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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(3) L. A. Branda, V. J. Hruby, and V. du Vigneaud, *Mol. Pharmacol.*, 3, 248 (1967).

(4) M. Bodanszky, *Nature*, **175**, 685 (1955); M. Bodanszky, M. Szelke, E. Tömörkeny, and E. Weisz, *Chem. Ind.* (London), 1517 (1955).

(5) M. Bodanszky, J. Meienhofer, and V. du Vigneaud, *J. Am. Chem. Soc.*, **82**, 3195 (1960).

(6) M. Bodanszky and V. du Vigneaud, *ibid.*, **81**, 5688 (1959).

tide N-carbobenzoxy-S-benzyl-L-cysteinyl-L-isoleucyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycinamide was obtained. This protected nonapeptide was then treated with sodium in liquid ammonia to remove the protecting groups by the method of Sifferd and du Vigneaud.[7] The dithiol so obtained was oxidized to the cyclic disulfide by treatment with potassium ferricyanide in aqueous solution at pH 8.2.[8] Purification of the analog was accomplished by partition chromatography on Sephadex G-25[9,10] in the solvent system 1-butanol–ethanol–pyridine–0.1 *N* acetic acid (4:1:1:7) in which it exhibited an R_F of 0.23. This compound was then further purified by gel filtration on Sephadex G-25[11] in 0.2 *N* acetic acid.

The highly purified [2-isoleucine]-8-lysine-vasopressin possessed about 0.5 unit/mg of avian vasodepressor activity,[12] less than 0.005 unit/mg of oxytocic activity,[13] about 1.8 units/mg of pressor activity,[14] and 3.7 units/mg of antidiuretic activity.[15] Lysine-vasopressin, on the other hand, possesses approximately 50 units/mg of avian vasodepressor activity, 7 units/mg of oxytocic activity, 265 units/mg of pressor activity, and 240 units/mg of antidiuretic activity.[16]

It may be recalled that 1-deamino-8-lysine-vasopressin ([1-β-mercaptopropionic acid]-8-lysine-vasopressin) is a highly active analog of lysine-vasopressin, being more potent than the parent hormone with respect to oxytocic, avian vasodepressor, and antidiuretic activities and less potent with respect to pressor activity.[17] It was of interest, therefore, to determine the effect of the replacement of the free amino group on the half-cystine residue at position 1 of [2-isoleucine]-8-lysine-vasopressin by hydrogen through the synthesis of 1-deamino-2-isoleucine-8-lysine-vasopressin ([1-β-mercaptopropionic acid,2-isoleucine]-8-lysine-vasopressin) and determination of its pharmacological properties. For this purpose the free octapeptide used in the preparation of [2-isoleucine]-8-lysine-vasopressing was coupled with *p*-nitrophenyl S-benzyl-β-mercaptopropionate.[8] The resulting protected polypeptide was freed of protecting groups by treatment with sodium in liquid ammonia, and the dithiol thus produced was oxidized with potassium ferricyanide. Purification of the resulting 1-deamino-2-isoleucine-8-lysine-vasopressin was effected by partition chromatography on Sephadex G-25 in the system 1-butanol–pyridine–0.2 *N* acetic acid (6:1:7) in which it exhibited an R_f of 0.26. The compound possessed 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 the formal replacement of the amino group of the half-cystine residue at position 1 of 2-isoleucine-lysine-vasopressin by hydrogen has no significant effect upon the pharmacological activities of this analog.

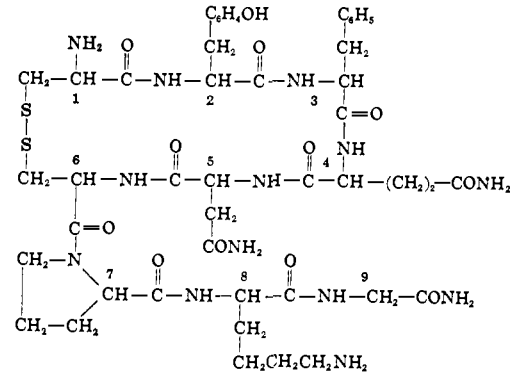

Figure 1. Structure of lysine-vasopressin with numbers indicating the position of individual amino acid residues.

