Dimerization of 3-Methyl-N-carbomethoxyazepine (33). An 850-mg (5.15 mmol) sample of **33**[1] was sealed in an ampoule and heated for 10 min at 200°. The resulting black gummy solid was recrystallized from methanol (charcoal decolorization) to give 205 mg (24%) of white crystals of **34**, mp 245–246.5°; $\nu_{max}^{CHCl_3}$ 1692 cm^{-1}; λ_{max}^{EtOH} 239 mμ (ε 14,615); $\delta_{TMS}^{CDCl_3}$ 5.62–6.08 (broad multiplet, 6 H, vinyl protons), 4.49–5.14 (multiplet, 4 H, >C*H*N<), 3.76 (singlet, 6 H, $-OCH_3$), 1.86–2.06 (multiplet, 6 H, $\geqslant CCH_3$).

Anal. Calcd for $C_{18}H_{22}N_2O_4$: C, 65.44; H, 6.71; N, 8.48. Found: C, 65.69; H, 6.64; N, 8.45.

Dimerization of 4-Methyl-N-carbomethoxyazepine (35). Heating of 1.10 g (6.65 mmol) of **35** in the above manner gave, after recrystallization from methanol, 248 mg (22.6%) of **36** as a highly crystalline white solid, mp 228–230°; $\nu_{max}^{CHCl_3}$ 1695 cm^{-1}; λ_{max}^{EtOH} 238 mμ (ε 14,190); $\delta_{TMS}^{CDCl_3}$ 5.50–5.91 (multiplet, 6 H, vinyl protons); 4.47–4.91 (multiplet, 4 H, >C*H*N<), 3.67 (singlet, 6 H, $-OCH_3$), and 1.81 (singlet, 6 H, $\geqslant CCH_3$).

Anal. Calcd for $C_{18}H_{22}N_2O_4$: C, 65.44; H, 6.71; N, 8.48. Found: C, 65.41; H, 6.75; N, 8.47.

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The Detection of a Schiff Base Intermediate in the Formation of Acetone-oxytocin[1]

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Abstract: Treatment of oxytocin with 60% aqueous acetone at 0° and at a pH of approximately 5 leads to the rapid formation of a Schiff base of oxytocin which has been trapped by reduction with sodium borohydride to give [1-(N-isopropylhemi-L-cystine)]-oxytocin (N-isopropyl-oxytocin). The reduction of the Schiff base was accomplished without reduction of the disulfide bond of oxytocin. Treatment of the hormone with acetone under the same conditions for 24 hr without the addition of borohydride affords acetone-oxytocin in aproximately 50% yield. N-Isopropyl-oxytocin possesses only a trace (~0.1 unit/mg) of oxytocic activity and does not exhibit avian vasodepressor activity. The preparation of N,N′-diisopropyl-L-cystine is also described.

The inactivation of oxytocin by aqueous acetone[3] with the formation of an isopropylidene derivative has been reported. This isopropylidene derivative, referred to as acetone-oxytocin, has been found to possess a 2,2-dimethyl-4-imidazolidinone ring structure in which the isopropylidene group from acetone forms a bridge between the nitrogen atom of the amino group of the half-cystine residue at position 1 of oxytocin and the nitrogen of the peptide bond between this half-cystine residue and the tyrosine residue at position 2, as shown in Figure 1.[4]

From a consideration of the mechanism of formation of the imidazolidinone derivative, it appeared likely that a Schiff base might be an intermediate. If the Schiff base were formed, it might be possible to detect its presence by treating the reaction mixture with sodium borohydride and trapping the Schiff base as [1-(N-isopropylhemi-L-cystine)]-oxytocin (N-isopropyl-oxytocin). It has been found that disulfide bonds in proteins are readily reduced by borohydride.[5] Since oxytocin contains a disulfide bond, it was necessary to find conditions suitable for reduction of the Schiff base without reduction of the disulfide bond. It was found that sulfhydryl formation was almost negligible at pH 5. Furthermore, at a pH of approximately 5 in 60% aqueous acetone, oxytocin is converted to acetone-oxytocin in 50% yield after 24 hr. The trapping experiment was therefore carried out in the following manner.

A solution of oxytocin in 60% aqueous acetone at pH 4.9 and 0° under nitrogen was treated over a 1-hr period with a large excess of sodium borohydride while the pH was maintained at 4.8–5.5 by addition of glacial acetic acid. Determination of the sulfhydryl content by the method of Ellman[6] was performed at various intervals during the addition of sodium borohydride. At no time was more than a trace of sulfhydryl detected.

