Cyanoethylation of α-Methylpyrrole. α-Methylpyrrole (2 g) was cyanoethylated as described above for the β-methyl isomer. The product was distilled at 84° (0.25 mm) to give 2.15 g of the expected nitrile **8B**; infrared spectrum: 4.45 μ (neat).

Anal. Calcd for $C_8H_{10}N_2$: C, 71.61; H, 7.51; N, 20.80. Found: C, 71.46; H, 7.66; N, 20.90.

2,3-Dihydro-5-methyl-1H-pyrrolizidin-1-one (8B). The cyclizations of 1 g of the above nitrile was carried out as described previously for the preparation of **5**. Upon sublimation of the crude product only a liquid film was obtained on the cold finger. The infrared spectrum showed absorption at 5.92 and 4.45 μ, which showed incomplete cyclization. Glpc analysis on a 10% Carbowax column (225°) showed the new ketone (**8B**) at 3 min, as compared with **5** at 6 min. The cyclization was not further studied since the ketone thus obtained was clearly different from the natural product.

Simultaneous Synthesis of 1-Hemi-D-cystine-oxytocin and Oxytocin and Separation of the Diastereoisomers by Partition Chromatography on Sephadex and by Countercurrent Distribution[1]

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Abstract: Oxytocin and 1-hemi-D-cystine-oxytocin have been synthesized simultaneously and separated by partition chromatography on Sephadex and by countercurrent distribution. *p*-Nitrophenyl N-carbobenzoxy-S-benzyl-DL-cysteinate was coupled with L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, and the resulting mixture of diastereoisomeric protected polypeptide amides was treated with sodium in liquid ammonia and then oxidized to yield a mixture of oxytocin and 1-hemi-D-cystine-oxytocin. The latter diastereoisomers were effectively separtated by partition chromatography on Sephadex G-25 in the solvent system 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9) and by countercurrent distribution in the same solvent system. Both procedures yielded oxytocin with a potency comparable to that previously reported for the highly purified hormone. The 1-hemi-D-cystine-oxytocin obtained by either method possessed an oxytocic potency of about 2 units/mg, which remained constant after submission of the compound to further chromatography.

In previous syntheses of 1-hemi-D-cystine-oxytocin,[2,3] S-benzyl-D-cysteine has been used as the starting material for the introduction of the hemi-D-cystine residue in the 1 position of oxytocin (Figure 1). In starting with S-benzyl-D-cysteine, if any traces of the L isomer are present or if any slight racemization should take place in the subsequent synthetic steps, oxytocin itself becomes a contaminant.

In preliminary experiments it was found that oxtocin could be cleanly separated from 1-hemi-D-cystine-oxytocin by partition chromatography on Sephadex in the solvent system 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9). It therefore occurred to us that by starting with S-benzyl-DL-cysteine one could prepare simultaneously both diastereoisomers and then separate one from the other. Thus one would obtain highly purified preparations of both oxytocin and 1-hemi-D-cystine-oxytocin. This has been accomplished and is being presented in this communication. This isolation of highly purified 1-hemi-D-cystine-oxytocin has also afforded a further examination of the pharmacological activities of this analog.

Previous efforts to separate oxytocin from 1-hemi-D-cystine-oxytocin by countercurrent distribution in the solvent system 1-butanol–1-propanol–0.05% acetic acid (3:2:5) at 4° were unsatisfactory.[3] The question arose as to whether this lack of separation was due to the nature of the solvent system that had been employed. We therefore thought it would be of interest to see whether these two diasteroisomers could be separated by countercurrent distribution in the solvent system that had been used on Sephadex. Such a separation did occur upon countercurrent distribution in the 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9) solvent system.

For synthesis the stepwise *p*-nitrophenyl ester method[4] was used, the last step being the coupling of *p*-nitrophenyl S-benzyl-N-carbobenzoxy-DL-cysteinate with the octapeptide L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. The resulting mixture of diastereoisomeric protected nonapeptides was treated with sodium in liquid ammonia for removal of the benzyl and carbobenzoxy groups according to the method of Sifferd and du Vigneaud.[5] The resulting disulfhydryl nonapeptides were oxidized to the cyclic octapeptides by the method of Weygand and Zumach[6] with the use of 1,2-diiodoethane in aqueous acetone, the disappearance of sulfhydryl groups being followed quantitatively by the method of Ellman.[7] It has recently been found in

(1) This work was supported in part by Grant HE-01675 from the National Heart Institute, U. S. Public Health Service. Dr. Gillessen wishes to acknowledge a Fulbright Travel Grant.

