Study of Pituitary Hormone Analogs for Their Inhibitory Properties and Resistance to Carboxamidopeptidases: [9-L-Proline]oxytocin, [9-L-Glutamic acid]oxytocin, and [8-L-Lysine,9-glycine methylamide]oxytocin†

Z. Grzonka, J. D. Glass, I. L. Schwartz, and Roderich Walter*

Department of Physiology and Biophysics, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029, and Medical Research Center, Brookhaven National Laboratory, Upton, New York 11973.
Received April 24, 1974

Syntheses, biological activities, and preliminary enzyme studies are reported for two oxytocin analogs in which the glycine residue in position 9 is replaced by glutamic acid or proline. Biological and enzymological studies of [8-lysine,9-glycine methylamide]oxytocin are also reported. [9-Glutamic acid]oxytocin was found to possess 0.05 ± 0.009 U/mg of oxytocic activity, <0.01 U/mg of avian vasodepressor activity, and 0.01 ± 0.001 U/mg of rat antidiuretic activity. [9-Proline]oxytocin exhibited 0.11 ± 0.02 U/mg of oxytocic activity and 0.03 ± 0.004 U/mg of antidiuretic activity. [8-Lysine,9-glycine methylamide]oxytocin showed 11.5 ± 0.8 U/mg of oxytocic activity, 3.44 ± 0.35 U/mg of rat antidiuretic activity, and 0.05 ± 0.01 U/mg of rat pressor activity. In accordance with conformational predictions the oxytocin analog containing the hydrophobic proline residue as well as the methylamide analog showed the properties of reversible and competitive inhibitors of oxytocin in the avian vasodepressor assay. Upon incubation of the analogs with an enzyme preparation from toad urinary bladder, which readily releases C-terminal glycinamide from oxytocin and arginine-vasopressin, there was a minimal release of isoglutamine from [9-glutamic acid]oxytocin and no detectable cleavage of [9-proline]oxytocin. From [8-lysine,9-glycine methylamide]oxytocin only a small amount of glycine methylamide was released.

In the proposed conformation of oxytocin,[2] one face of the molecule is hydrophobic, while the other side contains all of the hydrophilic amino acid side chains of the ring portion as well as the C-terminal tripeptide. The hydrophilic region in the "biologically active" structure of oxytocin contains the chemically reactive side-chain moieties which are thought to be intimately involved in the expression of agonistic activity of the hormone.[3] Modifications of the reactive groups of residues in position 2 (for a summary, see ref 4) and position 4 (see ref 5 and 6 and references cited therein) have led to an impressive list of competitive inhibitors of neurohypophyseal hormone-induced responses. Changes in position 5 have thus far not yielded any competitive inhibitors since replacement of the asparagine residue has resulted in analogs of extremely low affinity revealing the critical function of this moiety (*e.g.*, ref 3). From a conformational point of view we suggested that certain substitutions in the hydrophilic area of the biologically active conformation of oxytocin—particularly if these modifications are hydrophobic in nature—might yield analogs with reduced catalytic activity (partial agonists) or which lack agonistic properties completely and act instead as competitive inhibitors; in particular, side chains in positions 2, 4, and 9 appear suitable for modification to convert hormone to antagonist.[6]

Moreover, the inactivation of neurohypophyseal hormones by enzymes present in tissues is frequently observed to occur by hydrolysis of amide bonds located in the linear portion of the peptides.[7-11] Continued study of the substrate specificity of such enzymes is warranted.

With this as background, we describe the synthesis of [9-glutamic acid]- and [9-proline]oxytocin, their biological activities, and preliminary enzymological data using "carboxamidopeptidase" isolated and partially purified from toad urinary bladder. The biological and enzymological studies were also carried out with [8-lysine,9-glycine methylamide]oxytocin.[12] The two analogs in which the glycine residue in position 9 of oxytocin has been replaced by proline and glutamic acid were prepared by the solid-phase method of peptide synthesis.[13] Generally, the peptide chain was elongated with the respective *N*-Boc-protected amino acid by using dicyclohexylcarbodiimide[14] in the presence of 1-hydroxybenzotriazole[15] in dimethylformamide. The N-protected Asn and Gln were introduced into the peptide as *p*-nitrophenyl esters.[16-18] The fully protected nonapeptide of [9-proline]oxytocin, Z-Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Pro-NH_2, was obtained by ammonolysis of the acyclic peptide from the polymeric support.[19] In the case of [9-glutamic acid]oxytocin the protected nonapeptide was liberated from the resin with HBr in trifluoroacetic acid.[20] The two nonapeptide intermediates, characterized by amino acid analysis, were deprotected by reduction with sodium in liquid ammonia[21] and converted to the cyclic analogs by oxidation to the disulfide.[22] The resultant oxytocin analogs were purified by partition chromatography on Sephadex G-25 using appropriate solvent combinations.[23]

