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.

Acknowledgments. We wish to thank Dr. W. Y. Chan for the pharmacological studies on the compounds reported herein. We also wish to thank Mr. Joseph Albert for the elemental microanalyses.

Reaction of Hydroxocobalamin with Thiols

Norman Adler,[1] Thomas Medwick, and T. J. Poznanski

Contribution from the Merck Chemical Division, Merck and Company, Inc., Rahway, New Jersey. *Received June 21, 1966*

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.

(4) F. Wagner and K. Bernhauer, *Ann. N. Y. Acad. Sci.*, 112, 580 (1964).

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

of COH [*i.e.*, $C(H_2O)^+ \rightleftharpoons C(OH)^0$], p$K$ = 7.1, are converted readily into this species. Conversely, oxidation of GSH before or during chromatography resulted in the liberation of the conjugate forms of COH.

Table I. Chromatography[a] of Glutathionocobalamin

Compound	R_f	Detection: Ninhydrin	Detection: Cobalamin
CSG	0.33	+	+[b]
COH	0.57, 0.23		+[c]
GSH	0.48, 0.25	+	
GSSG	0.25[d]	+	
GSH/COH, 2:1	0.35[e]	+	+[b]
GSH/COH, 12:1	0.35[e]	+	+[b]

[a] System: 70% aqueous 1-propanol, Whatman No. 4 paper, circular. [b] No free COH was detected. [c] Both bands disappear on complexation. [d] Also showed additional bands with R_f <0.25. [e] Also showed ninhydrin-positive bands corresponding to GSH and trace GSSG.

Studies of the interaction of amino acids, summarized in Table II, show that the sulfhydryl group is the complexing moiety. Amino acids with –SS– and $-SCH_3$ groups were without effect on the chromatography or the absorption spectrum (in acid, neutral, or alkaline media) of COH. Cysteine, on the other hand, in slightly acidic solution at a molar ratio of 9, and when suitably protected against oxidation, gives a spectrum virtually identical with that of the GSH complex. A similar spectrum is found initially in the presence of a several hundredfold excess of ethanethiol in a nitrogen-flushed cell, but here the spectrum changes rapidly ($t_{1/2}$ of several minutes) to that of B_{12r}.[6]

Table II. Cobalamin Complexation of Thiols

Compound	Key functional group	Interaction[a]: UV	Interaction[a]: C
Glutathione	–SH	+	+
Oxidized glutathione	–SS–	–	–
Cysteine	–SH	+	+
Cystine	–SS–	–	–
Methionine	$-SCH_3$	–	–
Ethanethiol	–SH	+	

[a] UV = ultraviolet and visible absorption spectra; C = paper chromatography; + indicates complexation; – indicates no interaction.

An analogous corrinoid–sulfhydryl reaction is known: factor B changes color from reddish orange to purple upon addition of the hydrosulfide ion, but the reaction is readily reversed in the presence of oxygen.[7]

CGS is converted readily to dicyanocobalamin, as indicated qualitatively by paper chromatography and quantitatively (98.5%) by use of the Rudkin and Taylor[8] method. Spectrophotometric and paper chromatographic investigation did not reveal any interaction between CCN and GSH, indicating that the cyanide ion is a considerably stronger complexing agent than the sulfhydryl group.

(6) H. Diehl and R. Murie, *Iowa State J. Sci.*, **26**, 555 (1952).
(7) P. George, D. H. Irvine, and S. C. Glauser, *Ann. N. Y. Acad. Sci.*, **88**, 393 (1960).
(8) G. O. Rudkin, Jr., and R. J. Taylor, *Anal. Chem.*, **24**, 1155 (1952).