The solid material obtained after neutralization of the reaction mixture and removal of the solvents *in vacuo* was purified twice by partition chromatography on Sephadex G-25[7] in the solvent system 1-butanol–3.5% acetic acid in 1.5% aqueous pyridine (1:1). N-Isopropyl-oxytocin was obtained in 75% yield as a white, lyophilized powder from the fractions comprising the peak with R_f 0.35. Oxytocin and acetone-oxytocin have R_f values of 0.24 and 0.76, respectively,[3] in the solvent system used. No oxytocin was detected in the partition chromatogram and only a trace of material was present at the position of acetone-oxytocin.

N-Isopropyl-oxytocin gave satisfactory values in the elemental and amino acid analyses. For the amino acid analysis, a 48-hr hydrolysis time was required due to the difficulty in the hydrolysis of the N-isopropyl-cystinyl–tyrosine peptide bond. It is interesting to note the high negative rotation of $[\alpha]^{23}D$ −88.3° (*c* 0.5, 1 *N*

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acetic acid) for N-isopropyl-oxytocin. In comparison, oxytocin shows $[\alpha]^{22.5}D$ −23.1° (*c* 0.51, 1 *N* acetic acid).[8] This large change in optical rotation to more negative values at the sodium D line occurs in the case of a number of N-substituted analogs of oxytocin.

N-Isopropyl-oxytocin was subjected to bioassay for avian vasodepressor[9] and oxytocic[10] activities. It was found to have no avian vasodepressor activity and only a trace (~0.1 unit/mg) of oxytocic activity. This compares with 507 ± 23 units/mg of avian vasodepressor activity[11] and 546 ± 18 units/mg of oxytocic activity[12] for oxytocin. Other oxytocin analogs that are blocked in the N-terminal position, *e.g.*, N-methyl-oxytocin[13,14] and N-acetyl-oxytocin,[15] show very low or negligible avian vasodepressor and oxytocic activities.

Experimental Section[16]

N-Isopropyl-S-benzyl-L-cysteine. A stirred solution of 2.76 g (10 mmol) of ethyl S-benzyl-L-cysteinate hydrochloride[17] in 20 ml of isopropyl alcohol and 10 ml of acetic acid was cooled to 10°, and 20 ml (150 mmol) of acetone was added. Then 6.0 g (960 mmol) of sodium borohydride (Metal Hydrides, Beverly, Mass.) was slowly added over a 40-min period accompanied by just enough water (~5 ml total) to keep the solution clear. The pH of the solution was maintained at 4.2–4.8 by addition of acetic acid. An extra 10 ml of acetone and 10 ml of isopropyl alcohol were added to the reaction mixture in the middle of the borohydride addition period. The excess borohydride was destroyed by stirring of the solution for 2 hr. The pH was adjusted to 7 by addition of 4 *N* KOH (~120 ml) and then to 12 with concentrated KOH. The organic solvents were removed *in vacuo*, and about 100 ml of ethanol was added to give a clear single-phase system which was stirred for 1 hr at room temperature. The ethanol was removed *in vacuo*, and the solution was extracted with ether. The aqueous layer was diluted with 100 ml of water, and the pH was adjusted to 5.3 with concentrated HCl. The precipitate was filtered off and recrystallized from 135 ml of boiling water to give in three fractions 1.80 g of N-isopropyl-S-benzyl-L-cysteine, mp 227–228° dec, $[\alpha]^{24}D$ +63.6° (*c* 1.0, 1 *N* HCl).

Anal. Calcd for $C_{13}H_{19}NO_2S$: C, 61.6; H, 7.56; N, 5.53. Found: C, 61.6; H, 7.62; N, 5.59.

N,N′-Diisopropyl-L-cystine. A modified Weygand procedure[18] was used.[7b] A solution of 0.51 g (2 mmol) of N-isopropyl-S-benzyl-L-cysteine in 75 ml of freshly distilled anhydrous ammonia was treated with small pieces of sodium until a blue coloration persisted for 2 min. The excess coloration was destroyed with ammonium trifluoroacetate, and the ammonia was removed by evaporation and lyophilization. The resulting powder was dissolved in 75 ml of 50% aqueous acetone saturated with nitrogen. A solution of 0.620 g (2.2 mmol) of freshly recrystallized 1,2-diiodoethane (Aldrich Chemical Co., Milwaukee, Wis.) in 50 ml of acetone was added, and the pH was adjusted to 9.0 by addition of 1 *N* NaOH. After the mixture had been stirred for 20 min, the pH was adjusted to 5.5 by addition of trifluoroacetic acid, and the acetone was removed *in vacuo*. The light brown solution was extracted with two 25-ml portions of ethyl acetate, and the water was removed *in vacuo*. The residue was triturated with acetone, filtered off, and washed with acetone. The product (0.32 g) was crystallized from 6 ml of water–acetone–ethanol (1:1:1) and then recrystallized from acetone–water to give, after being dried *in vacuo*, 0.20 g of N,N′-diisopropyl-L-cystine, mp 231–232° dec, $[\alpha]^{24}D$ +19.9° (*c* 1.0, 1 *N* HCl).

