imidazoles has been described. 2. Anticoccidial activity was demonstrated for imidazole-4-carboxamide and certain related compounds. 3. The most potent compound was 4,5-imidazoledicarboxamide (glycarbylamide). 4. Replacement of one or both carboxamides in the 4,5 positions on the imidazole ring reduced or completely eliminated the anticoccidial activity of glycarbylamide. Substitutions at the 1 or 2 positions on the imidazole ring produced compounds of less activity than glycarbylamide.

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Received March 14, 1958. P.S.E.B.M., 1958, v98.

Further Properties of Isoglutamine-oxytocin; Inhibition of Pressor Activity of Vasopressin.* (23978)

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The synthesis of the isoglutamine isomer of oxytocin, the chief oxytocic principle of the posterior pituitary gland, has recently been described(1). This isomer, the cyclic disulfide of L-cysteinyl-L-tyrosyl-L-isoleucyl-L-isoglutaminyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-leucyl-glycinamide, differs from oxytocin by substitution of an isoglutamine residue for the glutamine residue in the hormone. In view of this difference in structure and our interest in the correlation of the structures and properties of such polypeptides, the isomeric compound was compared with oxytocin. Observations were made on distribution coefficients, optical rotation, electrophoretic mobility, molecular weight and infrared pattern(1). In addition, the isomer was tested for some physiological activities associated with oxytocin; namely, oxytocic activity, avian vasodepressor activity, and pressor activity in the rat. As has been already reported, these activities were not detected in the isomer. Further testing revealed that isoglutamine-oxytocin exerts a definite inhibitory effect on the pressor action of administered vasopressin in the anesthetized rat. Inhibition toward both purified arginine-vasopressin and the posterior pituitary standard powder was observed. It may be recalled that oxytocin can be converted into a crystalline flavianate derivative (2). Efforts were therefore made to determine whether the isoglutamine isomer could show similar behavior. It has now been found that isoglutamine-oxytocin also yields a crystalline flavianate, and that the latter differs in form from the corresponding oxytocin derivative. When the acetate of isoglutamine-oxytocin was rigorously dried, it underwent change, as indicated by decrease in solubility. Results of elementary analysis and molecular weight determination suggested that it had probably lost acetic acid.

Experimental. Inhibition of pressor action of vasopressin. Anesthetized rats weighing 290-480 g were injected intravenously with solutions of isoglutamine-oxytocin(1), vasopressin, and various mixtures of these. The

* This work was supported in part by a grant (H-1675) from the Nat. Heart Inst., P.H.S.

† Part of this work was done during tenure of Fellowship as Established Investigator of Am. Heart Assn.

conditions described by Lindquist and Rowe (3) for bioassay of posterior pituitary pressor activity were used. Mepesulfate 'Roche,' however, replaced the heparin. The U.S.P. Posterior Pituitary lobe powder with a stated potency of 2 U/mg, or a solution of highly purified arginine-vasopressin assayed against the latter, served as the reference pressor substance or standard. When a solution containing 180 γ of isoglutamine-oxytocin was injected, no pressor activity was observed. It was noticed, however, that a subsequent injection of standard containing 30 milliunits of vasopressin produced almost no effect on blood pressure. The response was only 6% of that given by the same dose of standard prior to administration of the isoglutamine compound. To evaluate this effect more carefully the following procedure was used. Isoglutamine-oxytocin was injected at the same time as the standard, *i.e.*, as a mixed solution containing both polypeptide and standard. Administration of the mixture was preceded and followed by injections of the standard alone. Injections were performed only after the blood pressure of the animal had returned to the baseline after the preceding one. The effect of isoglutamine-oxytocin on response to the standard in the mixture and on response to the subsequent standard was evaluated by comparing these responses with results of the initial injections of standard. If inhibition was observed, several subsequent injections of standard were made in an attempt to estimate the duration of the effect. When the results of the latter injections indicated that the animal had regained its sensitivity to the standard, the amount of isoglutamine-oxytocin in the mixture was then varied, and the series of injections was repeated. Because the inhibitory effect persisted for some time, only a limited amount of data was obtained from each rat. The mixtures of isoglutamine-oxytocin and standard were made up by adding different volumes (0.01-0.19 ml) of a 0.00067 molar solution of isoglutamine-oxytocin acetate to a given volume of the standard solution of vasopressin having a potency of 0.2 U/ml. Usually 0.15 ml of the latter solution, or 30 milliunits of pressor activity, was used. An attempt was made to work at a fixed level of vasopressin and vary only the amount of the isoglutamine compound. However, because of some difference in sensitivity to vasopressin among the animals, other levels of standard were also used to obtain responses of suitable magnitude. In each experiment, however, the level of standard administered in the mixture was always kept the same as that of the preceding and following standards.

