tected nonapeptide amide VII was reduced with Na in liq NH_3, the resulting product was oxidized with $K_3Fe(CN)_6$, and the analog was isolated and purified as described earlier; wt 31 mg; $[\alpha]^{23}D$ −37.6° (c 1, DMF). Amino acid analysis gave the correct ratios of amino acids and NH_3. The analog moved as 1 spot on silica gel tlc plates in the above solvents. *Anal.* ($C_{47}H_{73}N_{11}O_{12}S_2$) C, H, N.

Acknowledgments. The authors are greatly indebted to Dr. W. Y. Chan, Department of Pharmacology, Cornell University Medical College, for very helpful discussions relating to the biological studies, to Mrs. Janet Huisjen of this department for her highly capable performance in carrying out the bioassays, and to Dr. Louis L. Nangeroni, New York State Veterinary College at Cornell University, for use of his laboratory in this aspect of the work.

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Further Studies of the Role of the Asparagine Residue in Oxytocin. Synthesis and Biological Properties of [5-α,γ-Diaminobutyric acid]oxytocin†‡

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The synthesis of [5-α,γ-diaminobutyric acid]oxytocin, an analog of oxytocin which contains an α,γ-diaminobutyric acid residue in place of the asparagine residue in position 5, is reported. On assay for biological activity this analog was found to possess 0.03 unit/mg of oxytocic activity, approximately 0.03 unit/mg of avian vasodepressor activity, and less than 0.01 and 0.001 unit/mg, respectively, of mammalian pressor and antidiuretic activities. The analog was incapable of inhibiting the oxytocin-induced responses in the 4 biological systems tested. It is concluded that [5-α,γ-diaminobutyric acid]oxytocin possesses a low affinity for the neurohypophyseal hormone receptors, and that this effect is a result of an increase in the conformational flexibility of this analog as compared with oxytocin.

All naturally occurring neurohypophyseal hormones of known amino acid sequence possess an Asp(NH_2) residue in position 5. Oxytocin analogs which bear a Me,[1,2] β-carboxamideethyl,[3] hydroxymethyl,[1] γ-aminopropyl,[4] and *i*-Pr[5] side chain in position 5 instead of the carboxamidemethyl moiety all exhibit an exceedingly low potency with respect to the activities characteristic of oxytocin—a finding which led us to focus on the importance of position 5 for the conformational stability of the hormonal molecule.[6-8] The elucidation of the conformation of oxytocin[9] confirmed the central role of the Asp(NH_2) residue. It is critical for the maintenance of both β-turns comprised of the sequences -Tyr-Ile-Glu(NH_2)-Asp(NH_2)- and -Cys-Pro-Leu-Gly- (Figure 1).

In the present communication we report on the synthesis of [5-α,γ-diaminobutyric acid]oxytocin and on the determination of its biological properties in order to assess the capability of α,γ-diaminobutyric acid (Dbu) to replace successfully the Asp(NH_2). The free tetrapeptide, *S*-Bzl-Cys-Pro-Leu-Gly-NH_2,[10-13] was elongated with BOC-Glu(NH_2)-N^{γ}-Pht-Dbu (**5**) in the presence of 1.4 equiv of DCI and 2.0 equiv of *N*-hydroxysuccinimide according to the procedure of Weygand, *et al.*,[14] to yield BOC-Glu(NH_2)-N^{γ}-Pht-Dbu-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (**6**).

The dipeptide **5** was secured from N^{γ}-Pht-Dbu·H_2O (**1**), obtained after phthalylation of the dihydrochloride salt of the free acid by the general procedure of Nefkens, *et al.*,[15] in the following manner: the acid **1** was converted to its *p*-toluenesulfonate salt (**2**), which in turn was esterified with Ph_2CHN_2;[16-18] the resultant ester **3** was allowed to react with BOC-Glu(NH_2)-ONp[19] to give BOC-Glu(NH_2)-N^{γ}-Pht-Dbu diphenylmethyl ester (**4**), which was subsequently deesterified to give **5**. The hexapeptide (**6**) was elongated stepwise to yield the fully protected nonapeptide, *N*-Z-*S*-Bzl-Cys-*O*-Bzl-Tyr-Ile-Glu(NH_2)-N^{γ}-Pht-Dbu-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (**9**). Peptide **9** was successfully dephthalylated with either hydrazine hydrate[20,21] or hydrazine acetate[22] to yield **10**, which was fully deprotected by treatment with

†This work was supported in part by United States Public Health Service grants AM-13567 and AM-10080.
‡Abbreviations used have been suggested in *J. Med. Chem.*, 13(5), 8A (1970). The amino acids (except glycine) are of the L configuration.

