2. Conjugation with cystine-S^{35} or glucuronic acid was not demonstrable. 3. It was concluded that Rose Bengal is not conjugated by the liver in the same form reported for bilirubin (glucuronides) or bromsulfalein (mercaptides).

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Synthesis and Some Pharmacological Properties of a Peptide Derivative of Oxytocin: Glycyloxytocin.* (25940)

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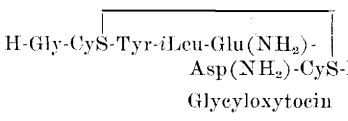
In a series of studies on the relationship between structure and biological activities of the posterior pituitary hormones and their analogs and derivatives, we became interested in the biological properties of peptide derivatives of these hormones, *i.e.* compounds in which the hormones remain unchanged except for the attachment of additional amino acids at the N- or C-terminal position. As a comparatively simple derivative of this type in which the amino group of oxytocin is involved in peptide linkage, glycyloxytocin has been synthesized.



Glycyloxytocin

Method of synthesis. The protected decapeptide, carbobenzoxyglycyl - S - benzyl - L - cysteinyl - L - tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl - L - prolyl - L - leucylglycinamide, which was employed as the intermediate for the glycyloxytocin, was prepared from the protected nonapeptide previously used for the synthesis of oxytocin(1), namely S-benzyl-N - carbobenzoxy - L - cysteinyl - L - tyrosyl-L - isoleucyl - L - glutaminyl - L - asparaginyl-S - benzyl - L - cysteinyl - L - prolyl - L - leucylglycinamide. For the present experiments, this protected nonapeptide was prepared by the stepwise synthesis recently described by Bodanszky and du Vigneaud(2). To obtain the protected decapeptide, the carbobenzoxy group was first removed from the protected nonapeptide by treatment with HBr in acetic acid and the resulting hydrobromide of the S,S'-dibenzyl derivative of reduced oxytocin

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(dibenzyloxytoceine)† was converted to the free base and allowed to react with *p*-nitrophenyl carbobenzoxyglycinate. Removal of the carbobenzoxy and benzyl groups from the protected decapeptide by treatment with sodium in liquid ammonia yielded the decapeptide in its sulfhydryl form, and oxidation of the latter compound by aeration gave glycyloxytocin. The observation was made that in the preparation of glycyloxytocin, oxidation to the disulfide requires a much longer time than in the case of oxytocin. In the preparation of oxytocin, 2 to 4 hours of aeration at pH 6.5 are usually sufficient to cause the disappearance of the reaction with sodium nitroprusside for sulfhydryl groups, whereas 4 days of aeration were consistently necessary for glycyloxytocin. This marked difference in the speed of the disulfide bond-forming reaction suggests that formation of the 20-membered ring may be hindered by the presence of the glycyl residue. Glycyloxytocin was subjected to countercurrent distribution in the system butanol-ethanol-0.05% acetic acid (4:1:5) for 950 transfers at which point the main component was obtained in a peak with a partition coefficient (K) of 0.36. The experimental curve plotted from the Folin color values was in agreement with a theoretical curve calculated for this K value. The glycyloxytocin obtained after evaporation and lyophilization of the solution showed a single ninhydrin spot on paper chromatography. Analysis of the material on the starch column showed the constituent amino acids to be present in the expected ratios, and the elementary analytical values were also satisfactory.