The *results* of a number of typical injections are given in Table I. Inhibition[‡§] is expressed in terms of percentage decrease in response to a dose of vasopressin in the presence of a dose of isoglutamine-oxytocin. The relative doses of isoglutamine compound and vasopressin in each experiment are given in terms of μg of the former/milliunit of pressor activity of the latter, *i.e.*, as a weight-activity ratio.

It was noticed that when isoglutamine-oxytocin was injected along with the standard at a ratio of approximately 0.7-1.8 γ/milliunit of pressor activity, the hypertensive response to the standard generally fell approximately 30-50%. In terms of relative weights of the 2 substances, this would correspond to 280-720 parts of isoglutamine peptide to one of vasopressin, based on an activity of approximately 400 U/mg for the latter. Some inhibition to subsequent injections of vasopressin persisted for about one-half to one hour. At a ratio of 0.4 γ/milliunit of pressor activity, no inhibitory effect was observed. At ratios above approximately 4-6 γ/milliunit, inhibition was

‡ Inhibition in the rat of the pressor activity of arginine-vasopressin by preparations of the latter that had been treated with acetic anhydride has been observed recently (Cash, W. D., Studer, R. O., and du Vigneaud, V., in preparation).

§ While this manuscript was in preparation, a nonapeptide having the structure CyS-Tyr-Tyr-Ileu-Glu(NH_2)-Asp(NH_2)-Cys-Pro-Leu-Gly(NH_2) was reported to inhibit the action of oxytocin on the isolated rat uterus and in lowering blood pressure of the chicken. (Guttmann, St., Jaquenoud, P. A., and Boissonnas, R. A., *Naturwiss.*, 1957, v24, 632). It is of interest that both the latter nonapeptide and isoglutamine-oxytocin possess cyclic disulfide rings that are larger than that which occurs in oxytocin and vasopressin.

TABLE I. Inhibition of Pressor Activity of Vasopressin.

Rat No.	Rat Wt, g	Dose of isoglutamine-oxytocin (I), γ	Dose of vasopressin (V), mU	Ratio I/V, γ/mU	Inhibition, %	Duration, min
1760*	360	14	20	.7	37	60
		18.7	31	.6	18	
		27.4	30	.9	24	
		19	21	.9	55	75
1761*	380	24	40	.6	16	<10
		22	30	.7	42	
		27	30	.9	30	
		36	40	.9	36	
1769*	350	55	31	1.8	55	
		121	51	2.4	70	
1768*	300	54	30	1.8	48	
		108	30	3.6	88	
1727*	320	102	30	3.4	88	
		136	20	6.8	Complete	>90
1733†	340	7	16	.4	None	
1731†	290	21	30	.7	60	
		48	30	1.7	62	
		68	30	2.3	85	
1816*‡	330	53	29	1.8	78	
		108	30	2.9	Complete	
1812*‡	390	53	29	1.8	55	
		108	30	3.6	62	<30

* Purified arginine-vasopressin served as standard. † U.S.P. posterior pituitary lobe powder served as standard. ‡ A Sanborn electromanometer was used.

usually complete; *i.e.*, there was no response to the administered vasopressin. Duration of the effect generally increased markedly with degree of inhibition initially observed. The results were similar when either purified arginine-vasopressin or the U.S.P. Posterior Pituitary lobe powder served as the pressor substance.

