References and Notes

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Inactivations of Oxytocin Suggesting Peptide Denaturation

Denaturation, a phenomenon associated with proteins (*1*), is frequently described by occurrences such as loss of biological activity, loss of crystallizability, change in solubility, increase in levorotation, and changes in parameters such as viscosity which indicate a change in the shape of the molecule. The term implies a subtle change in molecular structure, and the phenomenon, which is frequently base-catalyzed, is usually attributed to change in the configuration of the molecule due largely to a loss of hydrogen bonding, as is thought to occur with many proteins in concentrated solutions of urea. The role in the denaturation process in urea of sulfhydryl-disulfide interactions with resultant changes in cross-linking and molecular weight has also been recognized and has been studied for certain proteins (*2*).

Study of the inactivation of oxytocin, the cystine-containing octapeptide which is the chief oxytocic principle of the posterior pituitary, and of the material resulting from inactivation, suggests that denaturation, at least as defined by many of the criteria used with proteins, may occur with polypeptides of relatively small size. Two separate inactivations of oxytocin (molecular weight 1007) are described in this report (*3, 4*); both result in a large increase in levorotation.

Inactivation of oxytocin has been found to occur rather readily under the mildly alkaline conditions frequently used to form dinitrophenyl (-DNP) derivatives of peptides and proteins. A less soluble, higher molecular weight form of the hormone is obtained. This inactivation has been characterized as an intermolecular disulfide interchange. By treatment of the inactivation product with certain sulfhydryl reagents it has been found possible to regenerate the biological activity.

Mono-DNP-cystine (*5*), allowed to stand in 10.5 percent solution in the presence of five equivalents of $NaHCO_3$ at room temperature for 22 hours, was found to result in a mixture of approximately equimolar amounts of cystine, mono-DNP-cystine, and di-DNP-cystine (*6*), as indicated by paper chromatography. Oxytocin, represented by the structure (*7*)

Cys-Tyr-Ileu-Glu(NH_2)-Asp(NH_2)-Cys-Pro-Leu-Gly(NH_2)

is also, as are many proteins, an unsymmetrically substituted cystine derivative, or mixed disulfide. Under similar conditions, *p*H 8.3, a sample of the hormone with a potency of 350 unit/mg, as determined by the chicken depressor assay of Coon (*8*), gradually lost biological activity and deposited a white solid in 40 percent yield; $(\alpha)^{22.5}D$, $-104°$ (*c*, 0.5; 1*N* acetic acid); compare $(\alpha)^{22.5}D$, $-23.1°$ for oxytocin acetate. Loss of activity was not inhibited under these conditions by the addition of N-ethyl maleimide and proceeded much more rapidly than in the absence of $NaHCO_3$. The rate of inactivation was markedly decreased at low hormone concentrations.

Analysis (*9*) of a hydrolyzate (6*N* HCl, 14 hours, 120°C) of the solid showed approximately the same composition as oxytocin. Probably due to its low solubility, the material remained at the origin on paper electrophoresis in sodium acetate buffer, *p*H 5.5, or sodium glycinate buffer, *p*H 9.5, or on paper chromatography with *n*-BuOH–H_2O–HAc (5:5:1), while oxytocin traveled 6.8 cm and 3.5 cm, $R_f = 0.3$, respectively. On electrophoresis in 5*N* acetic acid it moved toward the cathode somewhat faster than oxytocin. It rapidly deposited an amorphous flavianate in contrast to the slowly formed, more soluble crystalline flavianate characteristic of oxytocin (*10*).

Treatment of the inactivated material (7 unit/mg) (*11*) at a concentration of 0.15 mg/ml at *p*H 5.7 with 8 μmole/ml of the following reagents led after 3 days to the following biological activities in units per milligram: cysteine, 235 (*12*); glutathione, 150; H_2S (saturated solution), 200; homocysteine, 140; thioglycollic acid, < 9; thiomalic acid, < 6; ergothioneine, < 5; ovalbumin, < 3; ascorbic acid, 5; oxidized glutathione, 4. Sodium in NH_3 led to 22 unit/mg; $Na(NH_3)$ followed by cysteine, to 42. It was also found that similar treatment of oxytocin (480 unit/mg) with cysteine, H_2S, or thioglycollic acid (*13*) led to approximately the same potencies as were regenerated from the inactivated material (7 unit/mg) with each of these reagents, suggesting that the same equilibrium mixture is attained starting from either oxytocin or the inactivated oxy-