Anal. Calcd for $C_{12}H_{24}N_2O_4S_2$: C, 44.4; H, 7.46; N, 8.63. Found: C, 44.5; H, 7.59; N, 8.52.

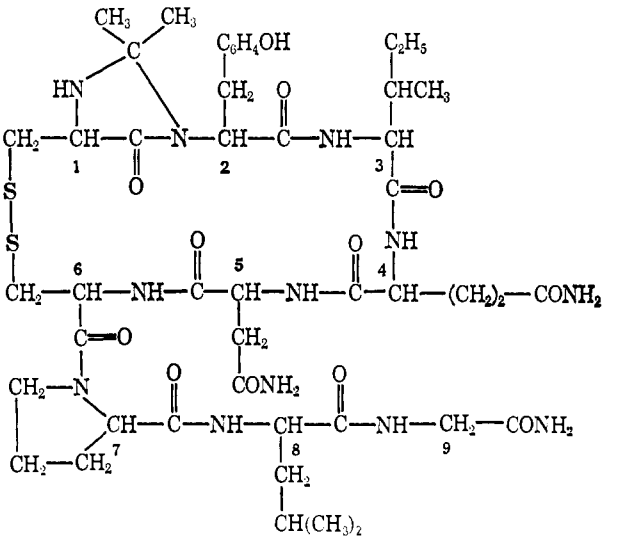

Figure 1. Structure of acetone-oxytocin with numbers indicating the position of the individual amino acid residues.

N-Isopropyl-oxytocin. A stirred solution of 50 mg (5×10^{-5} mol) of oxytocin in 25 ml of glass-distilled water was cooled to 0° under nitrogen; the pH was adjusted to 4.9 with acetic acid, and 37.5 ml (3.25×10^{-1} mol) of acetone was added. Then 5.0 g (1.25×10^{-1} mol) of sodium borohydride was added in small portions over a 1-hr period while the pH was maintained at 4.8–5.5 by addition of acetic acid. The sulfhydryl content was determined[6] at 15-min intervals, but at no time was more than a trace detected. After the addition of sodium borohydride was complete, the solution was stirred for 1 hr at 0°, the pH was adjusted to 6.5 with dilute NH_4OH, and the solvents were removed *in vacuo*. The residue was dissolved in 30 ml of the upper phase of the solvent system 1-butanol–3.5% acetic acid in 1.5% aqueous pyridine (1:1) and placed on a 2.8 × 65 cm column of Sephadex G-25 (100–200 mesh) that had been equilibrated with the lower and upper phases of the solvent system according to the method of Yamashiro.[7] The product was eluted with the upper phase, and 80 7.7-ml fractions were collected. After Folin–Lowry color values had been determined,[19] the fractions represented by the major peak (R_f 0.36) were pooled, 300 ml of water was added, the mixture was concentrated to 30 ml *in vacuo*, and the solution was lyophilized to give 46.6 mg of N-isopropyl-oxytocin. The product was dissolved in 7.5 ml of the upper phase of the same solvent system, and the entire procedure was repeated. In this instance, 90 6.6-ml fractions were collected. The material corresponding to the principal peak (R_f 0.35) was isolated in the manner already described to give 39.3 mg of N-isopropyl-oxytocin, $[\alpha]^{23}D$ −88.3° (*c* 0.5, 1 *N* acetic acid).

Anal. Calcd for $C_{46}H_{72}N_{12}O_{12}S_2$: C, 52.7; H, 6.92; N, 16.0. Found: C, 53.0; H, 7.08; N, 15.7.

The N-isopropyl-oxytocin was hydrolyzed for 48 hr in 6 *N* HCl at 110° and then analyzed[20] on a Beckman-Spinco amino acid analyzer. The following molar ratios were obtained with the value of glycine taken as 1.0: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.1; glycine, 1.0; cystine, 0.25; isoleucine, 1.0; leucine,

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1.0; tyrosine, 0.8;[21] mixed disulfide of N-isopropylcysteine and cysteine,[22] 0.41; and ammonia, 2.8.