(2) K. Jošt, J. Rudinger, and F. Šorm, *Collection Czech. Chem. Commun.*, **26**, 2496 (1961); **28**, 2021 (1963).

(3) D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *J. Am. Chem. Soc.*, **85**, 3686 (1963).

(4) M. Bodanszky and V. du Vigneaud, *Nature*, **183**, 1324 (1959); *J. Am. Chem. Soc.*, **81**, 5688 (1959).

(5) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).

(6) F. Weygand and G. Zumach, *Z. Naturforsch.*, **17b**, 807 (1962).

(7) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).

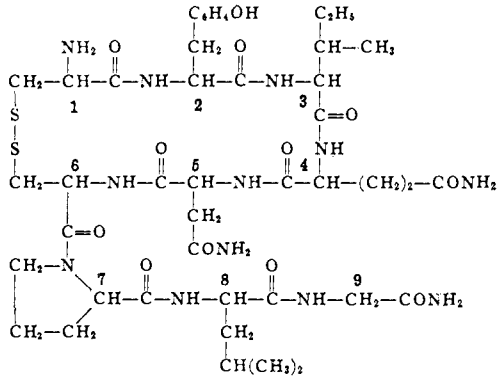

Figure 1. Structure of oxytocin with numbers indicating the position of the individual amino acid residues.

this laboratory[8] that oxytocin is inactivated by acetone with the formation of a new compound which was referred to as "acetone-oxytocin." Although some of this derivative is obtained when the procedure of Weygand and Zumach is used, the extent of reaction can be minimized by performing the operations as quickly as possible. Under these conditions the yields of 1-hemi-D-cystine-oxytocin and oxytocin obtained in the present work were comparable to those obtained by other methods of oxidation.

When the crude mixture of 1-hemi-D-cystine-oxytocin and oxytocin was subjected to partition chromatography on Sephadex[9] in the solvent system 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9) at 25°, the pattern shown in Figure 2 was obtained. The separation of the diastereoisomers was sharp, and the calculated values of R_f were 0.37 (1-hemi-D-cystine-oxytocin) and 0.24 (oxytocin).

When a mixture of equal parts of the purified diastereoisomers was subjected to countercurrent distribution[10] at 25° in the same solvent system as used in the partition chromatography, the 1-hemi-D-cystine-oxytocin (K = 1.12) and oxytocin (K = 0.63) were cleanly separated, as shown in Figure 3. The behavior of this pair of diastereoisomers on countercurrent distribution was similar to their behavior on Sephadex, the 1-hemi-D-cystine-oxytocin traveling faster in both cases.

The very low biological activity of 1-hemi-D-cystine-oxytocin and the very high activity of oxytocin offered a sensitive criterion of measuring the efficiencies of the separation processes. The activities followed were the avian vasodepressor activity[11] in the case of oxytocin and the oxytocic activity on the isolated rat uterus[12] in the case of 1-hemi-D-cystine-oxytocin. In regard to the latter compound, the oxytocic assay was preferred because of the erratic behavior of 1-hemi-D-cystine-oxytocin in the avian vasodepressor assay, protracted responses and/or inhibitory effects being observed. The

(8) D. Yamashiro, H. L. Aanning, and V. du Vigneaud, *Proc. Natl. Acad. Sci. U. S.*, **54**, 166 (1965).
(9) D. Yamashiro, *Nature*, **201**, 76 (1964).
(10) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).
(11) R. A. Munsick, W. H. Sawyer, and H. B. van Dyke, *Endocrinology*, **66**, 860 (1960).
(12) The assays for oxytocic activity were performed on isolated rat 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.

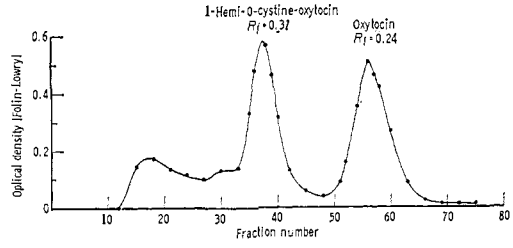

Figure 2. Partition chromatography of a crude mixture of 1-hemi-D-cystine-oxytocin and oxytocin on Sephadex G-25 in 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9).