For the preparation of the nonapeptide intermediate of [9-glutamic acid]oxytocin the isoglutamine residue was attached *via* its side chain to the polymeric support. Although for the synthesis of [9-glutamic acid]oxytocin the peptide was extended only in the N-terminal direction from the substituted glutamic acid residue, it should be noted that, generally speaking, the method is not limited to an isoglutamine residue in terminal position of a peptide. In principle, dicarboxylic amino acids lend themselves for "bidirectional solid-phase peptide synthesis," a method which has as its basis the attachment of the growing peptide chain to the polymer through the side chain of one of its amino acid components. Depending on the course of the synthesis, the dicarboxylic amino acid residue may eventually be in exo or endo position of the peptide as an acid or amide. The side chains of ornithine,[24] lysine,[25] histidine,[26,27] and cysteine[28] have been successfully attached to resins and, in the case of ornithine, used for the "bidirectional synthesis" of a cyclic peptide,[24] in the case of histidine, of thyrotropin-releasing hormone,[26,27] and in the case of cysteine, of oxytocin and its deamino analog.[28]

An interesting property of [9-glutamic acid]oxytocin is its ability to bind about 1 equiv of ammonia. In addition to the 3 equiv of ammonia found after acid hydrolysis of [9-glutamic acid]oxytocin another 1-1.2 equiv of *noncovalently* bound NH_3 was found to be associated with the

†Abbreviations are in accord with the IUPAC-IUB Tentative Rules on Biochemical Nomenclature.[1] Other abbreviations: Boc = *tert*-butyloxycarbonyl; Glu-NH_2 = isoglutamine; Gln = glutamine. All optically active amino acids are of the L configuration.

*Address correspondence to this author at the Department of Physiology and Biophysics, Mount Sinai School of Medicine of the City University of New York.

Table I. Inhibition of the Avian Vasodepressor Response to Oxytocin by [9-Proline]oxytocin and [8-Lysine,9-glycine methylamide]oxytocin

Dose of oxytocin, mol	Dose of inhibitor, mol	Per cent inhibition[a]	
		Simultaneous injection	Sequential injection
	[9-Proline]oxytocin		
2×10^{-10}	5.2×10^{-8}	17, 16, 20	0, 0, 0, 0
2×10^{-10}	10.4×10^{-8}	38, 40, 35	0, 0, 0
2×10^{-10}	20.8×10^{-8}	58, 61, 57	0, 0, 0
	[8-Lysine, 9-glycine methylamide]oxytocin		
1×10^{-10}	2.4×10^{-8}	11, 11, 13, 15	10, 7, 14, 14
1×10^{-10}	4.9×10^{-8}	55, 55, 61, 57	24, 24, 31, 31
1×10^{-10}	9.9×10^{-8}	100, 100, 100, 100	44, 42, 38, 44
1×10^{-10}	19.6×10^{-8}		56, 62, 55, 59

[a] The results of individual experiments for each dose of antagonist are given. For "simultaneous" injection, oxytocin was washed into the bird together with the inhibitor; for "sequential" injection, the analog was administered 1 min prior to oxytocin.

product. This was revealed by amino acid analysis of samples of unhydrolyzed analog. Several attempts to remove this excess ammonium ion by gel filtration on a Sephadex G-10 column (110 × 2 cm) in 0.2 *N* AcOH, by ion-exchange chromatography on a BioRad AG 3-X4 (OH form) column (*ca.* 20 × 1 cm), and by repeated lyophilization from dilute acetic acid solution were all unsuccessful. Moreover, storage of the analog at 100° *in vacuo* for 24 hr over P_2O_5 and NaOH failed to remove the ammonia. Such a strong association between NH_3 and a neurohypophyseal hormone analog possessing a free carboxylic acid group has not been observed previously and should be investigated further.

Bioassay of [9-glutamic acid]- and [9-proline]oxytocin revealed these analogs to be weak agonists of activities characteristic of the neurohypophyseal hormones. In line with considerations of the relationship between conformation and biological activities of neurohypohyseal hormones (see ref 6 and also introduction), the analog in which the glycine residue in position 9 is replaced by the hydrophilic glutamic acid residue exhibits no inhibitory properties, while the 9-proline analog (containing a hydrophobic substitution) acts as a competitive antagonist of the oxytocin-induced avian vasodepressor response.‡ Specifically, 9-glutamic acid]oxytocin possesses 0.05 ± 0.009 U/mg of oxytocic activity on the isolated rat uterus and has <0.01 U/mg of avian vasodepressor activity. The analog showed 0.01 ± 0.001 U/mg of antidiuretic activity in the rat but failed to increase the blood pressure in this animal at a dose of 0.09 mg per single injection. [9-Proline]oxytocin demonstrated a uterotonic activity of 0.11 ± 0.01 U/mg. The analog has no effect on fowl blood pressure when a dose of 0.97 mg is administered. However, [9-proline]oxytocin, when injected simultaneously with 2×10^{-10} mol of oxytocin, inhibits the avian vasodepressor response to the hormone. As shown in Table I, the inhibition increases with increasing concentrations of [9-proline]oxytocin. The response to oxytocin was inhibited by about 16% at a molar ratio of oxytocin to analog of 1:260, and inhibition increased to ~40 and ~60% at ratios of hormone to analog of 1:520 and 1:1040, respectively, The inhibition was fully reversible. Moreover, the inhibition was overcome by simultaneous injection of twice the dose of oxytocin along with a constant dose of inhibitor. When the inhibition experiments were repeated using identical conditions except that [9-proline]oxytocin was injected 1 min prior to oxytocin, no inhibition of the hormone-induced response was observed. [9-Proline]oxytocin exhibits a rat antidiuretic activity of 0.03 ± 0.004 U/mg. The compound has no effect on rat blood pressure at a dose of 0.22 mg.