Preparation of Acetone-oxytocin in 60% Aqueous Acetone at pH 5.1. The pH of a cool (0°) solution of 20 mg (2×10^{-5} mol) of oxytocin in 10 ml of glass-distilled water and 15 ml of acetone was adjusted to 5.1 with acetic acid. The solution was stirred for 24 hr at 0°, the pH was adjusted to 7.0 with dilute ammonia, and the acetone was removed *in vacuo*. After lyophilization of the aqueous solution, the resulting power (20.73 mg) was dissolved in 2 ml of the upper phase of the solvent system 1-butanol–3.5% acetic acid in 1.5% aqueous pyridine (1:1) and subjected to partition chromatography on a 1.9 × 38.3 cm column of Sephadex G-25 (100–200 mesh). Sixty 4.6-ml fractions were collected, and Folin–Lowry color values were determined. The fractions corresponding to the fast-moving peak with R_f 0.73 (lit.[3] R_f 0.76) were pooled, 80 ml of glass-distilled water was added, and the mixture was concentrated to a volume of about 20 ml *in vacuo*. Subsequent lyophilization gave 10.47 mg of acetone-oxytocin.[3] The fractions corresponding to the slow-moving peak with R_f 0.25 (lit.[3] R_f 0.24) were treated in a similar manner and yielded 9.20 mg of unreacted oxytocin.

(21) A value of 1.0 was found by ultraviolet determination.

(22) The position and color value for the mixed disulfide of cysteine and N-isopropylcysteine were obtained by analysis of an equimolar mixture of L-cystine and N,N′-diisopropyl-L-cystine after the mixture had been heated in 6 *N* HCl *in vacuo* at 110° for 48 hr. In the analysis of N-isopropyl-oxytocin, the mixed disulfide peak appeared just before the glutamic acid peak. N,N′-Diisopropyl-L-cystine has essentially no ninhydrin color value.

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The Synthesis and Pharmacological Properties of [2-Isoleucine]-8-lysine-vasopressin and Its 1-Deamino Analog[1]

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Abstract: [2-Isoleucine]-8-lysine-vasopressin, an analog of the pressor antidiuretic hormone in which the tyrosine residue is replaced by an isoleucine residue, has been synthesized and tested for pharmacological activity. This analog was prepared by synthesis of the required nonapeptide intermediate by the stepwise *p*-nitrophenyl ester method. Reduction of this protected nonapeptide with sodium in liquid ammonia and oxidation of the resulting dithiol with potassium ferricyanide yielded the [2-isoleucine]-8-lysine-vasopressin, which was then purified by partition chromatography and gel filtration on Sephadex G-25. [2-Isoleucine]-8-lysine-vasopressin was found to possess about 0.5 unit/mg of avian vasodepressor activity, less than 0.005 unit/mg of oxytocic activity, about 1.8 units/mg of pressor activity, and 3.7 units/mg of antidiuretic activity. [1-β-Mercaptopropionic acid,2-isoleucine]-8-lysine-vasopressin, in which the free amino group on the half-cystine residue at position 1 of [2-isoleucine]-8-lysine-vasopressin is replaced by hydrogen, was also prepared in a similar manner. It was found to possess about 1.0 unit/mg of avian vasodepressor activity, less than 0.1 unit/mg of oxytocic activity, about 1.5 units/mg of pressor activity, and about 3.0 units/mg of antidiuretic activity. Thus both analogs possessed extremely low pharmacological activities in contrast to comparable analogs of oxytocin, which exhibited an appreciable degree of oxytocic, avian vasodepressor, and milk-ejecting activity.

Recently it was found that [2-isoleucine]-oxytocin and its deamino analog, [1-β-mercaptopropionic acid,2-isoleucine]-oxytocin, possess the oxytocic and avian vasodepressor activities of oxytocin to a reduced but still appreciable degree, whereas analogs of oxytocin containing other aliphatic amino acid residues in place of the tyrosine residue at position 2 had been found to possess extremely weak oxytocic and avian vasodepressor activities.[3] We then became interested in determining the effect of the replacement of the tyrosine residue in lysine-vasopressin (Figure 1) and in its 1-deamino analog by isoleucine on their pharmacological activities. The synthesis of [2-isoleucine]-8-lysine-vasopressin and [1-β-mercaptopropionic acid,2-isoleucine]-8-lysine-vasopressin was therefore undertaken.

The synthesis of [2-isoleucine]-8-lysine-vasopressin was accomplished by use of the *p*-nitrophenyl ester method[4] as employed for the synthesis of lysine-vasopressin.[5] The reaction of *p*-nitrophenyl N-carbobenzoxy-L-isoleucinate[6] with L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycinamide[5] resulted in the required protected octapeptide intermediate. After removal of the carbobenzoxy group from the latter compound with HBr in acetic acid and treatment with *p*-nitrophenyl N-carbobenzoxy-S-benzyl-L-cysteinate,[6] the protected nonapep-

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