Table III. Quantities Used and Yields Obtained for Polymers of L-*p*-(Phenylazo)phenylalanine and γ-Benzyl-L-glutamate

Mole % azo content[a]	γ-Benzyl-L-glutamate N-carboxy Anhydride mg	mmole	L-*p*-(Phenylazo)-phenylalanine N-carboxy Anhydride mg	mmole	*A*/*I*[b]	Yield mg	Yield %
14.0	229	0.871	42.0	0.142	101	186	82
25.6	364	1.39	140	0.476	101	328	77
33.1	246	0.936	137	0.463	93.3	202	63
37.9	234	0.888	160	0.542	101	122	37
49.7	237	0.900	262	0.886	99.8	375	90
57.9	168	0.641	259	0.878	97.8	217	60
79.9	53.3	0.202	238	0.806	101	131	53

[a] Determined spectroscopically (see Results and Discussion). [b] Molar ratio of total anhydride to sodium methoxide initiator.

Table IV. Elemental Analysis of Polymers of L-*p*-(Phenylazo)phenylalanine and γ-Benzyl-L-glutamate

Mole % azo content	Calcd, % C	H	N	Found, % C	H	N	% residue of sample wt[a]
14.0	66.57	5.87	7.83	67.04	5.85	7.84	..
25.6	67.26	5.79	9.02	67.43	5.71	9.24	..
33.1	67.71	5.72	9.80	62.63	5.80	8.61	4.49
37.9	67.99	5.68	10.31	67.82	5.58	10.43	..
49.7	68.70	5.60	11.52	68.80	5.78	11.66	..
57.9	69.18	5.53	12.36	68.11	5.68	12.28	..
79.9	70.51	5.36	14.65	68.18	5.21	12.11	2.41

[a] Residues in the carbon and hydrogen analyses were reported for two of the copolymers.

spectrophotometer thermostated at 25.0 ± 0.5° and 1.00-cm fused silica cells. Infrared spectra were determined as Nujol mulls with Perkin-Elmer Models 21 or 137 spectrophotometers. Molecular weight determinations were carried out using a Beckman Model E analytical centrifuge. Optical rotatory dispersion data were obtained on a Cary Model 60 spectropolarimeter thermostated at 27.0 ± 0.5° with 0.100-mm (with TFA) and 1.00-cm (with dioxane) fused silica cells. Corrected residue rotations ([R']) were calculated from the equation

$$[R'] = 3MRW[\alpha]^{T}_{\lambda}/10^{2}(n^{2} + 2)$$

where MRW = mean residue weight of copolymer and n = refractive index of solvent (uncorrected for wavelength).

Acknowledgment. We wish to thank Mr. Martin Falxa of our laboratories for his helpful participation in these researches and for carrying out some of the important rotatory dispersion measurements contained in this paper.

The Effect of Replacing One of the Hydrogens of the β-Carbon of the β-Mercaptopropionic Acid Residue in Deamino-oxytocin by a Methyl Group on Its Oxytocic and Avian Vasodepressor Activity[1]

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Abstract: The effect of replacement of one of the hydrogens on the β-carbon of the β-mercaptopropionic acid residue in the 1 position of the highly potent deamino-oxytocin by a methyl group has been investigated. A diastereoisomeric mixture of 1-L-β-mercaptobutyric acid-oxytocin and 1-D-β-mercaptobutyric acid-oxytocin was first prepared with the use of DL-β-benzylmercaptobutyric acid as the starting material. This mixture of diastereoisomeric analogs possessed approximately 40 units per mg of oxytocic and avian vasodepressor activities, representing about $^{1}/_{20}$th of the corresponding activities of deamino-oxytocin. Attempts to separate these diastereoisomers by countercurrent distribution and by partition chromatography on Sephadex G-25 were not successful, and in order to ascertain whether one or both of the diastereoisomeric analogs possessed biological activity it was decided to synthesize one of these diastereoisomers. DL-β-Benzylmercaptobutyric acid was subjected to resolution with the use of quinine, and the optically active β-benzylmercaptobutyric acid obtained thereby was shown to possess the D configuration. 1-D-β-Mercaptobutyric acid-oxytocin was then prepared and found to possess approximately 35 units per mg of oxytocic activity and 55 units per mg of avian vasodepressor activity. From the biological activities of the diastereoisomeric mixture of 1-D- and 1-L-β-mercaptobutyric acid-oxytocin and those of 1-D-β-mercaptobutyric acid-oxytocin, it is evident that replacement of one of the hydrogens on the β-carbon of the β-mercaptopropionic acid residue in the 1 position of the highly potent deamino-oxytocin results in a considerable decrease in oxytocic and avian vasodepressor activity.

