Synthesis of [1-(L-2-Hydroxy-3-mercaptopropanoic acid)]oxytocin, a Highly Potent Analogue of Oxytocin

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An analogue of oxytocin containing a hydroxy-group in place of the primary amino-group was synthesized by coupling *p*-nitrophenyl L-2-acetoxy-3-benzylthiopropanoyl-L-tyrosinate with a heptapeptide. [1-(L-2-Hydroxy-3-mercaptopropanoic acid)]oxytocin possessed three times the oxytocic activity of oxytocin on the isolated rat uterus. Neurophysin did not bind the new analogue.

THE large number of analogues of oxytocin and vasopressin already synthesized [1] have given us some understanding of the relationship between chemical structure and biological activity. With three exceptions, (1) the deamino-analogues [2,3] of both hormones, (2) the 4-threonine analogue of oxytocin,[4] and (3) the mono-carba-analogues of deamino-oxytocin,[5] the synthetic analogues have proved to be considerably less active than the natural hormones.

The first synthetic analogue possessing greater activity than oxytocin was the deamino-analogue.[2,6] Here the primary amino-group at the *N*-terminal was replaced by a hydrogen atom. The oxytocic activity on the rat uterus was 795 ± 36 i.u. mg^{-1}. The introduction of an amino-group at the 1-position (see Figure) in either of the two possible configurations reduced biological activity. In the hormone, oxytocin, where the amino-group is in the L-configuration the oxytocic activity was 523 i.u. mg^{-1}.[7] In the D-configuration, the presence of an amino-group had a more profound effect, thus hemi-D-cystineoxytocin possessed only 1·9 i.u. mg^{-1}.[8,9]

The primary amino-group at the 1-position of the nonapeptide hormones, oxytocin and vasopressin, appears to have a precise role in their binding to the carrier proteins, the neurophysins.[10] Thus neurophysins bind neither deamino-oxytocin nor deamino[8-arginine]vasopressin.[11] An electrostatic linkage may be responsible for the formation of the neurophysin–hormone complex.[12] However the pK_a of the amino-group in oxytocin is *ca.* 6·3 according to Breslow,[13] so that only 10% of the molecules would be charged at physiological pH. Un-

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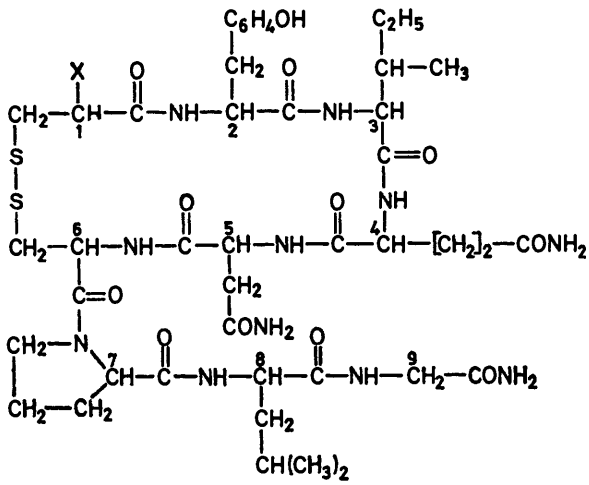
less the pH within the neurosecretory granules, where the neurophysin–hormone complexes are located, was appreciably lower than 7 or the pK_a effectively increased by a proton-donating group at the binding site on the



Formulae of oxytocin (X = NH_2), deamino-oxytocin (X = H), and [1-(L-2-hydroxy-3-mercaptopropanoic acid)]oxytocin (X = OH)

protein, the possibility of binding *via* hydrogen bonds must be considered.

To test this possibility we have synthesized [1-(L-2-hydroxy-3-mercaptopropanoic acid)]oxytocin, an analogue of the hormone in which the amino-group has been replaced by the strongly hydrogen-bonding hydroxy-group. The recent synthesis and resolution of 3-benzylthio-2-hydroxypropanoic acid [14] has enabled us to prepare peptides containing a residue of the α-hydroxy-analogue of cysteine in place of the α-amino-acid itself. A variety of derivatives of 3-benzylthio-2-hydroxypropanoic acid were prepared in preliminary attempts to incorporate it into a polypeptide chain.