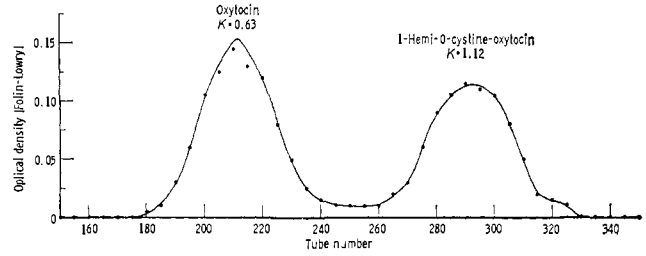

Figure 3. Countercurrent distribution of a mixture of purified 1-hemi-D-cystine-oxytocin (50 mg) and oxytocin (51 mg) after 550 transfers in 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9).

separation effected by countercurrent distribution as shown in Figure 3 gave 1-hemi-D-cystine-oxytocin with a potency of about 2 units/mg. From the chromatographic purification shown in Figure 2 the product isolated from the 1-hemi-D-cystine-oxytocin peak also possessed an oxytocic potency of about 2 units/mg. Both separation techniques gave oxytocin with an avian vasodepressor potency close to 500 units/mg, comparable to the value of 507 units/mg reported from this laboratory for a highly purified sample of synthetic oxytocin.[13]

The 1-hemi-D-cystine-oxytocin isolated by chromatography was subjected again to chromatography in the same solvent system and then assayed to see whether the oxytocic activity remained constant. No change in oxytocic potency was detected. The sample so obtained was then subjected to chromatography in another solvent system, 1-butanol–3.5% acetic acid in 1.5% aqueous pyridine (1:1), and again no change in oxytocic potency was observed. It would appear reasonable that these steps would have removed traces of oxytocin if they had been present. This highly purified 1-hemi-D-cystine-oxytocin was tested for some of the pharmacological properties associated with oxytocin. These results are tabulated in Table I along with the values previously established for the parent hormone.[13] The fact that the ratios of units per milligram of one activity to another for 1-hemi-D-cystine-oxytocin differ from the corresponding ratios for oxytocin provides a further indication that this diastereoisomer of oxytocin possesses inherent pharmacological activity, a conclusion that was reached by Rudinger and his co-workers[2] from their studies on this analog.

(13) W. Y. Chan and V. du Vigneaud, *ibid.*, **71**, 977 (1962).

Table I. Biological Activities of 1-Hemi-D-cystine-oxytocin and Oxytocin[a]

Compd	Oxytocic (rat)	Vasodepressor (fowl)	Antidiuretic (rat)	Milk-ejecting (rabbit)	Pressor (rat)
Oxytocin	486 ± 5	507 ± 23	2.7 ± 0.2	410 ± 16	3.1 ± 0.1
1-Hemi-D-cystine-oxytocin[b]	1.9 ± 0.1	~0.2	~0.02	6.2 ± 0.2	Nil

[a] In units per milligram. [b] The values for 1-hemi-D-cystine-oxytocin were obtained by the following methods: oxytocic, see ref 12; vasodepressor, see ref 11; antidiuretic, by 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); milk ejecting, by the method of B. A. Cross and G. W. Harris, *J. Endocrinol.*, **8**, 148 (1952), as modified by H. B. van Dyke, K. Adamsons, Jr., and S. L. Engel, *Recent Progr. Hormone Res.*, **11**, 1 (1955), and by W. Y. Chan, *J. Pharmacol.*, **147**, 48 (1965); pressor, as described in "The Pharmacopeia of the United States of America," 16th Revision, Mack Publishing Co., Easton, Pa., 1960, p 793.

Experimental Section[14]

Chromatographic Procedures. A. Preparation of Gel. Sephadex G-25 (medium grade block polymerizate) was used exclusively.[15] The material remaining on a No. 200 U. S. Standard Sieve was washed in the following manner. An 80-g portion was stirred with 750 ml of 0.5 *N* sodium hydroxide *in vacuo* at the water pump for 30 min. After being stirred for an additional 3 hr, the slurry was transferred to a 2-l. graduate cylinder and allowed to settle. The supernatant was decanted. Washing was performed by decantation, using successively, 1.5 l. of 0.5 *N* sodium hydroxide and five or more 1.5-l. portions of water until the pH was 8 or less. The gel was washed in the same manner in the cold (2°) with two 1.5-l. portions of 0.5 *N* hydrochloric acid followed by five or more 1.5-l. portions of water until the pH was no lower than 4. The gel was stored in approximately 0.2 *N* acetic acid by the addition of glacial acetic acid to the mixture. Equal volumes of settled sediment and supernatant gave a convenient slurry for the subsequent preparation of columns. This slurry was deaerated at the water pump with stirring for 10 min on the day that it was to be used.