It was recently found in this laboratory that replacement of the hydrogens on the β-carbon of the β-mercaptopropionic acid residue in the 1 position of the highly potent deamino-oxytocin[2] (Figure 1) by two methyl groups, as in deaminopenicillamine-oxytocin (1-β-mercaptoisovaleric acid-oxytocin), causes the total loss of oxytocic and avian vasodepressor activity.[3] In fact 1-deaminopenicillamine-oxytocin proved to be a highly

(1) This work was supported in part by Grant HE-01675 from the National Heart Institute, U. S. Public Health Service.

(2) D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *J. Biol. Chem.*, **237**, 1563 (1962); B. M. Ferrier, D. Jarvis, and V. du Vigneaud, *ibid.*, **240**, 4264 (1965).

(3) H. Schulz and V. du Vigneaud, *J. Med. Chem.*, **9**, 647 (1966).

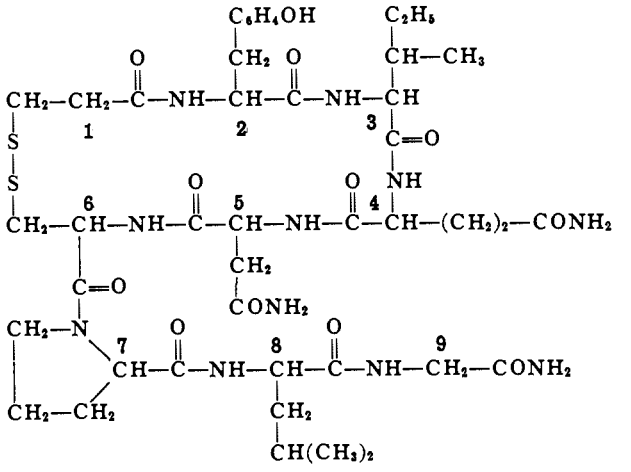

Figure 1. Structure of deamino-oxytocin with numbers indicating the positions of the component amino acid residues.

potent inhibitor of the oxytocic activity of oxytocin and possessed a slight inhibitory effect on the avian vasodepressor activity. This effect of the replacement of two hydrogens by methyl groups led us to investigate the effect of the replacement of only one of these hydrogens by a methyl group. We have therefore synthesized and investigated some of the pharmacological properties of 1-β-mercaptobutyric acid-oxytocin.

It was decided to synthesize the diastereoisomeric mixture of 1-L-β-mercaptobutyric acid-oxytocin and 1-D-β-mercaptobutyric acid-oxytocin starting with DL-β-benzylmercaptobutyric acid and to attempt to separate the diastereoisomers by countercurrent distribution and partition chromatography on Sephadex G-25. Separation of the diastereoisomers, oxytocin and 1-hemi-D-cystine-oxytocin,[4] has been accomplished in this laboratory by either countercurrent distribution or partition chromatography on Sephadex G-25, and 1-L- and 1-D-penicillamine-oxytocin[3] have also been separated from one another by partition chromatography on Sephadex G-25.