[8-Lysine,9-glycine methylamide]oxytocin, an analog of oxytocin in which the Leu residue in position 8 has been replaced by Lys and in which the primary carboxamide of the Gly residue has been monomethylated, has been synthesized by Schillinger, *et al.*, and was found to possess weak insulin-like activity in terms of glucose oxidation in the isolated fat cell.[12] This analog was subjected in this study to additional bioassays of activity characteristic of neurohypophyseal hormones. It exhibits an oxytocic activity of 11.5 ± 0.8 U/mg, an antidiuretic activity of 3.44 ± 0.35 U/mg, and a pressor activity of 0.05 ± 0.01 U/mg (activities are calculated on the basis of the anhydrous molecular weight of the peptide). Similar to the results found in this study with [9-proline]oxytocin, the [8-lysine,9-glycine methylamide]oxytocin does not reduce the blood pressure of the chicken when injected in a dose as high as 0.86 mg, but instead reversibly and competitively (the dose of oxytocin had to be quadrupled in order to overcome the inhibition) inhibits the oxytocin-induced avian vasodepressor response when injected simultaneously with 10^{-10} mol of oxytocin, indicating that substitution of a carboxamide proton in position 9 by a methyl group eliminates the agonistic properties of the peptide but not its binding capacity for the receptor. Specifically (Table I), at an oxytocin to analog ratio of 1:240, [8-lysine,9-glycine methylamide]oxytocin inhibits the hormonal response by about 15%. Inhibition increases at higher concentrations of antagonist, reaching ~60% at a ratio of 1:490, and 100% inhibition when the hormone to analog ratio is 1:990. It is clear from comparison of these results to those obtained with [9-proline]oxytocin that [8-lysine,9-glycine methylamide]oxytocin is a more potent inhibitor of the oxytocin-induced avian vasodepressor response. The higher affinity of [8-lysine,9-glycine methylamide]oxytocin is

‡ [9-Leucine]oxytocin and [9-methionine]oxytocin, two additional analogs in which the glycine residue has been replaced by hydrophobic residues, were found to be competitive and reversible inhibitors of the oxytocin-induced avian vasodepressor response (Walter, Sondheimer, and Glass, unpublished results). [9-Methionine]oxytocin is a stronger inhibitor (12 and 24×10^{-8} mol of antagonist inhibited the response to 10^{-10} mol of oxytocin by approximately 35 and 100%, respectively) than is [9-leucine]oxytocin (12 and 24×10^{-8} moles gave rise to 15 and 50% inhibition, respectively). In contrast, Aoyagi, *et al.*,[29] who tested a solution which was presumed to contain [9-leucine]oxytocin, observed a slight decrease in the blood pressure of the chicken.

also indicated by the fact that in contrast to [9-proline]oxytocin, injection of the methylamide analog 1 min *before* oxytocin still led to substantial inhibition (*i.e.*, ~40% at a ratio of 1:990, Table I). [1-Mercaptopropionic acid,9-glycine methylamide]oxytocin and its dimethylamide derivative have also been found to be devoid of any detectable avian vasodepressor activity but were not investigated for inhibitory properties of the oxytocin-induced response in this assay.[30]

It was reported earlier that the toad urinary bladder contains an enzyme which releases glycinamide from neurohypophyseal hormones and analogs irrespective of the nature of the side chain of the L residue in position 8 but only minimally or not at all attacks analogs terminating in glycine, glycine methylamide, or glycine dimethylamide.[7,31] In this study this carboxamidopeptidase[7] has been somewhat further purified and its action on [9-proline]-, [9-glutamic acid]-, and [8-lysine,9-glycine methylamide]-oxytocin was evaluated. While there was rapid release of glycinamide (~50% released in 3 hr) from oxytocin and arginine-vasopressin, which served as controls in these experiments, there was no detectable cleavage of [9-proline]oxytocin and only 4% of isoglutamine was released from [9-glutamic acid]oxytocin when these compounds were incubated with the same amount of enzyme and for the same time as controls. As was expected in the light of our previous study,[7] the enzyme preparation released only trace amounts of glycine methylamide from [8-lysine,9-glycine methylamide]oxytocin. The preliminary results described here suggest that the enzyme is also sensitive to the nature of the side chain of the amino acid residue in position 9.

