is identical with that reported in the literature (Walter *et al.*, 1968; Beychok and Breslow, 1968). The CD spectrum of [oxytocin·1Hg] at pH 2.5 (5b) exhibits three small positive bands in the 250–320-nm region and a larger negative band at 238 nm. On neutralization of the α-amino group of [oxytocin·1Hg] at pH 9.0 the positive bands above 250 nm disappear and there is a remarkable enhancement of the Cotton effect in the 240–260-nm region with a slight shift of the maximum toward longer wavelengths. It is pertinent to note that 8 M urea hardly affects the CD spectrum of oxytocin whereas it completely abolishes the CD bands of [oxytocin·1Hg] in the 240–325-nm region. The CD spectrum of [oxytocin·1Hg] reflects the inherent structural properties of this oxytocin derivative, as the CD spectrum of oxytocin in the 240–325-nm region is not affected by the addition of 2 mol of $HgCl_2$/mol of oxytocin.

Reduction of the disulfide bond of oxytocin, resulting in an open-form structure oxytoceine, is accompanied by the disappearance of the 240-nm positive Cotton effect (Figure 5d). Yet the negative band around 270 nm is still present. The CD spectrum of [oxytocin·2Hg] (Figure 5e) shows three bands in the 270–320-nm region and a strong negative cotton effect at about 240 nm.

Discussion

Dihydrolipoyl polyacrylamide was found to be a very efficient reducing agent for oxytocin, complete reduction being obtained within 3 hr. The low redox potential of the dihydrolipoyl moiety combined with the ease of separation of this insoluble reducing reagent from its substrate makes dihydrolipoyl polyacrylamide a highly potent reducing agent (Gorecki and Patchornik, 1973). The ease of separation of the substrate from the reducing reagent is especially useful in the case of oxytocin which tends to be oxidized to oxytoceine (Yamashiro *et al.*, 1966). Therefore the fast removal

of the insoluble reagent, followed by immediate acidification of the reduced peptide, enables maintenance of the product in its reduced form.

The reaction of oxytoceine with mercuric chloride in a 1:1 molar ratio resulted in the formation of a derivative with an S–Hg–S bridge as monitored by the potentiometric titration (see Figure 2). Column chromatography revealed that this derivative is monomeric indicating intramolecular cross-linking of the sulfhydryl groups by mercury. We may, therefore, conclude that this derivative, which we have designated [oxytocin·1Hg] is a 21-membered ring closed by an S–Hg–S bridge. Addition of 2 equiv of mercury/oxytoceine molecule led to the formation of an open peptide [oxytocin·2Hg] having two –S–Hg^+ bonds. Conversion of the disulfide bond of oxytocin into an S–Hg–S bridge caused a marked decrease in the hormone's biological activity. The open-chain mercury derivative [oxytocin·2Hg] is practically devoid of oxytocic activity.

The fluorescence intensity of [oxytocin·1Hg] is about half of that of oxytocin (see Table I). On the other hand, the fluorescence intensity of the tyrosine of [RNase·1Hg], in which disulfide bond IV–V was converted into an S–Hg–S bridge (Sperling *et al.*, 1969), was the same as that of native RNase. Thus the quenching of the tyrosine fluorescence of oxytocin by the mercury in the S–Hg–S bridge is most likely not a long-range effect. An external heavy atom can affect the fluorescence of tyrosine due to spin–orbit coupling, which causes a transition to the triplet state (McGlynn *et al.*, 1969). In this case the heavy atom and the tyrosine should be in close proximity to enable overlapping of orbitals. The tyrosine and the S–S bond are in close proximity in oxytocin as well, as the fluorescence of oxytocin is quenched relative to that of the open-chain derivative oxytoceine. This effect was explained by enhanced internal conversion, although intersystem crossing cannot be ruled out (Longworth, 1971).