The DL-β-benzylmercaptobutyric acid was prepared by a nucleophilic addition of benzyl mercaptan to crotonic acid in the presence of piperidine as used by Süs for preparing β-benzylmercaptoisovaleric acid from 3,3-dimethylacrylic acid.[5] It was then converted to the nitrophenyl ester and allowed to react with the S-benzyl-octapeptide amide, L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (I), obtained from its N-carbobenzoxy-O-benzyl derivative by treatment with hydrogen bromide in trifluoroethanol and subsequent neutralization with a basic anion-exchange resin. The protecting groups were removed from the mixture of the two protected diastereoisomeric polypeptide derivatives by reduction with sodium in liquid ammonia and the resulting sulfhydryl compounds were oxidized to the cyclic disulfides in neutral aqueous solution with potassium ferricyanide.[2] After the removal of the ferricyanide and ferrocyanide ions the crude material was purified by countercurrent distribution in the solvent system 0.5% aqueous acetic acid (containing 0.1% pyridine)–1-butanol–benzene (5:3:2) and by partition chromatography on Sephadex G-25 in the solvent system 3.5% aqueous acetic acid (containing 1.5% pyridine)–1-butanol (1:1), and in the solvent system 3.5% aqueous acetic acid (containing 1.5% pyridine)–1-butanol–benzene (2:1:1). In each case no separation of the diastereoisomers was achieved. The mixture of the diastereoisomeric analogs isolated after countercurrent distribution and partition chromatography possessed an oxytocic activity[6] of 41 units per mg (SE ±4) and an avian vasodepressor activity[7] of 42 units per mg (SE ±3). No inhibitory effect on the oxytocic or avian vasodepressor activity of oxytocin was observed.

To ascertain whether one or both of the diastereoisomeric analogs possessed biological activity, it was decided to synthesize one of these diastereoisomers. DL-β-Benzylmercaptobutyric acid was therefore subjected to resolution with the use of quinine and an optically active β-benzylmercaptobutyric acid was obtained. To establish the configuration of this isomer it was debenzylated with sodium in liquid ammonia to β-mercaptobutyric acid, since Levene and Mikeska[8] had established the configuration of the optically active forms of β-mercaptobutyric acid. The optical isomer obtained from the S-benzyl derivative was the levorotatory one which they had found to be of the D configuration. The nitrophenyl ester of the D-β-benzylmercaptobutyric acid was allowed to react with the S-benzyl-octapeptide amide (I) to give D-β-benzylmercaptobutyryl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, which was then converted to 1-D-β-mercaptobutyric acid-oxytocin in the manner described for the mixture of the diastereoisomeric forms. The crude material was purified by countercurrent distribution in the solvent system 0.5% aqueous acetic acid (containing 0.1% pyridine)–1-butanol–benzene (2:1:1), and also by partition chromatography on Sephadex G-25 in the solvent systems 3.5% aqueous acetic acid (containing 1.5% pyridine)–1-butanol–benzene (2:1:1) and 3.5% aqueous acetic acid (containing 1.5% pyridine)–1-butanol–benzene (3:2:1). The purified 1-D-β-mercaptobutyric acid-oxytocin possessed an oxytocic activity of 36 units per mg (SE ±3) and an avian vasodepressor activity of 56 units per mg (SE ±3). Comparison of these activities with those we obtained for the diastereoisomeric mixture of 1-D- and 1-L-β-mercaptobutyric acid-oxytocin indicates that 1-L-β-mercaptobutyric acid-oxytocin is also pharmacologically active.

Thus it is evident that the replacement of one of the hydrogens on the β-carbon atom of the β-mercaptopropionic acid residue in the 1 position of the highly potent deamino-oxytocin (1-β-mercaptopropionic acid-oxytocin) with a methyl group, yielding β-mercaptobutyric acid-oxytocin (β-methyl-β-mercaptopropionic acid-oxytocin), results in a considerable diminution of biological activity. When both hydrogens in this position are replaced with methyl groups, as in 1-de-

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

(5) O. Süs, *Ann. Chem.*, **559**, 92 (1948).