As mentioned previously, the replacement of the aromatic tyrosine residue in oxytocin and in deamino-oxytocin with the aliphatic isoleucine residue leads to analogs possessing the characteristic biological activities of oxytocin to an appreciable degree. On the other hand, it is apparent from the present study that the same replacement in lysine-vasopressin and in deamino-lysine-vasopressin leads to analogs with an extremely low degree of the activities characteristic of the parent hormone.

Experimental Section[18]

N-Carbobenzoxy-L-isoleucyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycinamide. The protected heptapeptide N-carbobenzoxy-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycinamide[5] (3.0 g) was dissolved in 24 ml of anhydrous acetic acid, and 14.4 ml of a solution of hydrogen bromide in acetic acid (5.3 *N*) was added. The mixture was stirred for 1 hr at room temperature and then poured into 150 ml of anhydrous ether. The precipitated hydrobromide was washed with ether (two 100-ml portions) by decantation and then dried *in vacuo* over P_2O_5, KOH. The residue was dissolved in 19.2 ml of dimethylformamide (DMF) and the solution was cooled to 0° before the addition of triethylamine (4 ml) and *p*-nitrophenyl N-carbobenzoxy-L-isoleucinate[6] (1.21 g). The mixture was then allowed to stand at room temperature for 2 days, during which time a gel-like mass was formed. After the addition of acetic acid (1.5 ml), the mixture was diluted with ethyl acetate (200 ml), which caused the formation of a filterable product. This material was washed on the filter with ethyl acetate, ethanol, ethyl acetate, DMF–ethyl acetate (1:5), and finally with ethyl acetate, and then dried *in vacuo* over P_2O_5, KOH; wt 2.4 g, mp 237–240°.

An analytical sample was prepared by three reprecipitations of the material from warm acetic acid by the addition of ethanol. The melting point was raised to 244–247°, $[\alpha]^{20}D$ −42.5° (*c* 1, DMF).

Anal. Calcd for $C_{62}H_{82}O_{14}N_{12}S_2$: C, 58.0; H, 6.44; N, 13.1. Found: C, 57.7; H, 6.48; N, 13.0.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-isoleucyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N$^{\epsilon}$-

(7) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935); V. du Vigneaud and O. K. Behrens, *ibid.*, **117**, 27 (1937).

(8) V. du Vigneaud, G. Winestock, V. V. S. Murti, D. B. Hope, and R. D. Kimbrough, Jr., *ibid.*, **235**, PC64 (1960); D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *ibid.*, **237**, 1563 (1962).

(9) D. Yamashiro, *Nature*, **201**, 76 (1964).

(10) D. Yamashiro, D. Gillessen, and V. du Vigneaud, *J. Am. Chem. Soc.*, **88**, 1310 (1966).

(11) J. Porath and P. Flodin, *Nature*, **183**, 1657 (1959).

(12) R. A. Munsick, W. H. Sawyer, and H. B. van Dyke, *Endocrinology*, **66**, 860 (1960).

(13) The assays for oxytocic activity were performed on isolated uteri from rats in natural estrus according to the method of P. Holton, *Brit. J. Pharmacol.*, **3**, 328 (1948), as modified by R. A. Munsick, *Endocrinology*, **66**, 451 (1960), with the use of magnesium-free van Dyke–Hastings solution. Bioassays were carried out against the USP Posterior Pituitary Reference Standard.

(14) The pressor assays were performed as described in "The Pharmacopeia of the United States of America," 17th Rev., Mack Printing Co., Easton, Pa., 1965, p 749.

(15) The antidiuretic assays were carried out according to the method of W. A. Jeffers, M. M. Livezey, and J. H. Austin, *Proc. Soc. Exptl. Biol. Med.*, **50**, 184 (1942), as modified by W. H. Sawyer, *Endocrinology*, **63**, 694 (1958).