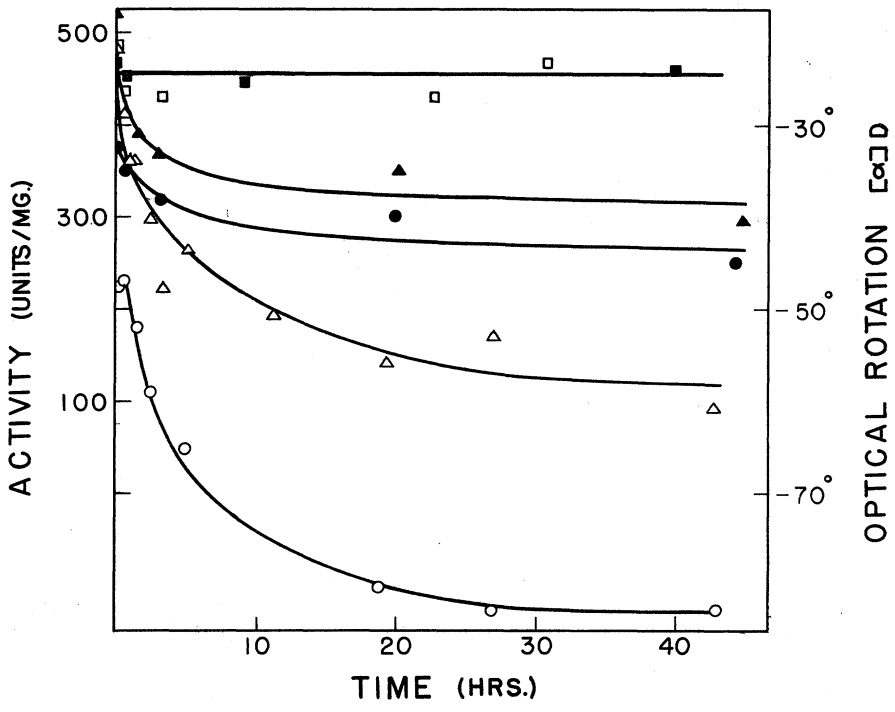

Fig. 1. Effect of urea on the biological activity and optical rotation $(\alpha)D$ of oxytocin, concentration 0.5 percent, at 22°C. Open circles, $(\alpha)D$; open triangles, activity, 7*M* urea, *p*H 8. Solid circles, $(\alpha)D$; solid triangles, activity, 7*M* urea, *p*H adjusted to 4.3 with acetic acid. Open squares, activity, 0.02*M* sodium barbital buffer, *p*H 8. Solid squares, activity, 0.02*M* sodium acetate buffer, *p*H 4.3.

tocin. The differences in the amount of activity regenerated from the inactivation product by the various —SH compounds may reflect differences in the reducing power of the —SH compounds and/or differences in the tendencies of the latter to form mixed disulfides with inactivated oxytocin (or oxytocin), as well as possible differences in the activity of the mixed disulfide species formed thereby. The unexpectedly low potencies obtained in the sodium-ammonia experiments indicate perhaps a greater lability, of undetermined type, toward the alkaline reagent than is the case with oxytocin or its S,S′-dibenzyl derivatives, which yield appreciable activity with this reagent (*14*).

A preliminary determination of molecular weight gave a calculated minimal value of 2250 (uncorrected for moisture) (*15*). The inactivated material is therefore a dimer or other higher molecular weight form of the hormone and may be represented at present as $(\text{oxytocin})_n$; $n \geqq 2$. The inactivation process would appear to involve cleavage of the intramolecular disulfide bond of oxytocin and the formation of intermolecular disulfide bonds between two or more molecules of the hormone. This inactivation of oxytocin, which may also be represented as an interchange between two or more molecules of an unsymmetrical disulfide, may be a prototype of some of the dimerization or aggregation reactions that occur with certain proteins, especially in concentrated solution. Whether it is related in mechanism to the interchange studied by several investigators between two different symmetrical disulfides (*6, 16*), which under neutral or alkaline conditions is thought to proceed through a reaction of the disulfide bond with a mercaptide ion, remains to be determined.