(6) Oxytocic assays were performed according to the method of P. Holton, *Brit. J. Pharmacol.*, **3**, 328 (1948), on uteri from rats in natural estrus with the use of magnesium-free van Dyke–Hastings solution as employed by R. A. Munsick, *Endocrinology*, **66**, 451 (1960).

(7) Avian vasodepressor assays were performed on conscious chickens according to the procedure employed by R. A. Munsick, W. H. Sawyer, and H. B. van Dyke, *Endocrinology*, **66**, 860 (1960).

(8) P. A. Levene and L. A. Mikeska, *J. Biol. Chem.*, 70, 365 (1926).

aminopenicillamine-oxytocin (β-β-dimethyl-β-mercaptopropionic acid-oxytocin), a complete loss of oxytocic and avian vasodepressor activity results. It may also be noted that no inhibitory effect on the oxytocic activity of oxytocin is observed with the monomethyl analog, whereas the dimethyl analog, 1-deaminopenicillamine-oxytocin, is a potent inhibitor of this activity of oxytocin on the virgin rat uterus both *in vitro* and *in vivo*.[3] A study on the effect of other substitutions on the β-carbon of the β-mercaptopropionic acid residue of deamino-oxytocin is indeed warranted.

Of additional interest is the fact that the 1-β-mercaptobutyric acid-oxytocins possess a fair degree of avian vasodepressor and oxytocic activity while 1-γ-mercaptobutyric acid-oxytocin[9] possesses no detectable avian vasodepressor activity and only 3 units per mg of oxytocic activity. In the case of 1-γ-mercaptobutyric acid-oxytocin an additional methylene group is incorporated in the 20-membered ring of deamino-oxytocin making a 21-membered ring, while in the case of 1-β-mercaptobutyric acid-oxytocin a methyl group is attached to the 20-membered ring. This is another manifestation of the importance of ring size to biological activity.[9-11]

Experimental Section[12]

DL-β-Benzylmercaptobutyric Acid. Crotonic acid (19 g, containing 10% water), benzyl mercaptan (23.6 ml), and piperidine (40 ml) were refluxed for 13 hr. The reaction mixture was acidified with concentrated hydrochloric acid and extracted three times with ether. The combined ether extracts were subsequently treated with a concentrated solution of sodium bicarbonate until the evolution of carbon dioxide ceased. The combined aqueous phases were acidified with hydrochloric acid and extracted with ether. After the extracts were dried over sodium sulfate the solvent was removed by evaporation and the oily residue was distilled under reduced pressure; bp 192° (12 mm), 30.3 g. To remove traces of benzyl mercaptan the substance was dissolved in a concentrated solution of sodium bicarbonate, washed with ether, acidified, and extracted with ether. The solvent was removed by evaporation *in vacuo* at 100°.

Anal. Calcd for $C_{11}H_{14}O_2S$: C, 62.8; H, 6.71. Found: C, 62.8; H, 6.65.

***p*-Nitrophenyl DL-β-Benzylmercaptobutyrate.** DL-β-Benzylmercaptobutyric acid (2.3 g) and *p*-nitrophenol (1.8 g) were dissolved in ethyl acetate (10 ml) and dicyclohexylcarbodiimide (2.3 g) was added. After the solution was stirred overnight, 1 *N* hydrochloric acid (5 ml) was added and the N,N'-dicyclohexylurea was filtered off. The aqueous phase was separated, and the organic phase was dried over magnesium sulfate and evaporated. The oily residue was dissolved in chloroform and passed through a silica gel column. The solvent was removed from the filtrate by evaporation *in vacuo* at 80°, and the product was crystallized from methanol, 3.1 g, mp 24–26°.

Anal. Calcd for $C_{17}H_{17}O_4NS$: C, 61.6; H, 5.17. Found: C, 61.8; H, 5.26.