The hydrazide of L-3-benzylthio-2-hydroxypropanoyl-*O*-benzyl-L-tyrosine was prepared as a crystalline compound. When used in the azide-coupling procedure with L-phenylalanine in a model experiment, the desired compound was obtained but the yield was low; possibly the presence of a free hydroxy-group is responsible. In subsequent work the hydroxy-group was protected by acetylation and *p*-nitrophenyl esters of 2-acetoxy-3-benzylthiopropanoic acid and of 2-acetoxy-3-benzylthiopropanoyl-L-tyrosine were prepared.

The *p*-nitrophenyl ester of DL-2-acetoxy-3-benzylthiopropanoic acid was obtained as a crystalline solid by a carbodi-imide condensation. However, this procedure gave only the same racemic *p*-nitrophenyl esters with the enantiomorphs of 2-acetoxy-3-benzylthiopropanoic acid. A similar observation was reported some time ago by Cash [15] with the corresponding amino-acid derivative *N*-acetyl-*S*-benzyl-L-cysteine. It is interesting that racemization occurs with the acetoxy-derivative where azlactone formation is not possible. The *p*-nitrophenyl ester of L-2-acetoxy-3-benzylthiopropanoyl-L-tyrosine could be obtained without racemization by a carbodi-imide condensation involving a *p*-nitrophenyl ester of an amino-acid salt (hydrobromide) as recommended by Goodman.[16]

The hydroxy-analogue of oxytocin was synthesized by a dipeptide–heptapeptide coupling. The sequence 3—9 was prepared as a protected heptapeptide by the stepwise *p*-nitrophenyl ester procedure.[17] Deprotection with hydrogen bromide in acetic acid gave the free heptapeptide, which was crystallized from water to remove diastereoisomerides.[18] Sequence 1—2 was synthesized as follows: L-3-benzylthio-2-hydroxypropanoic acid was protected by treatment with acetic anhydride to give L-2-acetoxy-3-benzylthiopropanoic acid, an oil, characterized first as the isothiouronium salt. It was later crystallized as the monohydrate. A dicyclohexylcarbodi-imide coupling of *p*-nitrophenyl L-tyrosinate hydrobromide with the *O*-acetyl derivative of L-2-hydroxy-3-benzylthiopropanoic acid in acetonitrile gave the desired *p*-nitrophenyl L-2-acetoxy-3-benzylthiopropanoyl-L-tyrosinate as a crystalline compound in 80% yield. Fragments 1—2 and 3—9 were condensed in dimethylformamide to give L-2-acetoxy-3-benzylthiopropanoyl-L-tyrosinyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide in a yield of 95%. Although the absence of racemization has not been demonstrated, the experiments of Cash [15] indicate that it is unlikely to occur, since an optically active dipeptide was obtained by coupling *p*-nitrophenyl *N*-acetyl-*S*-benzyl-L-cysteine and ethyl glycinate hydrochloride. The product was deacetylated with methanolic ammonia, and the *SS'*-dibenzyl peptide was debenzylated with sodium in liquid ammonia. The dithiol was oxidized with potassium ferricyanide [18] and the biologically active disulphide was subjected to countercurrent distribution. After 600 transfers the active material was isolated as a lyophilized powder. The oxytocic activity was assayed on the isolated rat uterus: [1-(L-2-hydroxy-3-mercaptopropanoic acid)]oxytocin had 1607 ± 20 ($n = 7$) i.u. mg^{-1}. Oxytocin, isolated under comparable conditions showed 523 ± 8 ($n = 3$) i.u. mg^{-1}. Thin-film dialysis experiments in the 'alternate' cell [19] showed that the hydroxy-analogue was not bound by neurophysin-II. This demonstrates that a hydroxy-group cannot be substituted for a primary amino-group in oxytocin and retain the affinity for neurophysin. We conclude that an electrostatic force is of primary importance for the formation of a protein–hormone complex. A brief account of this work has been previously published.[7]