B. Solvents. Reagent grade 1-butanol was further purified according to the procedure of Crocker[16] by distillation through a 2.5 × 90 cm glass helices packed column from a mixture of 4 l. of 1-butanol and 25 ml of 2-aminoethanol. All other solvents used in this work were redistilled. Components of all solvent systems were separately deaerated for 5 to 10 min each at the water pump at room temperature before their combination in order to reduce the possibility of the formation of bubbles in the columns.

C. Preparation of Columns. Columns were constructed of glass and Teflon, and lengths of 60 to 120 cm with diameters ranging from 1.0 to 4.7 cm have been used. Each column was packed in sections of about 30 cm in length in 0.2 *N* acetic acid. The slurry for each section was poured on a 30-cm height of solvent and allowed to settle under gravity. After each section had settled to constant height the column was further settled under a flow rate of about 30–50 ml/hr cm^{-2} until an equilibrium height was reached where additional shrinkage only resulted in expansion of the bed after the flow was stopped. The newly packed column was thoroughly washed with acetic acid–water (1:4) at a flow rate of about 15 ml/hr cm^{-2} until four bed volumes had been passed. This was followed by 1.3 bed volumes of 0.2 *N* acetic acid at the same flow rate.

D. Operation of Columns. Columns were operated at 25° in a cycle of five stages in the manner described previously.[9] These stages were: A, equilibration with the aqueous phase of the solvent system; B, equilibration with the organic phase of the solvent system; C, chromatography of the sample; D, regeneration of the spent column with either pyridine–0.2 *N* acetic acid or acetic acid–water; E, equilibration with 0.2 *N* acetic acid. The sample could also be applied to the column in the aqueous phase immediately preceding stage B. A column could be used for either partition chromatography or gel filtration after stage E. This last stage could often be eliminated if the column was used solely for partition chromatography. The hold-up volume was calculated by measuring the displacement of aqueous phase from the column during stage B and subtracting the shrinkage of the column during this stage. This volume with most solvent systems was about one-fourth of the bed volume.

Solvent systems were selected after preliminary experiments similar to those described for countercurrent distribution.[17] For optimum purification solvent systems were chosen in which the compound to be purified was eluted with an R_f in the range 0.5–0.15, where $R_f = V_H/V_E$, V_H is the hold-up volume and V_E is the elution volume of the peak. These limits of R_f corresponded to distribution constants of approximately 1.8 and 0.3, respectively, provided that no strong exclusion or adsorption effects occurred.[18,19]

The washing solvent used in stage D was chosen so as to give little or no shrinkage during the regeneration process. This was minimized by avoiding the simultaneous presence of excessive amounts of pyridine and acetic acid in the column.

E. Detection and Isolation of Products. Fractions were collected during stage C ranging in size from $^1/_{20}$ to $^1/_{10}$ of the hold-up volume at a flow rate of about 3–5 ml/hr cm^{-2}. Peptide materials were detected by the Folin–Lowry procedure[20] on suitable aliquots. If emulsions were obtained in this colorimetric method, the sample aliquots were partially evaporated at the water pump for about 20 min. Products were isolated by mixture of the selected fractions with twice their volume of water, evaporation to a low volume, and lyophilization.

N-Carbobenzoxy-S-benzyl-DL-cysteine. S-Benzyl-DL-cysteine[21] (25.0 g) was dissolved in 330 ml of ice-cold 2 *N* sodium hydroxide. Carbobenzoxy chloride (24 g) was added to the stirred solution over a period of 15 min at 0°. The cooling bath was removed and the milky suspension was stirred for 15 min. Water (410 ml) was added followed by an additional 15 min of stirring. The resulting clear solution was washed with three 250-ml portions of ether and then made acid to Congo Red with 5 *N* hydrochloric acid. The product was extracted with three 300-ml portions of ethyl acetate. The combined ethyl acetate extracts were washed with two 150-ml portions of saturated sodium chloride and dried over magnesium sulfate. After removal of the drying agent the solvent was removed *in vacuo*, and the oil was taken up in 100 ml of benzene from which the product separated as a solid. The material was filtered off, and dilution of the filtrate with 150 ml of petroleum ether (bp 30–60°) afforded a second crop. The total crude yield was 33.2 g, mp 75–78°. Three recrystallizations from benzene–petroleum ether gave 25.7 g, mp 76–78°.