Experimental Section

The protected nonapeptide intermediates were synthesized according to the general method described by Merrifield[13] with specific variations indicated below. Intermediates and products were dried *in vacuo* over P_2O_5 and NaOH. All melting points were determined with a Thomas-Hoover capillary melting point apparatus and are not corrected. Optical rotations were determined with a Carl Zeiss precision polarimeter (0.001°). Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn. Amino acid analyses were carried out according to the general method of Spackman, *et al.*:[32] samples were hydrolyzed in sealed evacuated tubes in 6 *N* HCl at 110° for 22 hr and dried over NaOH, and the residue was dissolved in 0.2 *M* sodium citrate buffer, pH 2.2, and analyzed on a Beckman 121C amino acid analyzer.

Boc-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Glu-(Resin)-NH_2. Chloromethylated copolystyrene-2% divinylbenzene (5 g, containing 1.7 mmol of Cl/g of resin), Boc-Glu-NH_2[33] (2.09 g, 8.5 mmol), and triethylamine (1.06 ml, 7.65 mmol) in 15 ml of absolute EtOH were refluxed for 42 hr. The Boc-Glu(resin)-NH_2 was washed several times with EtOH and Et_2O and dried: yield, 6.34 g. A sample of the esterified resin was N-deprotected with 1 *N* HCl in anhydrous AcOH and hydrolyzed in dioxane–12 *N* HCl (1:1)[20] at 110° for 22 hr. Amino acid analysis of the hydrolysate showed that the esterification product contained 0.21 mmol of isoglutamine/g of esterified resin. A 3.5-g sample of the Boc-Glu-(resin)-NH_2 was used for synthesis. A cycle for incorporation of each amino acid residue into the growing peptide chain involved the following washing and reaction steps: (1) three washings with 20 ml of AcOH; (2) 1 *N* HCl in anhydrous AcOH (20 ml) for 25 min (except in the case of Boc-Gln where the amino group was liberated by treatment with 20 ml of TFA for 20 min in order to avoid cyclization to <Glu); (3) three washings with 20 ml of AcOH; (4) three washings with 20 ml of EtOH; (5) three washings with 20 ml of DMF; (6) 5% triethylamine in DMF (20 ml) for 10 min; (7) three washings with 20 ml of DMF; (8) addition of *tert*-butyloxycarbonylamino acid, 1-hydroxybenzotriazole, and dicyclohexylcarbodiimide (DCC), all in 4 equiv with respect to Glu, in 20 ml of DMF (these compounds were mixed at 0° 15 min before addition to the resin), reaction for 16–22 hr [Boc-Gln and Boc-Asn were introduced through their *p*-nitrophenyl esters (3 equiv based on the substitution of the first amino acid residue to the resin) in 20 ml of DMF]: (9) three washings with 20 ml of DMF; (10) three washings with 20 ml of EtOH.

The completion of each coupling step was checked according to the ninhydrin method of Kaiser, *et al.*[34] In instances (after addition of Asn and Gln derivatives and of Ile in [9-proline]oxytocin) where small amounts of unacylated amino group were detectable, the resin was washed with 3 × 20 ml of DMF, treated with 20 ml of 5% triethylamine in DMF (5 min), again washed with 3 × 20 ml of DMF, and finally treated once again overnight with the 1-hydroxybenzotriazole derivatives generated *in situ* or the appropriate *p*-nitrophenyl derivative (2.0 or 1.5 equiv, respectively).

After addition of the Boc-Cys(Bzl) residue the nonapeptide derivative was washed successively with 4 × 20 ml of DMF, 4 × 20 ml of EtOH, and 3 × 20 ml of Et_2O and dried to yield 5.0 g of substituted resin.

H·Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Glu-NH_2·HBr. The protected nonapeptide resin (2.45 g) was suspended in CF_3COOH (25 ml) and anisole (2.16 ml). The suspension was saturated with HBr for 90 min.[20] The resin was filtered off and washed three times with CF_3COOH. The solvent was evaporated at room temperature, and the residue was washed with Et_2O. The precipitate was filtered, washed several times with Et_2O, and dried overnight to give 754 mg of white powder (this would amount to more than 100% of the theoretical yield of the partially protected nonapeptide, probably because of contamination with decomposition products of the resin formed during acidolysis).[20] Thin-layer chromatography on heavily loaded (50 μg of peptide) silica gel G layers developed with *n*-BuOH–H_2O–AcOH (4:1:1, v/v/v) or with 95% EtOH revealed one major and a very minor ninhydrin-active component. There was also a trace component developed with Cl_2–starch iodide. A sample of this material gave after acid hydrolysis[32] the expected ratios of ninhydrin-active components: NH_3, 3.2; Cys(Bzl), 2.0; Asp, 1.0; Glu, 2.2; Pro, 1.1; Ile, 1.0; Leu, 1.1; Tyr, 0.6.