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EXPERIMENTAL

M.p.s were determined on a Kofler block, optical rotations were measured on a Bellingham and Stanley Polarimeter (model A, type P3) with a 2-dm tube. Chromatographic data (R_F values) refer to descending chromatography on Whatman No. 1 paper with n-butanol–acetic acid–water,[20] and t.l.c. on Kieselgel G with methanol–chloroform (1 : 1 v/v). Ninhydrin-positive compounds were detected by spraying the dry plates and chromatograms with a solution of ninhydrin (0·1%, w/v) in acetone–water (95 : 5 v/v). Sulphur-containing substances were located with a solution of platinum(IV) iodide in acetone as described by Toennies and Kolb [21] (with one modification: the concentration of platinum(IV) chloride was increased ten-fold). Iodine was used as a general reagent for organic compounds. The ion-exchange columns were prepared from Dowex 2 × 8 (200—400 mesh) in the chloride form, from Bio–Rad Laboratories, Richmond, California. Elemental analyses were carried out by A. Bernhardt, Elbach, Germany. Amino-acid analyses were performed with an automatic analyser (Evans Electroselenium Ltd.) by the method of Spackman, Stein, and Moore.[22] Samples were hydrolysed *in vacuo* in constant-boiling hydrochloric acid [23] for 18 h. Counter-current distribution was run with a fully automatic train of 200 tube-units (H.O. Post and Co., Maspeth, New York). The hormone-binding ability was assessed by a modification of the thin-film dialysis.[24] The oxytocic activity was assayed on the isolated rat uterus [25] with Mg^{2+}-free van Dyke–Hastings solution suggested by Munsick [26] against synthetic oxytocin, standardized against the IIIrd International Oxytocic Standard.[27]

DL-2-*Acetoxy*-3-*benzylthiopropanoic Acid*.—A solution of racemic 3-benzylthio-2-hydroxypropanoic acid (5·0 g) in acetic anhydride (100 ml) was maintained at room temperature for 2 days. The volume was reduced *in vacuo* at 60° until an oil was obtained. Crystallization was induced by emulsifying the oil in hot water, adding ethanolic diethyl ether, cooling to 0°, and scratching with a glass rod. The white solid was filtered off and recrystallized from water. After drying *in vacuo* ($CaCl_2$) the *product* (5·67 g, 94·5%) had m.p. 57—58·5° (Found: C, 56·8; H, 5·6; S, 12·7. $C_{12}H_{14}O_4S$ requires C, 56·7; H, 5·6; S, 12·6%).

S-*Benzylisothiouronium* DL-2-*Acetoxy*-3-*benzylthiopropanoate*.—A solution of DL-3-benzylthio-2-hydroxypropanoic acid (1·0 g) in acetic anhydride (20 ml) was set aside at room temperature overnight and evaporated to an oil *in vacuo*. The product (1107 mg) was emulsified in water (20 ml) and the emulsion was neutralized with N-NaOH. By addition of N-HCl the solution was made faintly acidic and a solution of *S*-benzylisothiouronium chloride (4·415 g) in water (22 ml) was added at 0°. The mixture was kept at 0° for 5 h and then at room temperature for 2 days. The well-crystalline precipitate was filtered off and recrystallized from aqueous ethanol. After drying *in vacuo* ($CaCl_2$), the *material* (833 mg) had m.p. 110—112° (Found: C, 56·9; H, 6·0; N, 6·5; S, 15·0. $C_{20}H_{24}N_2O_4S_2$ requires C, 57·1; H, 5·8; N, 6·6; S, 15·2%).