Pharmacological tests. Glycyloxytocin was tested for avian depressor activity in comparison with a laboratory standard of highly purified oxytocin by measuring the drop in blood pressure of the chicken by the method of Coon(3), as modified by Munsick *et al.*(4). The glycyloxytocin in sufficient amount did produce a drop in blood pressure, but the variation in relative responses to oxytocin and to glycyloxytocin was so great from bird to bird as to preclude a definite assignment of units per mg of avian depressor activity for the derivative. Furthermore, the character of the response to glycyloxytocin differed from that to oxytocin. The drop in blood pressure after administration of oxytocin is usually complete within 10 seconds and a return to the normal level usually takes place within another 10 to 20 seconds. The response to glycyloxytocin is slower, about one minute being required for the pressure to reach the lowest level, and the return to normal level is much more gradual. Occasionally the blood pressure does not return to its original level. The ratio between the amount of oxytocin and glycyloxytocin necessary to produce comparable drops in blood pressure varied from 1:170 to 1:650 in different birds. After injection of glycyloxytocin the response to oxytocin or glycyloxytocin was greatly decreased. In fact, 0.68 μg of glycyloxytocin, which in itself produced no noticeable change in blood pressure, inhibited by 50% the normal response to oxytocin when injected simultaneously with 0.04 μg of oxytocin and had the same effect on subsequent doses of this amount of oxytocin administered 10 minutes later. When larger doses of the derivative were injected, almost complete inhibition of the effect of subsequent doses of oxytocin resulted. The inhibitory effect lasted for some hours, the exact length of time depending on the amount of glycyloxytocin injected. At higher dose levels the inhibitory effect lasted at least 6 hours. Obviously, further studies into the nature of the response are necessary for a full understanding of the behavior of this compound in the bird.

† In the original report of this compound by Gordon and du Vigneaud (PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 723), it was simply referred to as S,S′-dibenzyloxytocin, since it seemed obvious that it would be interpreted as the dibenzyl derivative of the reduced form of oxytocin. However, it now seems advisable to have a specific name for the reduced form in the nomenclature of the rapidly expanding number of compounds related to oxytocin and vasopressin. We therefore suggest the name *oxytoceine* for the reduced form of oxytocin and *vasopresseine* for the reduced form of vasopressin. This nomenclature would be analogous to that of such sulfhydryl compounds as cysteine and homocysteine. The dibenzyl derivative of reduced oxytocin would then be called *S,S′-dibenzyloxytoceine* and the corresponding derivative of reduced vasopressin, *S,S′-dibenzylvasopresseine.*

In the assay for pressor activity(5) in the male rat, 120 μg of glycyloxytocin exhibited a pressor effect equivalent to 0.018 μg of arginine-vasopressin and 1.2 μg of oxytocin, respectively. The character of the response again differed from that observed with both arginine-vasopressin and oxytocin in that the rise was much more gradual, taking 10 minutes to reach its peak and from 30 minutes to 1 hour to return to its former baseline. With both vasopressin and oxytocin the rise in blood pressure in the rat and the return to normal are complete within 15 minutes. At these dosages the glycyloxytocin had no inhibitory effect on the pressor response of oxytocin or arginine-vasopressin, whether the derivative was injected simultaneously or following injection of the hormone.

When assayed for oxytocic activity by the method of Holton(6) on isolated rat uteri, 1.7 μg of glycyloxytocin showed a potency equivalent to that of 0.004 μg of oxytocin. The dose-response curve of glycyloxytocin appeared to be somewhat different from that of oxytocin, the linear portion of the curve having a shorter range than that of oxytocin. No inhibitory effect on the uterine-contracting effect of subsequent doses of oxytocin was detected.

Experimental.‡ *p-Nitrophenyl Carbobenzoxyglycinate.* Carbobenzoxyglycine (10.4 g) and *p*-nitrophenol (8.4 g) were dissolved in ethyl acetate (150 ml) and treated with dicyclohexylcarbodiimide (10.3 g) at 0°. After 1.5 hours at room temperature the N,N′-dicyclohexylurea was filtered off and the solvent was removed from the filtrate *in vacuo*. The residue was recrystallized from ethanol (100 ml) and the crystals were washed with ethanol (50 ml); wt. 13.8 g, m.p. 126-128°. A recrystallized sample melted at 128°; lit. m.p. 128°(7), 124-125°(8).