[9-Glutamic acid]oxytocin. A sample of the partially deprotected peptide was dissolved in 250 ml of boiling ammonia, freshly distilled from sodium. A fresh sodium stick was introduced intermittently into the solution until a blue color remained for 20 sec. Glacial acetic acid (2 drops) was added, the ammonia was evaporated to a small volume, and the remainder of the ammonia removed by lyophilization. The residue was dissolved in water (2 ml of water per 1 mg of starting peptide) and the pH of the solution adjusted to 6.8 with acetic acid. The solution was aerated until the test for sulfhydryl groups according to Ellman[35] was negative. The pH was adjusted to 4.8 with AcOH and the solution lyophilized. The product was dissolved in a small volume of the upper phase of the solvent system *n*-BuOH–pyridine–AcOH–H_2O (1000:15:35:950) and subjected to partition chromatography on a Sephadex G-25 (100–200 mesh) column (2 × 52 cm) in the same solvent system. The fractions corresponding to the major peak (R_f 0.29) of Folin–Lowry[36] color values were pooled and lyophilized: yield, 66.5 mg. Purification of the material was repeated by partition chromatography in the same solvent system. Recovery of [9-glutamic acid]oxytocin was 53.7 mg (yield 47%, based on the original substitution of the resin by Boc-Glu-NH_2): $[\alpha]^{24}D$ −40.8° (*c* 0.88, 1 *N* AcOH). The product was homogeneous as judged by electrophoresis at pH 3.5 and development of the electrophoretogram with ninhydrin stain. Amino acid analysis[32] gave the following molar ratios: NH_3, 4.2 (regarding this high value, see Discussion); Asp, 1.0; Glu, 2.0; Pro, 1.1; ½Cys, 2.0; Ile, 0.9; Leu, 1.0; Tyr, 0.9.

Z-Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Pro-Resin. Boc-Pro-resin (3 g) containing 0.34 mmol of Boc-Pro/g of polymer, prepared by the standard procedure, was the starting material which gave, after addition of the appropriate amino acids as described, 4.74 g of protected nonapeptide-resin. The coupling step involving Boc-Ile-OH had to be repeated to obtain full acylation of the glutaminyl amino group.

Z-Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Pro-NH_2. Protected nonapeptide-resin (3 g) was suspended in a 1:1 mixture of DMF–EtOH (120 ml). The mixture was saturated at −20° with dry ammonia and stirred under pressure for 4 days at room temperature. The resin was removed by filtration (and kept in this case for further treatment, see below) and washed several times with DMF. The combined filtrates were evaporated, the residue was redissolved in a small amount of DMF, and the solution was filtered through glass wool into boiling EtOH (100 ml). After cooling, the precipitate was filtered, washed several times with ether, and dried: yield, 114.4 mg. A second batch was obtained after evaporation of the EtOH from the precipitation step to a small volume followed by precipitation with ether: yield, 95 mg. Be-

cause of the low yield of the peptide obtained by ammonolysis the substituted resin which had been retained was subjected twice more to a 4-day treatment with NH_3 in DMF-EtOH. Two batches of product were obtained after precipitation of peptide by EtOH (79 mg) and ether (15 mg).

The combined crude product (~300 mg) was dissolved in 3 ml of AcOH and the solution was added dropwise with stirring to 100 ml of boiling 50% aqueous EtOH to form a clear solution from which the peptide separated on cooling. The gelatinous product was filtered, washed several times with 50% EtOH and Et_2O, and dried overnight: yield, 140 mg (32% based on the substitution of the polymer by Boc-Pro); mp 231–232°; $[\alpha]^{24}D$ −62.2° (c 0.81, DMF). Thin-layer chromatography of approximately 50 μg of peptide on silica gel G plates revealed one major and one very minor Cl_2-starch iodide reactive component. Amino acid analysis of a hydrolyzed sample gave the following molar ratios: NH_3, 3.0; Cys(Bzl), 1.9; Asp, 1.0; Glu, 1.0; Pro, 2.0; Ile, 0.9; Leu, 1.0; Tyr, 0.9.

[9-Proline]oxytocin. The protected nonapeptide (118 mg) was converted to the disulfide as described for the glutamic acid analog. The crude product was purified by partition chromatography on a 57 × 2 cm column of Sephadex G-25 (100–200 mesh) in the solvent system *n*-BuOH-PrOH-pyridine-AcOH-water (600:300:13.5:31.5:855). Only one Folin–Lowry peak (R_f 0.52, symmetrical) was obtained. Its material was pooled and lyophilized: yield, 52.2 mg (20%); $[\alpha]^{23}D$ −71.5° (*c* 0.63, 1 *N* AcOH). High-voltage electrophoresis at pH 3.5 followed by development with ninhydrin spray revealed a major ninhydrin-active component along with a minor contaminant. Amino acid analysis of a hydrolyzed aliquot (6 *N* HCl, 110°, 24 hr) gave the following molar ratios: NH_3, 3.2; Asp, 1.0; Glu, 1.0; Pro, 2.0; ½Cys, 2.0; Ile, 1.0; Leu, 1.0; Tyr, 1.0.