(16) D. Gillessen and V. du Vigneaud, *J. Biol. Chem.*, **242**, 4806 (1967); D. Yamashiro, R. T. Havran, H. L. Aanning, and V. du Vigneaud, *Proc. Natl. Acad. Sci. U. S.*, **57**, 1058 (1967).

(17) R. D. Kimbrough, Jr., W. D. Cash, L. A. Branda, W. Y. Chan, and V. du Vigneaud, *J. Biol. Chem.* **238**, 1411 (1963).

(18) All melting points were done in capillary tubes and are corrected.

tosyl-L-lysylglycinamide. The protected octapeptide (mp 237–240°, 1.0 g) was converted into its hydrobromide as described in the preceding section. The powder was then dissolved in 12.7 ml of DMF and the solution was cooled to 0°; triethylamine (0.75 ml) and 0.435 g of recrystallized *p*-nitrophenyl N-carbobenzoxy-S-benzyl-L-cysteinate (Cyclo Chemical Corp., Los Angeles Calif.; Lot K-4585) were added. After 1 day at room temperature, acetic acid (1.5 ml) and ethyl acetate (125 ml) were added. The product was filtered off, washed with ethyl acetate (15 ml) and ethanol (three 25-ml portions), and dried; wt 0.85 g. This crude product (400 mg) was twice precipitated from acetic acid with ethanol yielding 220 mg, mp 253–254°. For an analytical sample the crude material was reprecipitated three times as just described, mp 258–261°, $[\alpha]^{20}D$ −41.0° (*c* 1, DMF).

Anal. Calcd for $C_{72}H_{93}O_{15}N_{13}S_3$: C, 58.6; H, 6.35; N, 12.3. Found: C, 58.3; H, 6.46; N, 12.4.

[2-Isoleucine]-8-lysine-vasopressin. Small amounts of sodium were added to a solution of 120 mg of the twice-reprecipitated protected nonapeptide in 300 ml of boiling anhydrous ammonia (freshly distilled from sodium) until a blue coloration persisted for 30 sec. The ammonia was removed by evaporation to a volume of 30 ml and then by lyophilization. The colorless residue was dissolved in 250 ml of water containing 0.10 ml of trifluoroacetic acid, the pH was adjusted to 8.2 by addition of 2 *N* NH_4OH, and the resulting solution was treated with excess 0.01 *N* potassium ferricyanide. After 30 min the pH was adjusted to about 6.5 with dilute trifluoroacetic acid and the ferrocyanide and excess ferricyanide ions were removed by treatment of the solution with AG3X4 resin (Bio-Rad Laboratories, Richmond, Calif.) in the chloride form. The solution obtained after removal of the resin was lyophilized. The colorless powder was taken up in 5 ml of the upper phase of the solvent system 1-butanol–ethanol–pyridine–0.1 *N* acetic acid (4:1:1:7), and placed on a column (6.37 × 58 cm) of Sephadex G-25 (100–200 mesh) that had been equilibrated with the upper phase according to the method of Yamashiro.[9,10] The column was eluted with the organic phase, and 100 10-ml fractions were collected. Folin–Lowry color values[19] of aliquots from every second fraction were determined, the fractions corresponding to the principal peak (R_f 0.23) on the chromatogram were pooled, and 300 ml of water was added. The mixture was concentrated to a volume of 50 ml and lyophilized. The dried material was dissolved in 4 ml of 0.2 *N* acetic acid and placed on a Sephadex G-25 (200–270 mesh) column (6.24 × 67 cm) that had been equilibrated with 0.2 *N* acetic acid. One hundred and fifty 4.15-ml fractions were collected. Folin–Lowry color values for every fourth tube were determined, and the fractions corresponding to the major peak which had its maximum at fraction 81 were pooled, concentrated, and lyophilized to give 49.4 mg of colorless powder, $[\alpha]^{20}D$ −36.0° (*c* 0.5, 1 *N* acetic acid).