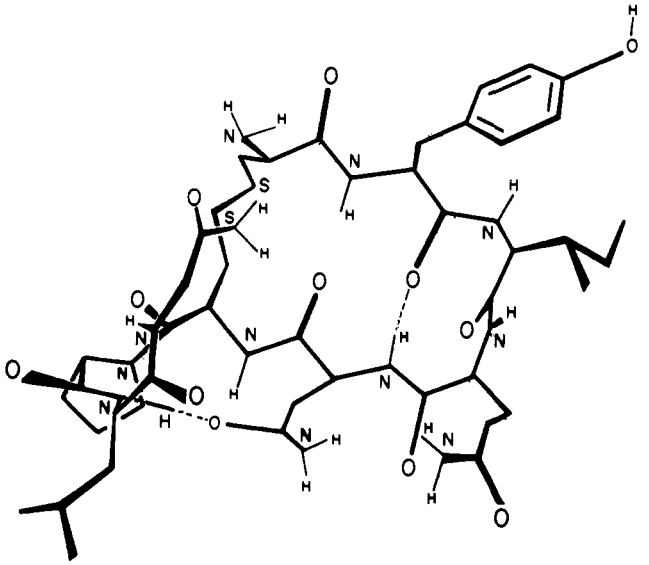

Figure 1. Conformation of oxytocin in solution as proposed by Urry and Walter.[9]

HF as described by Sakakibara, *et al.*[23,24] The intermediary dithiol was oxidized to the disulfide by action of aq $K_3Fe(CN)_6$.[25] [5-α,γ-Diaminobutyric acid]oxytocin was purified by partition chromatography[26] using 2 solvent systems.

Upon bioassay [5-α,γ-diaminobutyric acid]oxytocin was found to possess 0.03 unit/mg of rat uterotonic activity. It exhibited approximately 0.03 unit/mg of avian vasodepressor activity, less than 0.01 unit/mg of rat pressor activity, and less than 0.001 unit/mg of rat antidiuretic activity. The analog did not inhibit the oxytocin-induced responses in any of these 4 biological test systems. This indicates that the greatly diminished capability of this compound to evoke the responses characteristic of oxytocin resides in its low affinity for the oxytocin receptors. The predominant factor may be that Dbu—in contrast to Asp(NH_2)—lacks the carbonyl group in the side chain and thus is unable to form an H bond with the amide NH of the residue in position 8. This enhances the conformational ambiguity of the acyclic tripeptide, which factor in turn also appears to affect the conformation of the 20-membered ring adversely. It is concluded that Dbu, unlike the Asp(NH_2) residue, is unable to stabilize the oxytocin conformation, and hence is not an effective substitute for the Asp(NH_2) residue in neurohypophyseal hormones.

Experimental Procedure[♦]

N^{γ}-Pht-Dbu·H_2O (1). Dbu·2HCl (9.55 g, 0.05 mole) was dissolved in a mixt of H_2O (100 ml) and 4 *N* NaOH (37.5 ml), and an aq soln (20 ml) of $CuSO_4 \cdot 5H_2O$ (6.25 g, 0.025 mole) was added. Carboethoxyphthalimide[15] (13.1 g, 0.06 mole) and $NaHCO_3$ (5 g, 0.05 mole) were added to the mixt with strong stirring. After 60 min the Cu salt of N^{γ}-Pht-Dbu was filtered, washed successively with H_2O, EtOH, CH_2Cl_2, and Et_2O; yield, 13.0 g (92.2%). The Cu salt was suspended in a soln of EDTA (18.6 g, 0.05 mole) in H_2O (200 ml), and the mixt was stirred vigorously for 60 min and then refrigerated for 2 hr.[27,28] The white ppt was collected by filtration, washed successively with H_2O, EtOH, and Et_2O; yield, 11.55 g. The crude product was recrystd from boiling H_2O (250 ml) contg EDTA (1 g); yield, 7.76 g (58.9%); mp 205–206° dec, $[\alpha]^{20}D$ +14.2° (*c* 2, 1 *N* HCl). *Anal.* ($C_{12}H_{12}N_2O_4 \cdot H_2O$) C, H, N.