Isolation and Purification of Toad Urinary Bladder Carboxamidopeptidase. A carboxamidopeptidase preparation from toad urinary bladder[7] was obtained according to the procedure of Walter, *et al.*[31].§ In brief, toads were double-pithed and the urinary bladders removed and washed in 0.65% NaCl. Bladders were homogenized (4 ml/bladder) in 0.025 *M* phosphate buffer containing 1 m*M* EDTA, pH 7.0 ("phosphate buffer"), on a Virtis "45" homogenizer. The homogenate was diluted twofold with water and centrifuged at 0° at 100,000g for 30 min. The supernatant was absorbed onto a DEAE-cellulose column (1.5 × 21 cm) equilibrated with phosphate buffer. The column was washed with the same buffer and then eluted with a linear NaCl gradient (0–0.4 *M*); 5-ml fractions were collected. Enzyme activity was monitored by incubation of aliquots from alternate fractions with [9-glycine-*1*-^{14}C]arginine-vasopressin ([^{14}C]-AVP),[37] followed by electrophoretic separation and identification of radioactive products as previously described.[9,10] Fractions releasing [^{14}C]-Gly-NH_2 from AVP (fractions 33–54; corresponding to a peak with elution volume 220 ml) were pooled, concentrated, and subjected to gel filtration on a Sephadex G-200 column (2.5 × 32 cm) in phosphate buffer. Enzyme activity was detected as described. Fractions 56–62, corresponding to a peak at elution volume 75 ml, had the highest specific activity, *i.e.*, released greater than 0.5 nmol of Gly-NH_2/min/mg of protein, when protein was measured by the absorbence ratio at 280/260 mμ.[38] Therefore, these fractions were pooled and concentrated (to ~2 mg/ml), and aliquots were frozen at −20° for further studies.

Incubation of Neurohypophyseal Peptides with Carboxamidopeptidase and Identification of Products. Enzyme preparation (0.05 ml; ~0.1 mg) was incubated with substrate in a total volume of 0.2 ml of 0.07 *M* phosphate buffer containing 1 m*M* EDTA, pH 7.0, for 3 hr at 30°. Amount of substrate varied for each peptide: [^{14}C]-AVP and [^{14}C]oxytocin (specific activity *ca.* 6 mCi/mmol), 50 nmol; [9-glutamic acid]oxytocin, 80 nmol; [9-proline]oxytocin, 90 nmol. As controls, peptides were incubated without enzyme, enzyme was incubated in the absence of peptide, and isoglutamine and prolinamide were incubated with enzyme; all controls were subjected to procedures described below. In the case of AVP and oxytocin, products were separated by high-voltage paper electrophoresis as previously described;[10] radioactive products were detected on a Packard 7201 chromatogram scanner and glycinamide, the only product detected, was identified by comparison with an authentic sample. Products released from [9-proline]- and [9-glutamic acid]oxytocin were identified by subjecting an unhydrolyzed aliquot of the incubation mixture to amino acid analysis. Authentic isoglutamine (time of emergence 326 min after the start of the chromatogram on a 55 × 0.9 cm column of Beckman UR 30 resin; the column was eluted at 55° sequentially with 0.2 *M* sodium citrate buffers, pH 3.25 and 4.25, and the buffer change was programmed for 140 min after the start of the run) and prolinamide (time of emergence 67 min after the start of the chromatogram on a 6.2 × 0.9 cm column of Beckman PA35 resin eluted with 0.2 *M* sodium citrate buffer, pH 5.26, at 55°) were run as standards. In the digest mixtures isoglutamine was the only detectable product. Control incubations showed that prolinamide and isoglutamine were unaffected by the toad bladder enzyme. The methods used allowed quantification of products released from the substrates. In the case of [8-lysine,9-glycine methylamide]oxytocin the incubation was carried out and the reaction products were identified as dansylated derivatives as previously described.[7,39]

Bioassay Methods. At least three animals were used for determination of activities in each assay system. For bioassays a four-point design or matches were used, employing the USP Posterior Pituitary Reference Standard. When the inhibitory properties of the analogs were tested, oxytocin with an avian vasodepressor activity of 510 ± 23 U/mg[40] served as standard. Oxytocic activity was measured on isolated uterine horns from Sprague–Dawley rats in natural estrus, determined on the morning of the assay by vaginal smear, by the method of Holton[41] as modified by Munsick,[42] utilizing Mg^{2+}-free Van Dyke–Hastings solution. Avian vasodepressor assays were performed on conscious white Leghorn roosters[43] according to the procedure of Coon.[44] Inhibitory studies were performed using ten chickens for each peptide. The antidiuretic activity was determined in inactin- and ethanol-anesthetized, hydrated male Sprague–Dawley rats, according to the method of Jeffers, *et al.*,[45] with some modifications. Pressor activity was determined in atropinized, urethane-anesthetized male Sprague–Dawley rats following the procedure in the U. S. Pharmacopeia.[46] The specific activities of [8-lysine,9-glycine methylamide]oxytocin are expressed on the basis of the molecular weight of the anhydrous peptide. The inhibitory properties of the analogs in the avian vasodepressor assay were measured under two sets of conditions. In the first, solutions of oxytocin and inhibitor were washed *simultaneously* into the wing vein of the chicken with 1.0 ml of saline ("simultaneous injection"). For "sequential injection," the solution of analog was washed into the bird with 1.0 ml of saline *1 min before* the solution of oxytocin, which was injected in the same manner. In order to test for reversibility of the inhibition, a series of injections of oxytocin (1 × 10^{-10} mol) was given at 10-min intervals. These were followed by a combined dose of oxytocin (1 × 10^{-10} mol) plus inhibitor (5 × 10^{-8} mol) and 10 min later again by a series of injections of oxytocin standard (1 × 10^{-10} mol).