L-2-*Acetoxy*-3-*benzylthiopropanoic Acid*.—This compound was prepared from L-3-benzylthio-2-hydroxypropanoic acid [14] (5·0 g) and acetic anhydride (100 ml). After recrystallization from water, the air-dried *material* (5·63 g, 94%) had m.p. 45—47°, $[\alpha]_D^{23}$ −43·2° ± 1·5 (*c* 1 in ethanol). The anhydrous product is an oil, the crystalline material is the monohydrate (Found: C, 53·0; H, 6·0; S, 11·9. $C_{12}H_{14}O_4S,H_2O$ requires C, 52·9; H, 5·9; S, 11·8%).

D-2-*Acetoxy*-3-*benzylthiopropanoic Acid*.—The product obtained from D-3-benzylthio-2-hydroxypropanoic acid [14] (5·0 g) was crystalline as the monohydrate only (5·79 g, 96·7%), m.p. 45—46·5°, $[\alpha]_D^{23}$ +42·5° ± 1·3 (*c* 1 in ethanol) (Found: C, 52·8; H, 5·7; S, 11·7%).

Methyl L-3-*Benzylthio*-2-*hydroxypropanoate*.—(A) *From* L-2-*acetoxy*-3-*benzylthiopropanoic acid*. To a solution of thionyl chloride (0·3 ml) in methanol (5 ml) at −20°, L-2-acetoxy-3-benzylthiopropanoic acid (1·0 g) was added with stirring. The mixture was kept at room temperature for 2 h and was then heated under reflux for 30 min. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate (50 ml). The solution was washed with water (2 × 10 ml) and the organic phase was dried ($MgSO_4$). After filtration, it was evaporated under reduced pressure at 50°. The oil soon solidified on cooling to room temperature. It was dried *in vacuo* ($CaCl_2$ and NaOH) and the methyl ester (761 mg, 92%) had m.p. 65—67° (softening at 59°). The compound was recrystallized from light petroleum (b.p. 40—60°) and dried *in vacuo* ($CaCl_2$). The dry *needles* (730·5 mg, 96%) melted at 69·5—70·5° without softening, $[\alpha]_D^{21·5}$ −16·0 ± 0·3 (*c* 1 in ethyl acetate) (Found: C, 58·3; H, 6·2; S, 14·1. $C_{11}H_{14}O_3S$ requires C, 58·4; H, 6·2; S, 14·2%).

(B) *From* L-3-*Benzylthio*-2-*hydroxypropanoic acid*. Similar treatment of the acid (1·0 g) gave the methyl ester (872 mg, 82%), m.p. 69·5—70°, $[\alpha]_D^{21}$ −16·0° ± 0·5 (*c* 1 in ethyl acetate) (Found: C, 58·4; H, 6·1; S, 14·3%).

Methyl D-3-*Benzylthio*-2-*hydroxypropanoate*.—(A) D-2-*Acetoxy*-3-*benzylthiopropanoic acid*. This acid (1·0 g) gave the methyl ester (776 mg, 93%), m.p. 65—66·5° (softening at 59·5°). After recrystallization from light petroleum (b.p. 40—60°) and drying *in vacuo* ($CaCl_2$), the product (743 mg, 97%) had m.p. 69·5—70·5°, $[\alpha]_D^{21·5}$ +16·0° ± 0·2 (*c* 1 in ethyl acetate) (Found: C, 58·3; H, 6·1; S, 14·2%).

(B) D-3-*Benzylthio*-2-*hydroxypropanoic acid* (1·0 g) gave the methyl ester (921 mg, 87%), m.p. 69·5—70·5°, $[\alpha]_D^{21·5}$ +16·0 ± 0·3 (*c* 1 in ethyl acetate).