N^{γ}-Pht-Dbu *p*-Toluenesulfonate (2). Compd 1 (5.32 g, 20 mmoles) was dissolved in warm H_2O (15 ml) together with *p*-TsOH (3.99 g, 21 mmoles). The soln was concd to form a cryst residue, which was suspended in acetone and collected by filtration after cooling; yield, 7.81 g (92.9%); mp 176–177° dec; $[\alpha]^{20}D$ +9.2° (*c* 2.2, MeOH). *Anal.* ($C_{19}H_{20}N_2O_7S$) C, N; H: calcd, 4.81; found, 5.33.

N^{γ}-Pht-Dbu Diphenylmethyl Ester *p*-Toluenesulfonate (3). A soln of diphenyldiazomethane (3.70 g, 19 mmoles) in DMF (10 ml) was stirred into a soln of 2 (6.31 g, 15 mmoles) in DMF (5 ml) at 50°. After 10 min the reaction mixt was concd, and the resulting oil was crystd from a mixt of EtOAc (10 ml) and Et_2O (100 ml). The material was filtered and washed repeatedly with EtOAc; yield, 6.86 g; mp 148–150° dec. Recrystn from CH_3CN (100 ml) and then from DMF–EtOAc gave fine needles; yield 4.83 g (54.9%); mp 159–160° dec; $[\alpha]^{20}D$ +14.3° (*c* 1.1, MeOH). *Anal.* ($C_{32}H_{30}N_2O_7S$) C, H, N.

BOC-Glu(NH_2)-N^{γ}-Pht-Dbu Diphenylmethyl Ester (4). A soln of 3 (2.34 g, 4 mmoles) in DMF (10 ml) was allowed to react with BOC-Glu(NH_2)-ONp (1.83 g, 5 mmoles) in the presence of Et_3N (0.56 ml) for 24 hr. The reaction mixt was concd to an oily residue which was crystd from Et_2O; yield, 2.9 g. The crude product was recrystd from EtOAc; yield, 1.3 g (50.6%); mp 148–150° (sintg 146°); $[\alpha]^{20}D$ −12.3° (*c* 1, DMF). *Anal.* ($C_{35}H_{38}N_4O_8$) C, H, N.

BOC-Glu(NH_2)-N^{γ}-Pht-Dbu (5). A soln of 4 (1.22 g, 1.9 mmoles) in a mixt of MeOH (70 ml) and THF (20 ml) was subjected to catalytic hydrogenation using 0.5 g of 10% Pd/C as catalyst. After 3 hr the catalyst was removed by filtration and the filtrate was concd to a residue which was crystd from Et_2O. Recrystn from MeOH–Et_2O gave 0.83 g (92.1%) of product; mp 170–171° dec; $[\alpha]^{20}D$ +7.5° (*c* 1, DMF). *Anal.* ($C_{22}H_{28}N_4O_8$) C, H, N.

BOC-Glu(NH_2)-N^{γ}-Pht-Dbu-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (6). A soln of DCI (346 mg, 1.68 mmoles) in DMF (5 ml) was stirred into a soln of *S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (573 mg, 1.2 mmoles), 5 (572 mg, 1.2 mmoles), and *N*-hydroxysuccinimide (276 mg, 2.4 mmoles) in DMF (30 ml) at −22°. The reaction mixt was stirred at −22° for 1 hr and at room temp for 18 hr and then filtered and the filtrate was concd to an oily residue which solidified upon trituration with a mixt of EtOAc–Et_2O (1:2); yield, 1.17 g. The crude product was repeatedly pptd from 90% EtOH; yield, 615 mg (54.9%); mp 205–208° dec; $[\alpha]^{20}D$ −70.8° (*c* 1, AcOH). *Anal.* ($C_{45}H_{61}N_9O_{11}S$) C, H, N.

BOC-Ile-Glu(NH_2)-N^{γ}-Pht-Dbu-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (7). Compd 6 (562 mg, 0.6 mmole) was treated with F_3CCO_2H (1 ml) for 30 min. The volatile components were removed and the ppt, which resulted upon trituration of the oil with Et_2O, was filtered, washed with Et_2O, and dried over NaOH; yield, 570 mg. This salt was dissolved in DMF (3 ml) and allowed to react with BOC-Ile-ONp[29] (317 mg, 0.9 mmole) in the presence of *N*-methylmorpholine (0.01 ml). After 24 hr a mixt of EtOAc and Et_2O (1:1, 50 ml) contg AcOH (0.1 ml) was added to ppt the crude product, which was purified by repptn from 96% EtOH; yield, 395 mg (62.8%); mp 203–206° dec; $[\alpha]^{20}D$ −73.7° (*c* 1, AcOH). *Anal.* ($C_{51}H_{72}N_{10}O_{12}S$) C, H, N.