Acknowledgment. The authors wish to thank Dr. J. Friedland for carrying out the enzymological tests. We are also grateful to Ms. D. Surovec for invaluable help in performing bioassays and to Ms. M. Lin for technical assistance. The editorial help of Dr. P. Hoffman is appreciated. The [8-lysine,9-glycine methylamide]oxytocin was a gift of Dr. Klaus Lübke of Schering AG, West Germany. The work was supported by Grants GM-18752, AM-10080, and AM-13567 of the National Institutes of Health, U. S. Public Health Service, and by the U. S. Atomic Energy Commission.

References

(1) *Biochemistry*, **6**, 362 (1967); *J. Biol. Chem.*, **247**, 977 (1972).
(2) D. W. Urry and R. Walter, *Proc. Nat. Acad. Sci. U. S.*, **68**, 956 (1971).
(3) R. Walter, I. L. Schwartz, J. H. Darnell, and D. W. Urry, *Proc. Nat. Acad. Sci. U. S.*, **68**, 1355 (1971).
(4) J. Rudinger and I. Krejčí in "Handbuch der Experimentellen Pharmakologie," Vol. XXIII, B. Berde, Ed., Springer-Verlag, Berlin, 1968, p 748.
(5) (a) W. Y. Chan, V. J. Hruby, G. Flouret, and V. du Vigneaud, *Science*, **161**, 280 (1968); (b) V. Pliška, J. Vašák, M. Rufer, and J. Rudinger, *Experimentia*, **29**, 171 (1973).
(6) R. Walter, M. A. Kirchberger, and V. J. Hruby, *Experientia*, **28**, 959 (1972).
(7) J. D. Glass, I. L. Schwartz, and R. Walter, *Proc. Nat. Acad. Sci. U. S.*, **63**, 1426 (1969).
(8) M. Koida, J. D. Glass, I. L. Schwartz, and R. Walter, *Endo-*

§R. Walter, M. Koida, J. Friedland, and I. L. Schwartz, unpublished results.

crinology, 88, 633 (1971).
(9) R. Walter, H. Shlank, J. D. Glass, I. L. Schwartz, and T. D. Kerenyi, *Science*, 173, 827 (1971).
(10) R. Walter, *Peptides, Proc. Eur. Symp., 12th*, 363 (1973).
(11) L. Fruhaufová, E. Suska-Brzezińska, T. Barth, and I. Rychlík, *Collect. Czech. Chem. Commun.*, 38, 2793 (1973).
(12) E. Schillinger, O. Loge, E. Schröder, E. Klieger, and K. Lübke, *Eur. J. Biochem.*, 27, 473 (1972).
(13) R. B. Merrifield, *J. Amer. Chem. Soc.*, 85, 2149 (1963); *Advan. Enzymol.*, 32, 221 (1969).
(14) J. C. Sheehan and G. P. Hess, *J. Amer. Chem. Soc.*, 77, 1067 (1955).
(15) W. König and R. Geiger, *Chem. Ber.*, 103, 788 (1970).
(16) E. Sandrin and R. A. Boissonnas, *Helv. Chim. Acta*, 46, 1637 (1963).
(17) E. Schröder and E. Klieger, *Justus Liebigs Ann. Chem.*, 673, 208 (1964).
(18) H. Zahn, W. Danho, and B. Gutte, *Z. Naturforsch. B*, 21, 763 (1966).
(19) M. Manning, *J. Amer. Chem. Soc.*, 90, 1348 (1968).
(20) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," W. H. Freeman, San Francisco, Calif., 1969.
(21) R. A. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, 108, 753 (1935).
(22) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon, *J. Amer. Chem. Soc.*, 75, 4879 (1953).
(23) D. Yamashiro, *Nature (London)*, 201, 76 (1964).
(24) L. Y. Sklyarov and I. V. Shaskova, *Zh. Obshch. Khim.*, 39, 2779 (1969).
(25) J. Meienhofer and A. Trzeciak, *Proc. Nat. Acad. Sci. U. S.*, 68, 1006 (1971).
(26) J. D. Glass, I. L. Schwartz, and R. Walter, *J. Amer. Chem. Soc.*, 94, 6209 (1972).
(27) J. D. Glass, R. Walter, and I. L. Schwartz, *Peptides, Proc. Eur. Symp., 12th*, 135 (1973).
(28) J. D. Glass, A. Talansky, Z. Grzonka, I. L. Schwartz, and R. Walter, *J. Amer. Chem. Soc.*, in press.
(29) H. Aoyagi, M. Kondo, and N. Izumiya, *Bull. Chem. Soc. Jap.*, 41, 2772 (1968).
(30) H. Takashima, W. Fraefel, and V. du Vigneaud, *J. Amer. Chem. Soc.*, 91, 6182 (1969).
(31) R. Walter, J. D. Glass, B. M. Dubois, M. Koida, and I. L. Schwartz, *Peptides: Chem. Biochem., Proc. Amer. Peptide Symp., 2nd*, 327 (1972).
(32) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, 30, 1190 (1958).
(33) P. Lefrancier and E. Bricas, *Bull. Soc. Chim. Biol.*, 49, 1257 (1967).
(34) E. Kaiser, R. L. Colescott, C. D. Bessinger, and P. I. Cook, *Anal. Biochem.*, 34, 595 (1970).
(35) G. Ellman, *Arch. Biochem. Biophys.*, 82, 70 (1959).
(36) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193, 265 (1951).
(37) R. Walter and R. T. Havran, *Experientia*, 27, 645 (1971).
(38) O. Warburg and W. Christian, *Biochem. Z.*, 310, 384 (1941).
(39) J. D. Glass, B. M. Dubois, I. L. Schwartz, and R. Walter, *Endocrinology*, 87, 730 (1970).
(40) S. Hase and R. Walter, *Int. J. Protein Res.*, 5, 283 (1973).
(41) P. Holton, *Brit. J. Pharmacol.*, 3, 328 (1948).
(42) R. A. Munsick, *Endocrinology*, 66, 451 (1960).
(43) R. A. Munsick, W. H. Sawyer, and H. B. Van Dyke, *Endocrinology*, 66, 860 (1960).
(44) J. M. Coon, *Arch. Int. Pharmacodyn.*, 62, 79 (1939).
(45) W. A. Jeffers, J. J. Livezey, and J. H. Austin, *Proc. Soc. Exp. Biol. Med.*, 50, 184 (1942).
(46) "The Pharmacopeia of the United States," 17th revision, Mack Publishing Co., Easton, Pa., 1965, p 749.