L-3-*Benzylthio*-2-*hydroxypropanoic Acid Hydrazide*.—Methyl L-3-*benzylthio*-2-*hydroxypropanoate* (500 mg) was dissolved in methanol (3 ml) and heated under reflux together with hydrazine hydrate (99—100%; 1 ml) for 10 min. The hydrazide was precipitated with water (20 ml). The mixture was cooled and filtered off, washed with ice–water (15 ml), and dried *in vacuo* (P_2O_5). The needles (413 mg, 83%) were recrystallized from the minimum of hot water and dried *in vacuo* (P_2O_5). The dry *material* (370 mg, 90%) had m.p. 111—111·5°, $[\alpha]_D^{22}$ −19·9° ± 0·3 (*c* 1 in dimethylformamide) (Found: C, 52·9; H, 6·1; N, 12·4; S, 14·0. $C_{10}H_{14}N_2O_2S$ requires C, 53·0; H, 6·2; N, 12·4; S, 14·2%).

D-3-*Benzylthio*-2-*hydroxypropanoic Acid Hydrazide*.—From methyl D-3-benzylthio-2-hydroxypropanoate (500 mg) the corresponding hydrazide (436 mg, 88%) was iso-

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lated, recrystallized from hot water, and dried *in vacuo* (P_2O_5). The dry product (388 mg, 89%) had m.p. 111—112°, $[\alpha]_D^{21.5}$ +20·0° ± 0·2 (*c* 1 in dimethylformamide) (Found: C, 53·2; H, 6·1; N, 12·4; S, 14·0%).

L-3-*Benzylthio*-2-*hydroxypropanoyl*-O-*benzyl*-L-*tyrosine Hydrazide*.—(A). *From* L-2-*acetoxy*-3-*benzylthiopropanoic acid monohydrate*. Methyl *O*-benzyl-L-tyrosinate hydrochloride [28] (1·1817 g) was suspended in peroxide-free dioxan (50 ml) and triethylamine (0·511 ml) was added. At first a clear solution was obtained, then the hydrochloride of triethylamine separated out. L-2-Acetoxy-3-benzylthiopropanoic acid (1·0 g) (as the monohydrate) was added to the mixture, followed by *NN'*-dicyclohexylcarbodi-imide (757·7 mg) at −10°. It was stirred for 30 min and left at room temperature for 2·5 days. The solid material was filtered off, washed with dioxan (5 ml), and the filtrate and washings were evaporated under reduced pressure at 60° to yield methyl L-2-acetoxy-3-benzylthiopropanoyl-*O*-benzyl-L-tyrosinate as an oil. This was dissolved in methanol (10 ml) and heated under reflux together with hydrazine hydrate (99—100%; 3 ml) for 10 min. The hydrazide was precipitated with water (25 ml) at 0°, filtered off, washed with ice–water, and dried. The material (1·256 g, 72%) was recrystallized from methanol–water and dried *in vacuo* (conc. H_2SO_4). The dry *product* (900 mg, 71·5%) had m.p. 155·5—156·5°, $[\alpha]_D^{22}$ −10·5° ± 0·5 (*c* 1 in dimethylformamide) (Found: C, 65·0; H, 6·2; N, 8·8; S, 6·6. $C_{26}H_{29}N_3O_4S$ requires C, 65·1; H, 6·1; N, 8·8; S, 6·7%).

(B) *From* L-3-*benzylthio*-2-*hydroxypropanoic acid*. From this acid (779·5 mg) and methyl *O*-benzyl-L-tyrosinate hydrochloride (1·1817 g), crude L-3-*benzylthio*-2-*hydroxypropanoyl*-*O*-benzyl-L-tyrosine hydrazide (1·140 g, 65%) was obtained. After recrystallization from methanol–water the product (925 mg, 81%) had m.p. 154·5—156°, $[\alpha]_D^{20}$ −10·9° ± 0·4 (*c* 1 in dimethylformamide).