Anal. Calcd for $C_{18}H_{19}NO_4S$: C, 62.6; H, 5.54. Found: C, 62.8; H, 5.57.

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

(15) When a batch (Lot No. To4119) of the newer bead-type Sephadex G-25 was used under the conditions established for the block polymerizate, less favorable results were obtained. These results were improved by isolating a fraction with a narrow particle size range (20–44 μ, dry state) and performing the chromatography of the sample (stage C) at a relatively slow flow rate (2 ml/hr cm^{-2}).

(16) H. P. Crocker, British Patent, 919,178 (1963); *Chem. Abstr.*, **59**, 1487c (1963).

(17) T. P. King and L. C. Craig, *Methods Biochem. Analy.*, 10, 201 (1962).

(18) From preliminary estimates of the distribution constant (K) an approximate R_f value was calculated from the formula $R_f = 1/[1 + (V_S/V_H)(1/K)]$ where V_S is the volume of stationary phase and V_H is the hold-up volume of the column; see H. B. F. Dixon, *J. Chromatog.*, 7, 467 (1962). From the R_f and K values obtained for oxytocin and 1-hemi-D-cystine-oxytocin in this communication the calculated value of the ratio V_S/V_H is in agreement with preliminary experiments which had indicated that this ratio for Sephadex G-25 (block polymerizate) was in the range 1.5–2.0.

(19) Oxytocin is eluted very slowly as an extremely broad unsymmetrical peak in the solvent system 1-butanol–1-propanol–0.05% acetic acid (2:1:3). This indicates that strong adsorption occurs in such systems where the ionic strength is very low.

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

(21) J. L. Wood and V. du Vigneaud, *ibid.*, **130**, 109 (1939).

p-**Nitrophenyl N-Carbobenzoxy-S-benzyl-DL-cysteinate.** N-Carbobenzoxy-S-benzyl-DL-cysteine (10.4 g) and *p*-nitrophenol (4.2 g) were dissolved in tetrahydrofuran (50 ml). The solution was cooled to −10° and stirred while dicyclohexylcarbodiimide (6.2 g) was added. Stirring was continued for 30 min at −5° and then for 10 hr at room temperature. The N,N′-dicyclohexylurea was filtered off and washed with tetrahydrofuran. The combined filtrate and washings were evaporated *in vacuo*, and the crystalline residue was recrystallized from a mixture of tetrahydrofuran–ethanol (1:5), giving 12.4 g, mp 110–111°.

Anal. Calcd for $C_{24}H_{22}N_2O_6S$: C, 61.8; H, 4.75; N, 6.01. Found: C, 61.7; H, 4.78; N, 6.04.

N-Carbobenzoxy-S-benzyl-DL-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. N-Carbobenzoxy-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide[4] (3.5 g) was dissolved in trifluoroethanol (117 g) saturated with hydrogen bromide, and the solution was stirred for 1 hr at room temperature. The solvent was removed *in vacuo*, and the oily residue was triturated with 200 ml of anhydrous ether until a powder was obtained. The solid was washed by decantation with two additional 200-ml portions of anhydrous ether. The dried powder was dissolved in 100 ml of liquid ammonia, and the solvent was removed *in vacuo*. The residue was stirred with 50 ml of water for 2 hr at 4°, collected on a filter, and washed with 50 ml of cold water to give 2.51 g. An aliquot of this free octapeptide (845 mg) was dissolved in 10 ml of dimethylformamide, and *p*-nitrophenyl N-carbobenzoxy-S-benzyl-DL-cysteinate (436 mg) was added. After 24 hr the gelatinous mixture was stirred with ethyl acetate (42 ml), collected on a filter, and washed with ethyl acetate (40 ml) and absolute ethanol (20 ml) to give 1.05 g, mp 241–244°, $[\alpha]^{20}D$ −40° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{65}H_{86}N_{12}O_{14}S_2$: C, 59.0; H, 6.55; N, 12.7. Found: C, 58.7; H, 6.62; N, 12.6.