The uv absorption spectra of [oxytocin·1Hg] and [oxytocin·2Hg] reflects the conversion of an S–S bond into an S–Hg–S bridge and S–Hg^+ bonds, respectively. The increase in the uv absorption of [oxytocin·1Hg] upon raising the pH can also account for some interactions with neighboring groups.

The disulfide bond has a dihedral angle of about 90° and an energy barrier of rotation around this bond of 10–20 kcal/mol (Calvin, 1954; Bergson, 1962). This restriction is caused by repulsion of the nonbonded electrons of the sulfur atoms (Pauling, 1949; Bergson, 1958, 1962; Linderberg and Michl, 1970), which depends on the interatomic distance of the sulfur atoms. Thus elongation of the disulfide bridge by 1 Å will remove the energy barrier for rotation around this bond (J. Schellman, private communication). Insertion of a mercury atom into the disulfide bridge results in the formation of a linear S–Hg–S bridge (Grdenic, 1965), which is elongated by 3 Å with respect to the S–S bond (Pauling, 1960; Yakel and Hughes, 1954; Bradley and Kunchur, 1965). This elongation may result in the removal of the hindrance to rotation around the disulfide bond.

The CD spectrum of oxytocin in the 240–320-nm region is quite complex (see Figure 5) and the Cotton effects in this region are accounted for by the tyrosine and the disulfide bond (Beychok and Breslow, 1968; Walter *et al.*, 1968). The 280-nm band is attributed to both tyrosine and the disulfide bond and 240–260-nm shoulder is mainly ascribed to the disulfide bond. This band is indeed absent from the CD spectrum of oxytocin analogs in which one or both sulfur atoms are replaced by methylene groups (Fric *et al.*, 1973). Oxytocin has a positive Cotton effect at 240–260 nm, whereas [oxytocin·Hg] has a negative Cotton effect at this region (see Figure 5). Increasing the pH to pH 9.0 results in the intensification of the 240–260-nm negative Cotton effect of [oxytocin·1Hg] (Figures 5b,c); without any change of sign of this peak, whereas in 8 M urea [oxytocin·1Hg] has no Cotton effect in the 320–240-nm region.

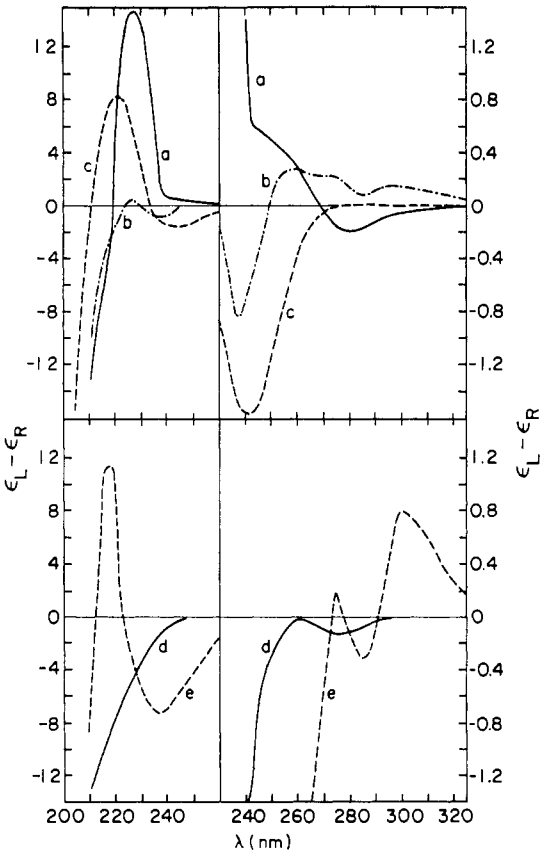


FIGURE 5: Circular dichroism spectra of oxytocin derivatives. Closed-ring structures: oxytocin (curve a), [oxytocin·1Hg] at pH 2.5 (curve b), and [oxytocin·1Hg] at pH 9.0 (curve c), are presented on the upper figure. Open-chain structures: oxytoceine (curve d) and [oxytocin·2Hg] (curve e) are presented on the lower figure. The spectra are presented as $\Delta\epsilon = \epsilon_L - \epsilon_R$, where ϵ_L and ϵ_R are the molar extinction coefficients for left and right circularly polarized light, respectively. (The molecular weight of oxytocin is taken as 1008 formula weight.)