Diastereoisomeric Mixture of the D- and L-β-Benzylmercaptobutyryl Derivatives of 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[13] (1.2 g) was suspended in 20 ml of trifluoroethanol and hydrogen bromide was bubbled through the suspension for 0.5 hr. The clear solution was kept at room temperature for another half hour. Then the solvent was removed by evaporation at 40°. The residue was washed three times with ether, dissolved in methanol (50 ml), and passed through a column containing the exchange resin IRA-410 in the hydroxide form. The filtrate was evaporated to dryness *in vacuo* at 50°; 0.73 g.

The free S-benzyl-octapeptide amide (I) (0.23 g) was dissolved in dimethylformamide (3 ml), and *p*-nitrophenyl DL-β-benzylmercaptobutyrate (0.23 g) was added. After the solution was stirred for 4 days the reaction product was precipitated by addition of ethyl acetate (50 ml) and filtered off. The substance was successively treated with ethanol (40 ml), ethyl acetate (40 ml), and finally washed with ethyl acetate (20 ml), 0.22 g, mp 241–244°, $[\alpha]^{20}D$ −41.5° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{58}H_{81}O_{12}N_{11}S_2$: C, 58.6; H, 6.87; N, 13.0. Found: C, 58.6; H, 6.87; N, 12.7.

Diastereoisomeric Mixture of 1-D- and 1-L-β-Mercaptobutyric Acid-oxytocin. The diastereoisomeric mixture of the D- and L-β-benzylmercaptobutyryl derivatives of L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (0.18 g) was dissolved in anhydrous liquid ammonia (50 ml) and reduced with sodium until the blue color lasted for a few seconds. The ammonia was removed by evaporation and lyophilization. The resulting white residue was dissolved in deaerated water (180 ml), the pH was adjusted to 6.8–7.0, and the theoretical amount of 0.01 *N* potassium ferricyanide solution (33 ml) was added. The solution was deionized by passage through a column containing an ion-exchange resin in the chloride form [Fisher's Rexyn CG (8) Cl]. The deionized solution was concentrated to a volume of 60 ml, placed in the first six tubes of a countercurrent distribution machine, and subjected to 200 transfers in the solvent system 0.5% aqueous acetic acid (containing 0.1% pyridine)–1-butanol–benzene (5:3:2). The main peak as determined by Folin–Lowry[14] color values had a partition coefficient of 1.35. No separation of the two diastereoisomers was observed. From the tubes of the main peak 73 mg of material was obtained after concentration and lyophilization. This material was then subjected successively to partition chromatography on Sephadex G-25 in the solvent systems 3.5% aqueous acetic acid (containing 1.5% pyridine)–1-butanol (1:1) (R_f 0.73) and 3.5% aqueous acetic acid (containing 1.5% pyridine)–1-butanol–benzene (2:1:1) (R_f 0.28). In both procedures no separation of the two diastereoisomers was observed. After the last chromatographic procedure, 58 mg of 1-DL-β-mercaptobutyric acid-oxytocin was obtained; $[\alpha]^{20}D$ −85° (*c* 0.5, 1 *N* acetic acid).

A small amount of this compound was subjected to gel filtration on Sephadex G-25 in 0.2 *N* acetic acid. A single peak emerged at the position of oxytocin. On paper chromatography the compound showed only one spot. For analysis a sample was dried *in vacuo* at 100° over phosphorus pentoxide and a loss in weight of 5% was observed.

Anal. Calcd for $C_{44}H_{67}O_{12}N_{11}S_2$: C, 52.5; H, 6.71; N, 15.3. Found: C, 52.6; H, 6.83; N, 15.1.

Resolution of DL-β-Benzylmercaptobutyric Acid. DL-β-Benzylmercaptobutyric acid (10.5 g) was dissolved in acetone (50 ml), and quinine (16.4 g) was added with warming. The clear solution was mixed with hexane (80 ml) and kept at 4° until the crystallization was complete. The compound was recrystallized from ethyl acetate–hexane until the optical rotation remained constant at $[\alpha]^{20}D$ −111.8° (*c* 1, ethanol), mp 69.5–73.5°, 4.25 g.