p-*Nitrophenyl* DL-2-*Acetoxy*-3-*benzylthiopropanoate*.—To a stirred solution of DL-2-acetoxy-3-benzylthiopropanoic acid (1·0 g) in ethyl acetate (16 ml), *p*-nitrophenol (657 mg) was added (20% excess). It was cooled to 0° and *NN'*-dicyclohexylcarbodi-imide (811·3 mg) was added. The mixture was kept at 0° for 1 h and then at room temperature for 2 days. The separated urea was filtered off, washed with ethyl acetate (7 ml), and the filtrate and washings were evaporated under reduced pressure at 60°. The resulting oil was crystallized by emulsifying in light petroleum (b.p. 40—60°; 5 ml), addition of ether (2 ml) and ethanol (10 ml), cooling, and scratching with a glass rod. The crystals were filtered off, washed with cold ethanol, and the product (1·240 g, 84%) was recrystallized from hot ethanol. It was dried *in vacuo* ($CaCl_2$), and the dry *material* (943 mg, 76%) had m.p. 61—62° (Found: C, 57·9; H, 4·2; N, 3·9; S, 8·4. $C_{18}H_{17}NO_6S$ requires C, 57·6; H, 4·5; N, 3·7; S, 8·5%).

Attempted Preparation of p-*Nitrophenyl* L-2-*Acetoxy*-3-*benzylthiopropanoate*.— L-2-Acetoxy-3-benzylthiopropanoic acid (1·0 g) and *p*-nitrophenol (657 mg) were reacted with *NN'*-dicyclohexylcarbodi-imide (811·3 mg). The resulting oil was crystallized by addition of water (1 ml) and ethanol (7 ml), cooling and scratching with a glass rod. The solid (382 mg, 26%) was recrystallized from hot ethanol and dried *in vacuo* ($CaCl_2$). The product (310 mg, 81%) had m.p. 61—62°, $[\alpha]_D^{20}$ 0° (*c* 1 in dimethylformamide). This method yields only racemic crystalline material (Found: C, 57·5; H, 4·7; N, 3·8; S, 8·4%).

p-*Nitrophenyl* L-*Tyrosinate Hydrobromide*.—*p*-Nitrophenyl *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosinate [17] (10·0 g) was suspended in 2N-hydrogen bromide in glacial acetic acid (200 ml) and the mixture was maintained at room temperature for 1 h. The solution was poured into vigorously stirred dry ether (600 ml) and after 1 h the hydrobromide salt was washed with ether (3 × 150 ml) by decantation. The solid was briefly dried *in vacuo* ($CaCl_2$) and solid NaOH, dissolved in the minimum amount of hot ethanol, reprecipitated with ether, and dried *in vacuo* (P_2O_5). The product (6·1 g, 84%) had m.p. 199—201°, $[\alpha]_D^{22}$ +15·0° ± 0·4 (*c* 1 in water) (Found: C, 46·9; H, 4·1; Br, 20·7; N, 7·2. $C_{15}H_{15}BrN_2O_5$ requires C, 47·0; H, 4·0; Br, 20·8; N, 7·3%).

p-*Nitrophenyl* L-2-*Acetoxy*-3-*benzylthiopropanoyl*-L-*tyrosinate*.—*p*-Nitrophenyl L-tyrosinate hydrobromide (2·205 g) and L-2-acetoxy-3-benzylthiopropanoic acid monohydrate (1·269 g) were suspended in acetonitrile (40 ml). The mixture was cooled to −20° and triethylamine (0·7 ml) was added, followed by *NN'*-dicyclohexylcarbodi-imide (961 mg). After 1 h at −20°, it was left at room temperature for 5 h. The urea was filtered off, washed with acetonitrile (6 ml), and the combined washings and filtrate were evaporated under reduced pressure. The oily residue was dissolved in hot acetone (6 ml) and left at room temperature where soon a semi-solid mass was formed. Ethyl acetate–ether (1 : 1; 10 ml) was added, producing a fine separation of solid. This was filtered off, washed with ether, and dried *in vacuo* ($CaCl_2$). The compound (2·51 g, 81%) was recrystallized from the minimum of hot methanol and dried *in vacuo* (P_2O_5) at 40°. The product (1·76 g, 70%) had m.p. 137—139°, $[\alpha]_D^{18}$ −34·3° ± 0·3 (*c* 1 in dimethylformamide), R_F 0·85 (t.l.c.) (Found: C, 60·1; H, 4·7; N, 5·1; S, 6·1. $C_{27}H_{26}N_2O_8S$ requires C, 60·2; H, 4·9; N, 5·2; S, 5·9%).