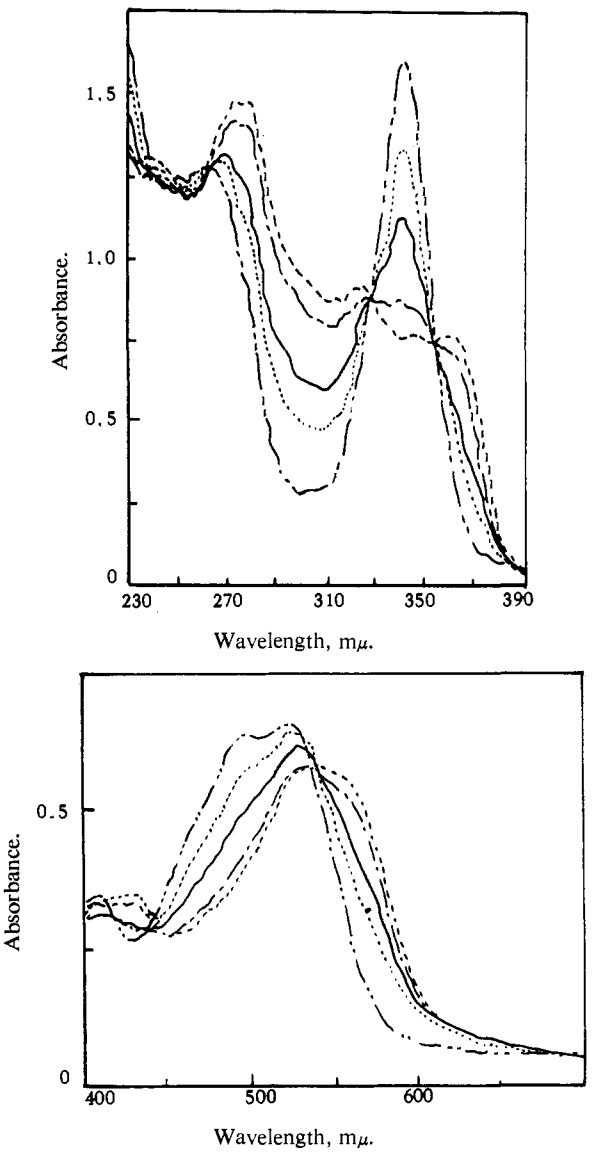


Figure 1. Interaction of glutathione and hydroxocobalamin at pH 4.70. COH concentration constant at 7×10^{-5} *M*. Molar ratios, GSH/COH: —·—·—, 0/1; – – – –, 0.45; ———, 0.8; —·—, 1.1; — — —, 2, 5, 9.

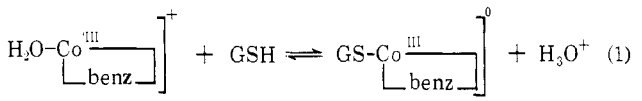
The stabilizing effect of COH on the –SH group of unbuffered GSH is shown by the following data. Solutions of GSH in distilled water containing various amounts of COH were flushed with nitrogen, stored in the dark at room temperature for 120 hr, and analyzed for –SH by a colorimetric ferrocyanide method. When the initial COH/GSH ratios were 0, 0.1, and 1, respectively, the residual mercaptan values (expressed as % of initial GSH) were 8.5 ± 2.5, 16, and 78%, respectively. In the absence of any interaction, the addition of alkali (as COH) would be expected to increase oxidation of GSH. These results thus suggest that principally only that amount of GSH in equilibrium bonding to COH is stabilized.

Discussion

In view of the many functional groups of GSH, the formation of a 1:1 complex does not in itself preclude the possibility of multiple bonding. However, multiple coordination to the cobalt ion is excluded by steric,

thermodynamic, and kinetic considerations:[7] four coordination positions of the cobalt complex are occupied by the extremely stable, more or less planar corrin ring, and the remaining two coordination sites that complete the octahedral configuration lie on opposite sides of the fairly wide corrin plane. Bonding elsewhere in the chromophoric system is eliminated by the formation of a complex with the same spectral features as the CSG complex by the small, monofunctional ethanethiolate ion and the absence of any interaction of corresponding amino acids in which the sulfhydryl group is blocked. Bonding anywhere else is eliminated by the electrophoretic demonstration[4] of the proper charge distribution as a function of pH[9] for the remaining one amine and two carboxy groups of GSH. The presence of a new, doublet band in the spectrum in the 270–290-mμ region, although reminiscent of the benzimidazole uncoupling effect noted by Beaven, *et al.*,[10] is perhaps best explained as an intrinsic feature of a cobalt–sulfhydryl bond.[11]