The quinine salt (4 g) was treated with 20% sulfuric acid (50 ml) and the oil that separated was extracted three times with ether. The combined ether extracts were dried *in vacuo* over sodium sulfate, and evaporation of the solvent left a colorless oil, 1.5 g, $[\alpha]^{20}D$ +16.2° (*c* 1.4, ethanol).

Anal. Calcd for $C_{11}H_{14}O_2S$: C, 62.8; H, 6.71. Found: C, 63.0; H, 6.71.

Determination of the Configuration of the Optically Active β-Benzylmercaptobutyric Acid. The optically active β-benzylmercaptobutyric acid (0.3 g) was dissolved in anhydrous ammonia (50 ml) and sodium was added until the blue color remained for 1 min. The excess sodium was destroyed by means of ammonium chloride.

(9) D. Jarvis, B. M. Ferrier, and V. du Vigneaud, *J. Biol. Chem.*, **240**, 3553 (1965).

(10) D. Jarvis, M. Bodanszky, and V. du Vigneaud, *J. Am. Chem. Soc.*, **83**, 4780 (1961).

(11) D. Jarvis and V. du Vigneaud, *Science*, **143**, 545 (1964); M. Manning and V. du Vigneaud, *Biochemistry*, **4**, 1884 (1965).

(12) Capillary melting points were determined and are corrected. Paper chromatography was performed on Whatman No. 1 in 1-butanol–acetic acid–water (4:1:5, descending), and Pauly reagent was used for development.

(13) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

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

The ammonia was removed by evaporation and lyophilization. The white residue was dissolved in water (10 ml) and extracted twice with ether. The aqueous phase was acidified with 20% sulfuric acid and extracted five times with ether. The combined ether extracts were dried *in vacuo* over magnesium sulfate, filtered, and evaporated to an oily residue; $[\alpha]^{20}D$ −33° (*c* 2, 80% methanol–20% water) for the monosodium salt [lit.[8] $[\alpha]^{20}D$ −27.7° (*c* 8, water) for the monosodium salt of the D-β-mercaptobutyric acid].

***p*-Nitrophenyl D-β-Benzylmercaptobutyrate.** The compound was prepared as described for *p*-nitrophenyl DL-β-benzylmercaptobutyrate in approximately the same yield but with D-β-benzylmercaptobutyric acid serving as starting material, $[\alpha]^{20}D$ +19.7° (*c* 1.8, dimethylformamide).

Anal. Calcd for $C_{17}H_{17}O_4NS$: C, 61.6; H, 5.17. Found: C, 61.7; H, 5.25.

D-β-Benzylmercaptobutyryl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. The compound was prepared as described for the comparable preparation involving the use of DL-β-benzylmercaptobutyric acid and was obtained in approximately the same yield, mp 240–244°, $[\alpha]^{20}D$ −38.4° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{58}H_{81}O_{12}N_{11}S_2$: C, 58.6; H, 6.87; N, 13.0. Found: C, 58.5; H, 6.96; N, 12.8.