L-2-*Acetoxy*-3-*benzylthiopropanoyl*-L-*tyrosinyl*-L-*isoleucyl*-L-*glutaminyl*-L-*asparaginyl*-S-*benzyl*-L-*cysteinyl*-L-*prolyl*-L-*leucylglycinamide*.—L-Isoleucyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide [18] (432 mg) was dissolved in dimethylformamide (6·5 ml) and *p*-nitrophenyl L-2-acetoxy-3-benzylthiopropanoyl-L-tyrosinate (310 mg) was added (10% excess). The solution was left at room temperature for 4 days. Ethyl acetate (20 ml) was added to the mixture and the precipitate was filtered off. It was washed with ethyl acetate (5 ml), ethyl acetate–ether (1 : 1; 4 ml), ethanol–ether (1 : 1; 2 ml), and dried *in vacuo* (P_2O_5) at 40°. The *peptide* (604 mg, 94·5%) had m.p. 231·5—233°, $[\alpha]_D^{23}$ −42·5° ± 0·4 (*c* 1 in dimethylformamide) (Found: C, 57·3; H, 6·8; N, 12·3; S, 5·0. $C_{59}H_{81}N_{11}O_{14}S_2$ requires C, 57·5; H, 6·6; N, 12·5; S, 5·2%). Amino-acid analysis of a hydrolysate gave the following molar ratios (with the value for isoleucine taken as 1): *S*-benzylcysteine, 1·1; ammonia, 2·9; aspartic acid, 1·1; glutamic acid, 1·1; proline, 0·9; glycine, 1·0; isoleucine, 1·0; leucine, 1·1; tyrosine, 1·0.

L-3-*Benzylthio*-2-*hydroxypropanoyl*-L-*tyrosinyl*-L-*isoleucyl*-L-*glutaminyl*-L-*asparaginyl*-S-*benzyl*-L-*cysteinyl*-L-*prolyl*-L-*leucylglycinamide*.— L-2-Acetoxy-3-benzylthiopropanoyl-L-tyrosinyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (494 mg) was dissolved in anhydrous liquid ammonia (300 ml) and methanol (40 ml) was added with stirring. The ammonia was allowed to evaporate spontaneously overnight. The residual solution was evaporated to dryness under reduced pressure at 50° and the solid residue was taken up in absolute ethanol (10 ml). The insoluble material was filtered off, washed

[28] E. Wünsch, G. Fries, and A. Zwick, *Chem. Ber.*, 1958, **91**, 542.

with ethanol (3 × 4 ml), and dried *in vacuo* (P_2O_5) at 40°. The *peptide* (320 mg, 67%) had m.p. 228—228·5°, $[\alpha]_D^{19·5}$ −38·5° ± 0·9 (*c* 1 in dimethylformamide) (Found: C, 57·3; H, 6·7; N, 12·8; S, 5·3. $C_{57}H_{79}N_{11}O_{13}S_2$ requires C, 57·5; H, 6·7; N, 12·9; S, 5·4%). Amino-acid analysis showed the following molar ratios (with the value for isoleucine taken as 1): *S*-benzylcysteine, 1·1; ammonia, 2·9; aspartic acid, 1·1; glutamic acid, 1·1; proline, 1·0; glycine, 1·0; isoleucine, 1·0; leucine, 1·0; tyrosine, 1·0.