The occurrence of varying degrees of disulfide interchange during the handling of the posterior pituitary hormones may account for some of the losses in biological activity that take place, and may well explain some of the difficulties encountered in obtaining preparations of vasopressin of a uniform high potency. It is possible that the inactivation of these hormones in the body might also proceed through an intermolecular disulfide exchange, where it could be catalyzed enzymically. The inactivated forms of oxytocin and vasopressin could serve perhaps as physiological storage products for these hormones; when needed, activity could be generated through the action of body —SH compounds.

Because the conditions under which these hormones undergo molecular rearrangement with resultant increase in molecular weight are those which are useful for forming -DNP derivatives, it is apparent with cystine-containing peptides and proteins that structural interpretations based on data obtained through the dinitrophenylation procedure, and perhaps through other procedures as well, must be made with caution.

A second inactivation of oxytocin was observed when the polypeptide was allowed to stand in 7*M* urea solution. Changes in optical rotation were also followed (Fig. 1). The potency after 7 days at *p*H 8 was not increased by addition of cysteine to the solution, either before or after dialysis. The findings would suggest that secondary forces such as hydrogen bonding are of importance for biological activity in oxytocin. However, the possibility of chemical change should be ruled out before this inactivation in urea is ascribed to change in the spatial configuration of the oxytocin molecule.

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3. Part of this work was done during the tenure of an established investigatorship of the American Heart Association. This work was also supported in part by a grant (H-1675) from the National Heart Institute, U.S. Public Health Service.
4. I express appreciation to Dr. V. du Vigneaud for the samples of arginine-vasopressin and some of the natural oxytocin, as well as for encouragement and stimulating discussion. Thanks are given to Mr. R. L. Tostevin and Miss D. W. Tull for the bioassays and to Mrs. L. S. Abrash for the chromatography on starch.
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11. For assay the solid (1 mg) was dissolved in 50 μl of 1*N* acetic acid and the solution was diluted with water. Whether or not the low activity is due to the presence of traces of residual oxytocin has not been determined.
12. Qualitatively similar results were obtained with arginine-vasopressin. After 3 days its potency in the $NaHCO_3$ reaction mixture fell from 309 pressor units to 17 units per milligram. Treatment with cysteine raised the potency to 225 unit/mg.
13. The decrease in potency observed on treatment of oxytocin with H_2S is in substantial agreement with earlier observations using the guinea pig uterus [J. M. Gulland and S. S. Randall, *Biochem. J.* 29, 378 (1935)]. The results obtained with cysteine or thioglycollic acid are similar to those obtained by H. B. van Dyke, B. F. Chow, R. O. Greep, and A. Rothen [*J. Pharmacol. Exptl. Therap.* 74, 190 (1942)] with their protein-hormone complex. Under other conditions no change in potency was found on treatment of oxytocin with cysteine [R. R. Sealock and V. du Vigneaud, *ibid.* 54, 433 (1935)].
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Airborne Immunization against Tuberculosis

Abstract. Inhalation of very small numbers of living attenuated (BCG) organisms and their multiplication in guinea pigs results in the development of acquired resistance against subsequent airborne infection with virulent tubercle bacilli. Different strains of BCG have differing capacities to immunize by this means.

It has long been known that certain attenuated mutant strains of tubercle bacilli are capable of limited, though definite, multiplication in animals susceptible to tuberculosis. The strain selected by Calmette and Guérin, BCG, for purposes of immunization, and some of its descendants, manifest this behavior. A wide variety of routes has been used to introduce such organisms into experimental animals or man (*1*), but introduction by the airborne route has not previously been investigated. During the course of such studies (*2*) we have observed that the viable units in certain cultures of BCG, even when inhaled in very small numbers, are able to "infect" and produce tuberculin allergy and immunity against subsequent airborne challenge with virulent tubercle bacilli in guinea pigs.

The apparatus and techniques employed were the same as those previously described (*3, 4*). Three different BCG strains have been used: a commercial BCG vaccine (*5*) prepared for purposes of immunization of human beings; and two different stock laboratory cultures of BCG, "D" and "T," derived from BCG 317, originally obtained from the Henry Phipps Institute (*6*). The "D" strain was maintained for long periods in the Deep-freeze and infrequently transferred in artificial media. The "T" strain had been frequently passed through Tween-albumin liquid medium for several years.

Suspensions of the commercially available BCG vaccine were prepared from glass vials of freeze-dried material: the