1-D-β-Mercaptobutyric Acid-oxytocin. D-β-Benzylmercaptobutyryl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (0.2 g) was reduced and oxidized as described for the preparation of 1-DL-β-mercaptobutyric acid-oxytocin. The deionized solution was concentrated to a volume of 50 ml, placed in the first five tubes of a countercurrent distribution machine, and subjected to a total of 400 transfers in the solvent system 0.5% aqueous acetic acid (containing 0.1% pyridine)–1-butanol–benzene (2:1:1). The main peak as shown by the Folin–Lowry color values had a partition coefficient of 0.6. From the tubes in the central part of the main peak 35 mg of 1-D-β-mercaptobutyric acid-oxytocin was obtained. In a second preparation (0.13 g) the reduced and oxidized material was subjected to partition chromatography on Sephadex G-25 in the solvent system 3.5% aqueous acetic acid (containing 1.5% pyridine)–1-butanol–benzene (2:1:1). The central part of the main peak was rechromatographed in the solvent system 3.5% aqueous acetic acid (containing 1.5% pyridine)–1-butanol–benzene (3:2:1). The substance emerged as a sharp, single peak with an R_f of 0.6. 1-D-β-Mercaptobutyric acid-oxytocin (37 mg) was obtained with an optical rotation of $[\alpha]^{20}D$ −96.5° (*c* 0.5, 1 *N* acetic acid). A small sample was subjected to gel filtration on Sephadex G-25 and emerged as a single peak at the position of oxytocin. On paper chromatography it behaved as a homogeneous compound. For analysis a sample was dried *in vacuo* at 100° over phosphorus pentoxide and a loss in weight of 4.8% was observed.

Anal. Calcd for $C_{44}H_{67}O_{12}N_{11}S_2$: C, 52.5; H, 6.71; N, 15.3. Found: C, 52.2; H, 6.61; N, 15.2.

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Reaction of Hydroxocobalamin with Thiols

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Abstract: Hydroxocobalamin reacts with thiol compounds, as exemplified by glutathione, to form relatively weak 1:1 inner coordination complexes. Previously reported inconsistencies in the generality of this reaction are explained in terms of the simultaneous role of thiol compounds as complexing and reducing agents.

The exact function of the cobalamins in biological systems is not as yet known. Prominent among the proposed mechanisms of their action are the moderation of enzymatic sulfhydryl disulfide oxidation–reduction systems by the protection of sulfhydryl groups[2a] and a role in methyl group transfer reactions.[3] Recently, Wagner and Bernhauer[4] found that glutathionocobalamin (CSG) upon alkylation gives coenzyme analogs with the alkyl group bound directly to cobalt. Dubnoff[5] has reported that the complexation with hydroxocobalamin is unique to glutathione: homocysteine, cysteine, and mercaptoethanol converted hydroxocobalamin to B_{12r}. CSG would thus appear to be of special importance as a potential link between both mechanisms. This study reexamines the reactivity of thiol compounds with hydroxocobalamin, with particular emphasis on glutathione.

Results

Addition of GSH to an aqueous solution of COH changes the color from red to violet virtually instantaneously. Subsequent addition of acetone yields a reddish violet precipitate. The average GSH/COH ratio for two independently prepared precipitates was found to be 0.97 by tracer and microbial assays for cobalamin and a ferrocyanide colorimetric assay for GSH. A ratio of unity has also been inferred from electrophoretic behavior[4] and from reaction with *p*-mercuribenzoate.[5]

A spectrophotometric investigation of the stoichiometry in solution (Figure 1) indicates the formation of only one complex. Reasonably sharp isosbestic points are noted at 542, 443, 364, 337, and 273 mμ. Extrapolation of both sections of plots of absorbance *vs.* molar ratio (0 to 10) at various wavelengths gave lines intersecting at a ratio of unity, again indicating a 1:1 interaction.

A chromatographic study of the reaction, summarized in Table I, also indicates that only one interaction species is formed. Both the conjugate acid–base forms

(1) Arthur D. Little, Inc., Cambridge, Mass. The authors wish to express their appreciation to Mr. E. P. Schulz and to Mrs. Cynthia Kaye for obtaining some of the data and to Merck and Co., Inc., for permission to publish this report.

(2) (a) E. Lester Smith, "Vitamin B_{12}," John Wiley and Sons, Inc., New York, N. Y., 1960: (a) Chapter 15; (b) p 2; (c) p 55.

(3) H. R. V. Arnstein, "The Biochemistry of Vitamin B_{12}," Biochemical Society Symposium No. 13, Cambridge University Press, Cambridge, England 1955, p 92.

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(5) J. W. Dubnoff, *Biochem. Biophys. Res. Commun.*, **16**, 484 (1964).