Carbobenzoxyglycyl - S - benzyl - L - cysteinyl - L - tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl - L - prolyl - L - leucylglycinamide. The protected nonapeptide, S-benzyl-N-carbobenzoxy - L - cysteinyl - L - tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl - L - prolyl - L -leucylglycinamide (2.6 g)(2), was dissolved in acetic acid (20 ml) and 3 *N* HBr in acetic acid (40 ml) was added to the solution. After 1.5 hours at room temperature the hydrobromide was precipitated with ether (400 ml), filtered off, and washed with ether. After being dried briefly over calcium chloride and sodium hydroxide at room temperature *in vacuo* the hydrobromide was dissolved in dimethylformamide (25 ml) and treated with triethylamine (3 ml) and *p*-nitrophenyl carbobenzoxyglycinate (0.80 g). After one day at room temperature the reaction mixture was diluted with ethyl acetate (300 ml) and the precipitate was washed with ethyl acetate (100 ml), ethanol (200 ml), and again with ethyl acetate (50 ml); wt. 2.71 g, m.p. 242-245° (dec. 246°), $[\alpha]^{20}D$ −41.5° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{67}H_{89}O_{15}N_{13}S_2$: C, 58.3; H, 6.50; N, 13.2. Found: C, 58.0; H, 6.52; N, 13.0.

Glycyloxytocin. The protected decapeptide (300 mg) was dissolved in liquid ammonia (500 ml) and sodium was added until a blue color persisted. Ammonium chloride (0.2 g) was then added and the ammonia was allowed to evaporate. The residue was dissolved in water (500 ml) and the solution was aerated at pH 7 until the reaction with sodium nitroprusside was negative, about 4 days being required. Acetic acid (0.5 ml) was added and the solution was pooled with another similar batch. The combined solutions were evaporated *in vacuo* below room temperature to a volume of 30 ml. This solution was placed in the first 5 tubes of a 200-tube countercurrent distribution apparatus. A solvent system of butanol-ethanol-0.05% acetic acid (4:1:5) was used. After 377 transfers the contents of all the tubes except Tubes 80-120 were replaced with fresh solvent and the material was recycled to effect a total of 950 transfers. The plotting of the curve from the Folin color values(9) indicated the presence of one component ($K = 0.36$). The contents of Tubes 14-45 were pooled and 46-80 were pooled, and both fractions were evaporated

‡ The melting points reported are corrected, capillary melting points.

to small volumes, then lyophilized to give 60 mg and 33 mg of white solid, respectively. On paper chromatography in the system butanol-acetic acid-water (4:1:5) both fractions exhibited a ninhydrin-positive spot with an R_F value of 0.40. A sample of the glycyloxytocin was hydrolyzed in 6 N HCl for 16 hours at 110° and subjected to chromatography on the starch column (10). The molar ratios between the constituent amino acids and ammonia were as follows (with the value for leucine plus isoleucine taken arbitrarily as 2): tyrosine 0.9, proline 0.7, glutamic acid 1.0, aspartic acid 1.0, glycine 2.0, cystine 1.0 and ammonia 3.1.

Anal. Calcd. for $C_{45}H_{69}O_{13}N_{13}S_2$: C, 50.8; H, 6.54; N, 17.1. Calcd. for $C_{45}H_{69}O_{13}N_{13}S_2 \cdot C_2H_4O_2$: C, 50.2; H, 6.54; N, 16.2. Found: C, 50.2; H, 6.59; N, 16.7.

Summary. 1) The protected decapeptide carbobenzoxyglycyl - S - benzyl - L - cysteinyl - L - tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl - L - prolyl - L - leucylglycinamide was synthesized by coupling of the *p*-nitrophenyl ester of carbobenzoxyglycine with S-benzyl-L - cysteinyl - L - tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl - L - prolyl - L - leucylglycinamide. Removal of the protecting groups followed by oxidation to the disulfide gave glycyloxytocin which was isolated by countercurrent distribution. 2) The pharmacological effects of this peptide derivative of oxytocin were investigated by testing it for avian depressor, pressor, and rat uterine-contracting activities. The sample of glycyloxytocin possessed only a slight degree of these 3 activities. It was found to exert a marked inhibitory effect on the avian depressor activity of oxytocin. On the other hand, the glycyloxytocin did not show inhibitory action against oxytocin on the isolated rat uterus or against oxytocin or vasopressin in the assay for pressor activity in the rat. 3) Thus, the attachment of a glycyl residue to the free amino group of oxytocin causes almost complete loss of its avian depressor, rat uterine-contracting, and pressor effects. In addition, it is of considerable interest that this peptide derivative of oxytocin exerts a marked inhibitory action upon the avian depressor effect of the hormone.

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