1-Hemi-D-cystine-oxytocin and Oxytocin. The product (263 mg) described in the preceding section was dissolved in 60 ml of liquid ammonia (distilled from sodium) and treated at the boiling point with sodium until a blue color persisted throughout the solution for 1 min. The solution was evaporated *in vacuo* to about 15 ml and dried from the frozen state. The loose powder was dissolved in 100 ml of 50% aqueous acetone. The sulfhydryl content, as measured by the method of Ellman,[7] was 360 μmoles. A solution of 68 mg of 1,2-diiodoethane in 10 ml of acetone was added dropwise over a period of 1 min with stirring. After being stirred for 3 additional min the solution had a sulfhydryl content of 3 μmoles. The pH of the solution was adjusted to 8 with trifluoroacetic acid and 0.1 *N* sodium hydroxide. Water (50 ml) was added, followed by glacial acetic acid (0.1 ml) to bring the pH to 4.7. The acetone was removed *in vacuo*, and the aqueous solution was washed with 25 ml of ethyl acetate. The solution was frozen and lyophilized to give 329 mg of a coarse white solid.

The crude product was dissolved in 5 ml of the upper phase of the solvent system 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9) and applied to a Sephadex G-25 column (2.15 × 117 cm) which had been equilibrated with both phases. The chromatogram was developed with the organic phase at a flow rate of about 30 ml/hr. After 75 fractions of 7.9 ml each were collected, two main peaks (R_f values of 0.37 and 0.24) were detected by the Folin–Lowry method as shown in Figure 2. Isolation of the material in fractions 33–42 gave 45.6 mg of 1-hemi-D-cystine-oxytocin with a potency on the isolated rat uterus close to 2 units/mg. In a similar manner fractions 52–62 gave 50 mg of oxytocin having a potency of about 490 units/mg in the avian vasodepressor assay. The column was regenerated with pyridine–0.2 *N* acetic acid (3:5).

Subjection of 1-Hemi-D-cystine-oxytocin to Further Partition Chromatography. A sample of 1-hemi-D-cystine-oxytocin (43.9 mg having 2 units/mg of oxytocic activity) was chromatographed on Sephadex G-25 in the solvent system 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9) under the following conditions: column size, 2.17 × 118 cm; hold-up volume, 123 ml; fraction volume, 7.5 ml; flow rate, 27–31 ml/hr; regenerating solvent, pyridine–0.2 *N* acetic acid (3:5). Only one peak was detected, with an R_f of 0.44. Isolation of the material represented by this peak gave 37.3 mg, with an oxytocic potency close to 2 units/mg. A portion of this product (36.2 mg) was rechromatographed in the solvent system 1-butanol–3.5% acetic acid in 1.5% aqeous pyridine (1:1) under the following conditions: column size, 2.17 × 117 cm; hold-up volume, 110 ml; fraction volume, 4.8 ml; flow rate, 16–21 ml/hr; regenerating solvent, acetic acid–water (1:5). A single peak was detected with an R_f of 0.35 (under these conditions oxytocin appears at an R_f of about 0.24). Recovery of highly purified 1-hemi-D-cystine-oxytocin was 32.2 mg, with an oxytocic potency of 2 units/mg, $[\alpha]^{20}D$ −56° (*c* 0.5, 1 *N* acetic acid). For analysis a sample was dried at 100° over phosphorus pentoxide *in vacuo* with a loss in weight of 7.6%.

Anal. Calcd for $C_{43}H_{66}N_{12}O_{12}S_2$: C, 51.3; H, 6.60; N, 16.7. Found: C, 51.7; H, 6.52; N, 16.5.

A sample was hydrolyzed in 6 *N* hydrochloric acid at 110° for 22 hr and analyzed by the method of Spackman, Stein, and Moore[22] in the 30–50° system on a Beckman–Spinco amino acid analyzer. The following molar ratios of amino acids and ammonia were obtained, with the value of glycine taken as 1.0: aspartic acid 1.0, glutamic acid 1.0, proline 1.0, glycine 1.0, half-cystine 1.0, half-mesocystine 1.0, leucine 1.0, isoleucine 1.0, tyrosine 0.9, and ammonia 3.1.

Separation of 1-Hemi-D-cystine-oxytocin and Oxytocin by Countercurrent Distribution. A mixture of 1-hemi-D-cystine-oxytocin (50 mg) and oxytocin (51 mg) was placed in the first five tubes of a 200-tube Craig countercurrent machine in the solvent system 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9). After 550 transfers (with a recycling adjustment) Folin–Lowry analysis on selected tubes gave the pattern shown in Figure 3. Isolation of material represented by the peak with a *K* value of 1.12 gave 50 mg of 1-hemi-D-cystine-oxytocin with an oxytocic potency of about 2 units/mg, while the material represented by the second peak ($K = 0.63$) gave 43 mg of oxytocin with an avian vasodepressor potency of about 500 units/mg.

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