Although only a limited number of observations were made in each rat, there frequently appeared to be a fair degree of discrimination between doses of isoglutamine peptide in any one animal. Some responses, however, were irregular. This may be due in part to irregularity in response to vasopressin. In the rat preparation used, successive injections of a constant dose of vasopressin yield responses that may differ by 15-20%. Moreover, levels of vasopressin used were somewhat high and may not have been optimum for regular responses(4). Other factors which may have had some bearing on the results are possible variations in depth of anesthesia among the animals (see discussion) and variations in animal weight.

In preliminary experiments, isoglutamine-oxytocin also appeared to show some inhibition of the action of oxytocin on the rat uterus. This observation, however, needs further study and confirmation.

Formation of a crystalline flavianate. To a solution of 4 mg of polypeptide acetate in 0.19 ml of water was added 0.12 ml of a 5% solution of flavianic acid. Slow evaporation of the solution in the cold to a small volume yielded highly refractive rectangular and elongated plates. Under similar conditions oxytocin yields a flavianate in the form of fine, silky needles(2). The crystals were separated by centrifugal filtration in the cold, washed in a similar manner with 2 drops of 0.25% flavianic acid, and dried in an evacuated desiccator in the cold. After drying, the material became irregular in shape but remained highly refractive. It decomposed gradually above 205°. A sample was hydrolyzed in 6 N HCl for 14 hours at 120°. Paper chromatography of the hydrolyzed material in *n*-butyl alcohol-water-acetic acid (5:1:5) followed by 75% phenol, and development with ninhydrin, showed the

presence of the expected amino acids.

Change on Drying. Isoglutamine-oxytocin acetate had been obtained by lyophilization of the polypeptide from a dilute solution of acetic acid(1). The residual white, fluffy powder was highly water-soluble. Because of the variable amounts of volatile material usually present in lyophilized preparations, a sample was dried to constant weight at 100° at 0.2 mm before analysis and molecular weight determination. The loss in weight was 11.5%. The material was then no longer appreciably soluble in water. The free base of the peptide, which was obtained by passage of a solution of the acetate through an anion exchange column, Amberlite IR-45 in the hydroxyl form, followed by lyophilization of the effluent, also was not appreciably water-soluble. The dried material, like the free base, dissolved rather easily in 0.1 N acetic acid, however, and lyophilization of such a solution again yielded highly soluble material.

The dried material gave the following analysis: Calcd. for $C_{43}H_{66}O_{12}N_{12}S_2 \cdot C_2H_4O_2$: C, 50.6; H, 6.61; N, 15.8. Calcd. for $C_{43}H_{66}O_{12}N_{12}S_2$: C, 51.3; H, 6.61; N, 16.7. Found: C, 51.6; H, 6.77; N, 16.3 (determined on similar, separate sample).

Molecular weight determinations were carried out by use of a thermoelectric osmometer (5). Solutions approximately 0.05 molal with respect to sucrose in 0.1 *M* acetic acid served as standards. The values obtained on the original, lyophilized material and on the dried material were 969 (cor. for moisture) and 1220, respectively, which agree within the expected range with the theoretical values of 1068 and 1007 for the acetate and base, respectively, of isoglutamine-oxytocin. Derivation of molecular weight by this method, which is based on a colligative property, requires consideration of the number of particles/molecule afforded by the solute in solution. The calculated values are based on the assumption that the dried material provided one particle/molecule, but that the original acetate provided two.

A determination was also carried out after the dried material had been redissolved in dilute acetic acid and recovered by lyophilization of the solution. A value of 1108 (uncor. for moisture) was derived for the molecular weight, based on the assumption that the material now provided 2 particles/molecule in solution. These results are therefore consistent with the other data which suggest that acetic acid is lost on drying. That perhaps other changes have also occurred in the process cannot be precluded by the present data.