Anal. Calcd for $C_{43}H_{67}O_{11}N_{13}S_2 \cdot C_2H_4O_2$: C, 50.7; H, 6.71; N, 17.1. Found: C, 50.7; H, 6.86; N, 17.0.

A sample was hydrolyzed in 6 *N* HCl *in vacuo* at 110° for 22 hr and analyzed[20] in the Beckman-Spinco amino acid analyzer. The following molar ratios were obtained, glycine being taken as 1.0: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.0; glycine, 1.0; cystine, 1.0; isoleucine, 1.0; phenylalanine, 1.0; lysine, 1.0; and ammonia, 3.10.

S-Benzyl-β-mercaptopropionyl-L-isoleucyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N^{ϵ}-tosyl-L-lysylglycinamide. N-Carbobenzoxy-L-isoleucyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N^{ϵ}-tosyl-L-lysylglycinamide (0.515 g) was deprotected with HBr and converted to the free peptide with triethylamine as previously described. The free octapeptide was then coupled with *p*-nitrophenyl S-benzyl-β-mercaptopropionate (0.20 g) in 3 ml of DMF. After 2 days, water was added to the mixture. The precipitate was filtered off and washed twice with 20-ml portions of ethanol; wt 0.480 g. A sample was crystallized from acetic acid–ethanol solution and then melted at 238–240°, $[\alpha]^{25}D$ −44.0° (*c* 1, DMF).

Anal. Calcd for $C_{64}H_{86}O_{13}N_{12}S_3$: C, 57.9; H, 6.53; N, 12.7. Found: C, 57.7; H, 6.57; N, 12.7.

[1-β-Mercaptopropionic acid,2-isoleucine]-8-lysine-vasopressin. The preceding protected polypeptide (0.26 g) was reduced, oxidized, deionized, and lyophilized in the manner described for the preparation of [2-isoleucine]-8-lysine-vasopressin. The resulting product was dissolved in 7.5 ml of upper phase and 2 ml of lower phase of the solvent system 1-butanol–pyridine–0.2 *N* acetic acid (6:1:7) and placed on a column (6.37 × 58 cm) of Sephadex G-25 (100–200 mesh) that had been equilibrated with both lower and upper phase. The column was eluted with upper phase, and 100 9.0-ml fractions were collected. Folin–Lowry color values were determined, and a major peak at R_f 0.29 was found. The fractions corresponding to this peak were pooled, concentrated to a small volume after dilution with water, and lyophilized. This material, 101 mg, was redissolved in 2 ml of the upper phase of the same solvent system and rechromatographed under the same conditions. One peak with R_f 0.26 was obtained. The solutions represented by this peak were pooled, concentrated, and lyophilized to give 104 mg of material which was then subjected to gel filtration under the same conditions and on the same column as described for [2-isoleucine]-8-lysine-vasopressin. One symmetrical peak appeared, and the material represented by this peak was isolated and lyophilized to a white powder. The [1-β-mercaptopropionic acid,2-isoleucine]-8-lysine-vasopressin weighed 92 mg: $[\alpha]^{25}D$ −92.0° (*c* 0.5, 1 *N* acetic acid).

Anal. Calcd for $C_{43}H_{66}O_{11}N_{12}S_2$: C, 52.1; H, 6.71; N, 17.0. Found: C, 52.0; H, 6.83; N, 16.8.

A sample was hydrolyzed in 6 *N* HCl *in vacuo* at 110° for 22 hr and analyzed in the Beckman-Spinco amino acid analyzer. The following molar ratios were obtained, glycine being taken as 1.0: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.0; glycine, 1.0; isoleucine, 0.9; phenylalanine, 1.0; half-cystine, 0.5; mixed disulfide of β-mercaptopropionic acid and cysteine, 0.55; lysine, 1.0; and ammonia, 3.16.

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(19) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(20) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).