Mercury is known to have a characteristic digonal coordination, additional ligand approaches leads to effective coordination, generally octahedral (Grdenic, 1965). Thus mercury bound in an S–Hg–S linear bridge can still bind neighboring coordination groups. For example, unprotonated neighboring amino groups can coordinate to the mercury in the S–Hg–S bond. In case of mercurated cystine, coordination of amino groups to the mercury in the S–Hg–S bond will result in a closure of a five-membered ring. As the S–Hg–S chromophore absorbs at the 280–240-nm region, the increase in the absorption of [oxytocin·1Hg] in the near-uv region and the intensification of the Cotton effect at the 260–240-nm region, upon raising the pH from 2.5 to 9.0 may be the result of such a coordination. Formation of a five-membered close ring may lead to a frozen configuration. Some restriction on the free rotation around the S–Hg–S bond of [oxytocin·1Hg] may also be imposed by the polypeptide ring.

The biological activities of oxytocin are intimately connected with the intramolecular ring size and especially with its geometry (Rudinger, 1971): An open-chain derivative such as [Ala^1,Ala^6]oxytocin has a very low oxytocic activity

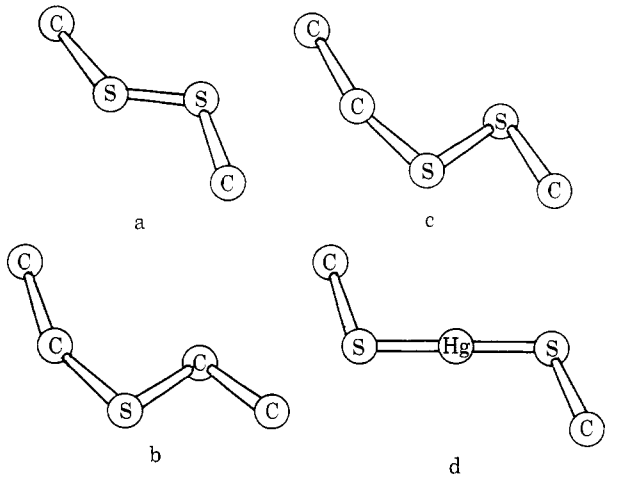
(Polacek *et al.*, 1970). However, small changes in the spatial arrangement of the disulfide bond induced by replacement of one sulfur atom by methylene group in position 1 or 6 in desamino-"carba"-oxytocin analogs (Jost and Sorm, 1971a,b), or of both sulfur atoms of the bridge by methylene group in desamino-"dicarba"-oxytocin (Jost and Rudinger, 1967) has given rather biologically potent analogs.

Enlargement of the 20-membered ring by replacing the disulfide bond by an CH_2–S–CH_2 group in desamino[1,6-homolanthionine]oxytocin yielded an analog of relatively high oxytocic activity (Prohazka *et al.*, 1972). Substitution of hemihomocystine for hemicystine in position 1 (Jarvis *et al.*, 1961) left only minute activity. [1-(ω-Mercaptoundecanoic acid)]oxytocin with a 28-membered ring is devoid of oxytocic activity (Fraefel and Du Vigneaud, 1970).

Replacement of one of the sulfur atoms by selenium resulted in biologically active desamino[1-seleno]oxytocin and desamino[6-seleno]oxytocin (Walter *et al.*, 1968). The latter compound has a CD spectrum similar to that of [oxytocin·1Hg] with a negative Cotton effect at 240 nm. In this case elongation of the loop by 0.15 Å (Pauling, 1960), due to replacement of one sulfur atom by selenium, had only small effect on the activity.