***N*-BOC-*O*-Bzl-Tyr-Ile-Glu(NH_2)-N^{γ}-Pht-Dbu-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (8).** The BOC group of 7 (525 mg, 0.5 mmole) was removed with F_3CCO_2H (2.5 ml) as described above. The peptide trifluoroacetate (535 mg) dissolved in DMF (5 ml) was allowed to react with *N*-BOC-*O*-Bzl-Tyr-ONp[30] (370 mg, 0.75 mmole) in the presence of *N*-methylmorpholine (0.1 ml). After 23 hr EtOAc (50 ml) contg a small amount of AcOH (0.1 ml) was added to the reaction mixt to ppt the crude product; yield, 660 mg. The crude product was purified by boiling with 80% EtOH (50 ml) and collected by filtration after cooling; yield, 563 mg (86.5%); mp 236–240° dec, $[\alpha]^{20}D$ −54.1° (*c* 1, AcOH). *Anal.* ($C_{67}H_{87}N_{11}O_{14}S$) C, H, N.

***N*-Z-*S*-Bzl-Cys-*O*-Bzl-Tyr-Ile-Glu(NH_2)-N^{γ}-Pht-Dbu-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (9).** The BOC group of 8 (521 mg, 0.4 mmole) was removed with F_3CCO_2H (2.5 ml) as described in the synthesis of 7. The resulting trifluoroacetate (525 mg) was dissolved in DMF (5 ml) and was allowed to react with *N*-Z-*S*-Bzl-Cys-ONp[31] (268 mg, 0.6 mmole) in the presence of *N*-methylmorpholine (0.08 ml) for 20 hr. The crude product (615 mg), pptd with EtOAc (35 ml) contg AcOH (0.1 ml), was purified by boiling with 80% EtOH; yield, 572 mg (93.5%); mp 265–268° dec; $[\alpha]^{20}D$ −42.8° (*c* 1, DMSO). *Anal.* ($C_{80}H_{96}N_{12}O_{15}S_2$) C, H, N.

♦All reactions were carried out at room temp and all products were dried at room temp over P_2O_5 *in vacuo* unless otherwise noted. Evaporations were performed under reduced pressure. All melting points were detd with a Thomas-Hoover capillary melting point apparatus and are not corrected. Optical rotations were detd with a Carl Zeiss photoelectric precision polarimeter set at 0.005°. Peptide hydrolysates were chromatographed on a Beckman/Spinco Model 120C amino acid analyzer, using Beckman custom research resin pA-28. Elementary analyses were carried out by Galbraith Laboratories, Knoxville, Tenn. Where analyses are indicated only by symbols of the elements, analytical results obtd for the elements were within ±0.4% of the theoretical values.

N-Z-*S*-Bzl-Cys-*O*-Bzl-Tyr-Ile-Glu(NH_2)-Dbu-*S*-Bzl-Cys-Pro-Leu-Gly-$NH_2 \cdot H_3CCO_2H$ (10). A soln of 1 *M* hydrazine hydrate in DMSO (0.4 ml) was added to a suspension of 9 (200 mg, 0.13 mmole) in DMSO (3 ml). The reaction mixt was stirred vigorously at 75° for 3 hr. A small amt of insol materials was removed by filtration and AcOH (0.2 ml) was added to the filtrate. The product pptd by addn of H_2O (20 ml) was filtered, washed with H_2O, and dried; yield, 147 mg (77%); mp 211–216° dec.

In an alternative experiment a soln of 2 *M* hydrazine acetate in DMF (2 ml) was used for the removal of the phthalyl group of 9 (306 mg, 0.2 mmole) and the reaction was carried out at room temp for 6 hr. The product was pptd with H_2O and then applied to a short column of CM-Sephadex (H^+ form) in a mixt of DMSO–MeOH–H_2O (3:3:1). After washing the column well with H_2O, the product was eluted with aq AcOH increasing from 50 to 80%. The eluates were dild with H_2O and lyophilized; yield, 155 mg (53%); $[\alpha]^{20}D$ −45.6° (*c* 0.8, DMF). *Anal.* ($C_{72}H_{94}N_{12}O_{13}S \cdot C_2H_4O_2$) C, H, N.

Both methods gave identical product showing an R_f 0.55 by tlc on silica gel G (Merck) in a solvent system of BuOH–AcOH–H_2O (4:1:1), although in the former case a small amount of by-product was detected.