For the ligand replacement reaction shown in eq 1



the value of the stability constant, $K_{CSG} = (CSG)\cdot(H^+)/(COH)(GSH)$, is 10 when evaluated in 0.05 *M* acetate buffer at pH 4.70. CSG is thus a considerably weaker complex than CCN, based on the estimate of 10^{12} M^{-1} for the stability constant of the latter complex.[7] Although the simple application of the mass action law can hardly be expected to be an adequate representation of behavior in a physiological environment, it nevertheless indicates that CSG is completely dissociated, whereas CCN remains completely associated, at a physiological pH and concentration level [*e.g.*, in liver, <1 ppm CCN,[2b] 1750 ppm GSH[12]].

The GSH–COH reaction, in which the sulfhydryl group is the complexing moiety, appears to be a prototype of the general reaction of COH with thiol compounds. Dubnoff,[5] however, has indicated that the reaction is unique to GSH, an exactly opposite conclusion. Smith[2c] has pointed out other reported inconsistencies in the behavior of thiol compounds. These apparent inconsistencies are readily resolved by consideration of the dual role of thiols.

Thiol compounds simultaneously function as complexing agents and as reducing agents. There is consequently a thermodynamic and kinetic competition between these functions, the final outcome of which must obviously depend, among other parameters, on the absolute and relative concentration of the thiol, on its oxidation–reduction potential, and on the relative strengths and concentration of all the cobalamin-complexing ligands in solution. To the extent that thiol complexes form in any solution (and complexation is usually a rapid reaction), the concentration of the reducing agent is decreased, and the resistance of the cobalamin to reduction is simultaneously increased by its incorporation into a more stable species. Low relative concentrations of thiol thus favor complexation and potentially greater stability of the cobalamins, whereas high relative concentrations ultimately favor reduction of the cobalamins. The rate of reduction, however, to the extent that it depends on the concentration of uncomplexed cobalamin, *i.e.*, aquocobalamin, would still depend on the thermodynamic stability of the various cobalamin complexes, including the thiol complex, in solution.

Experimental Section

Chemicals. Cyanocobalamin (CCN) and hydroxocobalamin [COH, aquocobalamin, cobalaminium hydroxide, vitamin B_{12a}, B_{12b}, α-(5,6-dimethylbenzimidazolyl)hydroxocobamide] were obtained within Merck and Co., Inc. Glutathione (GSH) was obtained from National Biochemical Corp. and oxidized glutathione (GSSG) from Schwartz Laboratories, Inc. Preboiled, nitrogen-saturated distilled water was used for the preparation of solutions. Operations were performed in dim light and solutions were stored in the dark.

Techniques and Equipment. A Cary Model 11 spectrophotometer and 1-cm quartz cells were used to obtain the spectra. Chromatography was performed in a darkroom maintained at 25 ± 1°, using a circular paper technique.[13] Cobalamin bands were located by their red color, whereas amino acids were located after a ninhydrin spray.

(9) F. A. Isherwood, "Glutathione," Biochemical Society Symposium No. 17, Cambridge University Press, Cambridge, England, 1959, p 3.
(10) G. H. Beaven, E. R. Holiday, E. A. Johnson, B. Elllis, and V. Petrow, *J. Pharm. Pharmacol.*, **2**, 944 (1950).
(11) M. P. Schubert, *J. Am. Chem. Soc.*, **53**, 3854 (1931).
(12) H. Waelsch, *Advan. Enzymol.*, **13**, 273 (1952).
(13) T. Medwick, R. G. Johl, and N. Adler, *J. Chromatog.*, **9**, 118 (1962).