It has been shown that the disulfide bond of oxytocin is not directly involved in the biological activity of the hormone, as analogs of oxytocin with a ring structure but lacking a disulfide bond exhibit biological activity (Rudinger and Jost, 1964; Jost and Rudinger, 1967). The role of the disulfide bond is probably to stabilize the active conformation of the polypeptide chain. This is achieved by the disulfide bond due to the closure of the ring and the hindrance of the rotation around the disulfide bond.

With the aid of models (using known bond lengths and angles and the disulfide dihedral angle (Yakel and Hughes, 1954; Bradley and Kunchur, 1965)), one can say that the disulfide bridge in oxytocin stabilizes a conformation in which the two β-carbon atoms of cysteine-1 and -6 are within 4-Å distance (a). (We have considered one disulfide rotomer. The same argumentation apply to the second rotomer as well.)



Using models we have compared the bridge elements of the two oxytocin analogs with a 21-membered ring, namely, desamino[1,6-homolanthaionine]oxytocin (b) and [1-hemihomocystine]oxytocin (c) with that of oxytocin. In both cases, owing to the bond angles of carbon and sulfur which are close to 100°, it is possible to obtain a configuration in which the orientation of the two β-carbon atoms of cysteine-1 and -6 are the same as with a cystine bridge elements, and the distance between them is ~4.2 Å for both (b) and (c). Both desamino[1,6-homolanthionine]oxytocin (Prohazka *et al.*, 1972) and [1-hemihomocystine]oxytocin (Jarvis *et al.*, 1961) exhibit biological activity, albeit with a different level. If the role of the disulfide bond is to stabilize the active conformation of the hormone, it is not surprising that analogs with bridge elements like (b) and (c) can stabilize a conformation similar to that of oxytocin and exhibit biological activity. This is in agreement with Fric *et al.* (1973) who have shown by CD spectral measurements that the conformation of the peptide chain of desamino[1,6-homolanthionine]oxytocin in solution is similar to that of oxytocin. The level of activity obtained with analogs (b) and (c) may depend on the probability of obtaining the drawn conformation out of all the other possible ones as well as on other factors. The open-chain oxytocin analog [Ala^1,Ala^6]oxytocin can acquire a conformation which is similar to that of oxytocin except for the bridge element. Yet without the stabilizing effect of the bridge element the probability of obtaining this conformation out of all the other possible ones is very low. This is in agreement with the very low activity found (Polacek *et al.*, 1970).

In the case of [oxytocin·1Hg] with an S–Hg–S bridge elements (d), because of the linearity of the S–Hg–S bond (Grdenic, 1965; Bradley and Kunchur, 1965) and the S–Hg bond length of 2.45 Å (Bradley and Kunchur, 1965) there is a net elongation of the bridge elements. It can be shown by model building that the distance between the two β-carbon atoms of cysteine-1 and -6 ranges between 6.2 and 7.1 Å in this case. When the two β-carbon atoms of cysteine-1 and -6 are kept in the same conformation of a disulfide bridge element the distance between these two carbon atoms is 6.7 Å. Thus conversion of an S–S bond into an S–Hg–S bridge resulted in a net elongation of the bridge element by about 2–3 Å. In [oxytocin·1Hg] the extra 2–3 Å probably causes changes in the conformation of the polypeptide ring resulting in a marked decrease in the biological activity.

Elongation of the small loop of RNase (Sperling *et al.*, 1969) and insulin (Sperling and Steinberg, 1974) forming an S–Hg–S bond had no influence on the activity of these proteins, and did not seem to affect their overall molecular conformation. Possibly the rest of the molecule in the large proteins helps retain the native conformation. However, in the case of oxytocin, with isolated 20-membered ring, the extra 2–3 Å obtained by converting an S–S bond into an S–Hg–S bridge affect the conformation in such a way that the biological activity is seriously damaged.

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