[1-(L-2-*Hydroxy*-3-*mercaptopropanoic acid*)]*oxytocin*.—A solution of L-3-benzylthio-2-hydroxypropanoyl-L-tyrosinyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl L-prolyl-L-leucylglycinamide (257 mg) in boiling liquid ammonia (500 ml) was reduced with sodium by the stick method until a blue colour persisted for 10 s. Glacial acetic acid (1 ml) was added and the ammonia was removed at 35° by passage of a rapid stream of dry nitrogen through the flask. The white, solid residue was dissolved in water and the volume was brought up to 1 l. A clear solution, pH 6·8, was obtained, and bioassay of this solution for oxytocic activity (rat uterus) showed a total of 113,750 i.u. The dithiol was oxidized by dropwise addition of 0·02M-potassium ferricyanide solution and vigorous stirring; in all 14·3 ml were added to produce a persistent yellow colour. The nitroprusside test ensured complete absence of thiol. With slightly reduced pressure the solution was passed through a column (2 × 4 cm) of Dowex 2 × 8 (200—400 mesh) in the chloride form, and the column was washed several times with water. Assay of the combined eluates (1·082 l) for oxytocic activity showed a total of 147·290 i.u. The solution was concentrated in a flash evaporator at 30° to a volume of *ca.* 40 ml. A slight amount of a sticky precipitate was formed at this stage, but it was not removed. This concentrate was diluted to 50 ml with lower phase of the system n-butanol–toluene–acetic acid (0·05%) [2] and divided between the first five tubes of a 200-tube countercurrent train. The distribution was carried through a total of 600 transfers at room temperature. After 200 transfers a partial separation into three main peaks with *K*-values of 0·012 (medium), 0·33 (large), 0·42 (medium), and a number of small peaks with *K*-values of 1·0; 1·22; 1·86; 2·33; 3·44; and 5·0 had been accomplished as was detected by the Folin–Lowry colour reaction.[29] Determination of the oxytocic activity in the lower phase of every fifth tube showed that the material in the peak with $K = 0·33$ contained all the biological activity. After 400 transfers the Folin–Lowry tests defined five bands travelling with *K*-values of 0·012 (medium), 0·22 (small), 0·33 (large), 0·47 (small), and 0·66 (small). The most satisfying separation had been effected after 600 transfers, showing a medium-sized peak ($K = 0·012$) and one main peak ($K = 0·33$), accompanied by a small peak on both sides ($K = 0·22$ and 0·47). The curves of the Folin–Lowry colour values and oxytocic activity were in excellent agreement with the theoretical curve. The contents of tubes 130—170 containing the [1-(L-2-hydroxy-3-mercaptopropanoic acid)]oxytocin were pooled, evaporated in a flash evaporator at 30° to a volume of *ca.* 25 ml, and the concentrate was diluted with water to 50 ml. The solution was filtered to remove mechanical impurities and the clear liquid was lyophilized. The white, non-crystalline *material* (86·7 mg) had $[\alpha]_D^{18}$ −91·5° ± 2·5 (*c* 0·05 in N-acetic acid), R_F 0·70 (PC). The moisture content of the lyophilized powder was found to be 6·2% (P_2O_5; 100° at 2 mmHg) (Found: C, 51·0; H, 6·7; N, 15·0; S, 6·2. $C_{43}H_{65}N_{11}O_{13}S_2$ requires C, 51·2; H, 6·5; N, 15·3; S, 6·4%). Amino-acid analysis of a hydrolysate gave the following molar ratios (with the value for tyrosine taken as 1): ammonia, 2·8; aspartic acid, 1·1; glutamic acid, 1·0; proline, 1·0; glycine, 1·0; isoleucine, 1·0; leucine, 1·1; tyrosine, 1·0. In addition, cystine (0·3) and the mixed disulphide of L-cysteine and L-2-hydroxy-3-mercaptopropanoic acid [30] (0·5) were present. These two sulphur compounds fully account for the half-cystine residue in the hormone analogue. [1-(L-2-Hydroxy-3-mercaptopropanoic acid)]oxytocin gave an oxytocic activity of 1607 ± 20 ($n = 7$) i.u. mg^{-1}.

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