Discussion. The foregoing data show that isoglutamine-oxytocin significantly inhibits the pressor activity of vasopressin administered to the anesthetized rat. The minimum effective dose under the experimental conditions described is approximately 50-60 γ/kg for 30 mμ of vasopressin. Because the pressor response to vasopressin is affected in the presence of anesthesia(6), these results cannot be extended directly to the unanesthetized animal without further investigation.

It may be recalled that vasopressin and oxytocin are closely related structurally, and this is manifested by the fact that many of the biological activities of the 2 hormones overlap. For example, vasopressin possesses some oxytocic activity, and oxytocin has a low degree of pressor activity(7). The isoglutamine isomer of oxytocin may therefore be considered structurally related to vasopressin as well as to oxytocin. This suggests that the inhibitory relationship that exists between vasopressin and isoglutamine-oxytocin may resemble in type many well established cases of inhibition based on the similarity in structure between the inhibitor and active compound. Despite the fact that the exact mode of action of hormones is not known, if this concept of inhibition does apply to the hormone under consideration, then one might picture in a general way the inactive isoglutamine compound as replacing vasopressin at the site of its action. Moreover, one might expect the corresponding isoglutamine isomer of vasopressin perhaps to inhibit vasopressin activity more effectively than does isoglutamine-oxytocin, since it would be closer in structure to the hormone than the latter.

The indication that acetic acid is lost from lyophilized preparations of isoglutamine-oxytocin acetate on rigorous drying is not without general precedence among salts of simpler bases. In the case of polypeptides and pro-

teins, however, it may have some special significance in relation to the phenomenon of inactivation. On drying solutions of the posterior pituitary hormones, as well as solutions of many enzymes, loss of activity is occasionally observed. It is well known that specific conditions of pH and charge favor maximum stability of these biologically active substances. The situation encountered with isoglutamine-oxytocin acetate suggests that, in general, a drying process may involve varying degrees of change in bound acid and charge, as well as in hydration, with the result that these biological substances may on occasion be left unexpectedly in a state less favorable with respect to stability.

Summary. 1) The isoglutamine isomer of oxytocin has been found to inhibit significantly the pressor activity of administered vasopressin in the anesthetized rat. 2) Various data suggest that acetic acid is lost when the polypeptide acetate is rigorously dried. Isoglutamine-oxytocin forms a crystalline flavianate salt.

Appreciation is expressed to Dr. V. du Vigneaud for his interest and suggestions. The authors would also also like to thank Dr. H. B. van Dyke for his helpful discussion and Dr. W. D. Cash for his interest in connection with the assays. The elementary analyses were performed by Mr. Joseph Albert.

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Received March 19, 1958. P.S.E.B.M., 1958, v98.

Specific Function of Bile Salts in Cholesterol Absorption.* (23979)

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It has been demonstrated that bile salts are an obligatory requirement for passage of cholesterol from lumen of the intestine to the lymph(1). However, the mechanism by which bile salts facilitate absorption of cholesterol is not clear. Several explanations have been offered. One is that bile salts dissolve cholesterol by virtue of their hydrotropic action and that this process is facilitated when cholesterol is dissolved in fat(2). It has also been suggested that bile salts are necessary for cholesterol absorption because they function as cofactor for cholesterol esterase activity(3,4). Recently(5) it was demonstrated that a metabolic pool of free cholesterol exists in the intestinal wall with which cholesterol from the lumen is mixed prior to its esterification and transfer to the lymph. The role which bile salts might have in the entrance of exogenous cholesterol-4-C^{14} into the metabolic pool of the intestinal mucosa and its subsequent transfer to the lymph was investigated.

Methods and materials. Rats with thoracic and bile duct fistulae were prepared and given saline to drink(5). Twenty-four hours after operation, each animal received by gastric intubation, 3 ml of aqueous emulsion containing cholesterol-4-C^{14} (0.5 μc) alone or in combination with one or more of blood albumin, glucose, oleic acid, sodium dehydrocho-

* This study was supported in part by grants from Am. Heart Assn. and the PHS.