[5-α,γ-Diaminobutyric acid]oxytocin (11). Compd 10 (146 mg, 0.1 mmole) was dissolved in anhyd HF (*ca.* 15 ml) together with anisole (0.5 ml). The reaction mixt was stirred at 20° for 60 min. HF was evapd with N_2 to result in a residue, which was dried in a desiccator over NaOH for 3 hr. The dried material was dissolved in 0.1% AcOH (100 ml) and the soln was extd with Et_2O (100 ml). The aq layer was passed through a column (3 × 8 cm) of Dowex 1-X2 (acetate form). Effluent and washings of the same solvent were combined (*ca.* 250 ml). The soln was adjusted to pH 8.2 with 1 *N* NH_4OH and treated with excess 0.01 *N* $K_3Fe(CN)_6$. After 15 min the pH was adjusted to 6.5 with 1 *N* AcOH, and ferro- and excess ferricyanide ions were removed by treatment of the soln with AG3X4 (Bio-Rad) in the Cl^- form. Following the removal of resin the filtrate was concd to *ca.* 10 ml and applied to a column (3 × 63 cm) of Sephadex G-25 (100–200 mesh), which had been equilibrated with the lower phase of BuOH–EtOH–H_2O contg 3.5% AcOH and 1.5% pyridine (7:2:9). The column was eluted with the upper phase and 90 9.3-ml fractions were collected. Aliquots from every second fraction were taken for detn of Folin-Lowry color values.[32] The fractions corresponding to the principal peak (R_f 0.36) were pooled and dild with excess H_2O. The mixt was concd to a small vol and the product was isolated by lyophilization; yield, 50 mg. This material was dissolved in the upper phase (3 ml) of the solvent system BuOH–PrOH–H_2O contg 3.5% AcOH and 1.5% pyridine (7:2:9). The soln was applied to the same column of Sephadex G-25 which had been equilibrated with both phases of the solvent system. After elution with the upper phase a single peak (R_f 0.21) was detected by Folin-Lowry color detn. Fractions 71–83 were combined, dild with H_2O, concd and, after azeotropic removal of the org phase, lyophilized; yield of [5-α,γ-diaminobutyric acid]oxytocin as monoacetate, 19.3 mg (18%). A sample for analysis was dried at 100° for 8 hr; $[\alpha]^{20}D$ −34.0° (*c* 0.6, 1 *N* AcOH). *Anal.* ($C_{43}H_{68}N_{12}O_{11}S_2 \cdot C_2H_4O_2$) C, H, N.

Amino acid analysis[33] after hydrolysis in 6 *N* HCl at 110° for 23 hr gave the following molar ratios with glycine taken as 1.00: Cys (1.02); Tyr (0.89); Ile (0.96); Glu (1.00); Dbu (1.02); Pro (1.02); Leu (1.01); Gly (1.00); NH_3 (2.04).

R_f values on tlc on silica gel G (Merck) with different solvent systems gave the following values: 0.34 with upper phase of BuOH–AcOH–H_2O (4:1:5) (0.49 for Tyr); 0.08 with BuOH–AcOH–H_2O (4:1:1) (0.42 for Tyr); 0.32 with upper phase of BuOH–EtOH–H_2O contg 3.5% AcOH and 1.5% pyridine (7:2:9) (0.46 for Tyr).

Bioassay Methods. Oxytocic assays were performed on 6 isolated uterine horns from 3 rats in natural estrus according to the method of Holton,[34] modified by Munsick[35] with the use of Mg-free van Dyke–Hastings soln as the bathing fluid. Avian vasodepressor assays were performed on 3 conscious chickens according to the procedure employed by Munsick, *et al.*[36] Assays for antidiuretic activity were performed on anesthetized, hydrated Sprague-Dawley male rats according to the method of Jeffers, *et al.*,[37] as modified by Sawyer;[38] maximal depression of urine flow, in contrast to average duration of the response, was used to measure the antidiuretic activity. Assays were carried out on 6 rats; not more than 6 hormone injections were given to each animal. Rat pressor assays were carried out on 5 atropinized, urethane-anesthetized male rats as described in the United States Pharmacopeia.[39] The biological activities were measured against the USP Posterior Pituitary Reference Standard.

Acknowledgments. The authors are indebted to Mr. J. Bitter, who was involved in the early phase of this work. They are also grateful for the technical assistance rendered by Mrs. I. Mintz and Mrs. A. Silverman.

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