Effect of Lipophilic Substituents on Some Biological Properties of Indoles

Calvert W. Whitehead* and Celia A. Whitesitt

The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46206. *Received May 1, 1974*

Simple indole derivatives were screened for antimicrobial activity. Although the majority of these indoles failed to inhibit the growth of any of the test microbes, some compounds were effective inhibitors at relatively low concentrations. Indoleacetic acids and carboxylic acids were the least active, aminomethyl derivatives were moderately active, and 5-, 6-, and 7-haloindoles were the most active of the simple indoles. Diarylmethyl substituents further enhanced the potency of the simple indoles including the active halo derivatives and also converted some inactive indoles into effective growth inhibitors. Toxicity, behavioral effects, antiviral activity, antiinflammatory properties, antispasmotic activity, and general endocrine properties of the (diarylmethyl)indoles are described.

Simple indole derivatives demonstrate numerous biological effects. Indole, 3-methylindole, 3-indoleacetic acid, and tryptophan delay the spread of tuberculosis in guinea pigs and 1-methylindole-2,3-dione 3-thiosemicarbazone is useful in the prophylaxis of smallpox.[1] Indolylalkylamines induce responses in the central nervous system and in peripheral organs and tissues of higher animals.[2-9] Carboxylic acid derivatives of indole are effective antiinflammatory agents.[10] A study of the antimicrobial activity of indoles, therefore, would not be complete nor practical without a knowledge of the compounds effects upon the host animal.

In this investigation, the antimicrobial structure-activity relations were correlated for 400 simple indole derivatives. The correlations served as references for establishing the effect of lipophilic groups on this activity. (Diarylmethyl)indoles were prepared by carbonium ion alkylations of selected indoles. The modified indoles were then screened for antibacterial, antifungal, antiprotozoal, and antiviral activities. Finally, they were studied for biological reactions that might appear as clinical manifestations in a host animal. These included toxic effects, behavioral changes, antiinflammatory activity in animals, antagonist reactions on isolated smooth muscle, and general endocrine properties.

The antimicrobial screening procedure employed by W. Wick and associates of these laboratories was essentially that described by Johnson.[11] Each of the 51 microbes (Table I) was inhibited by at least one of the indole compounds at a concentration of 200 μg/ml. Indole and 46% of the 400 variously substituted indoles that were tested failed to inhibit any of the organisms. A partial, but representative list of the inactive compounds is given in Table II.† The remaining 54% inhibited at least one species, either a bacterial or fungal organism. Of all the compounds tested, 36% inhibited at least two species and finally 8% of the compounds inhibited at least 15 species.

In order to establish substituent-activity relationships, the indoles were classified into groups having similar substituents. Furthermore, all active compounds were reviewed so that each important substituent-activity effect,

† See paragraph at end of paper regarding supplementary material.