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The Formation of Hydridocobalamin and Its Stability in Aqueous Solutions

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Hydridocobalamin, the two-equivalent reduction product of cyanocobalamin, was prepared employing chromous chloride reagent and by electrolysis at a mercury pool cathode. Amperometric titration and electrolysis with measurement of the amount of electricity passed have shown that it is also the product of the reduction of cyanocobalamin at the dropping mercury electrode. Hydridocobalamin is oxidized to B_{12r} at the dropping mercury electrode with a half-wave potential at -0.87 v vs. saturated calomel electrode. It slowly decomposes water to yield hydrogen and B_{12r}. In aqueous solution hydridocobalamin has a half-life which varies from 355 minutes at *p*H 10.0 to 87 minutes at *p*H 8.0.

The stepwise reduction of vitamin B_{12}, (cyanocobalamin), first yields vitamin B_{12r}, an orange-brown product which contains +2 cobalt, and finally a green product, which possesses remarkable reducing properties. Vitamin B_{12r} was obtained by Diehl and Murie (1952) by catalytic hydrogenation of vitamin B_{12}, and its polarographic behavior has been fully investigated by Jaselskis and Diehl (1954). Because of its hydridelike properties, the green product has been called *hydridocobalamin* by Smith and Mervyn (1963), a name which we shall employ herein. Boos *et al.* (1953) doubtless produced B_{12r} as the brown product of one-equivalent reduction of vitamin B_{12} by chromous ion in EDTA solution. In some experiments, however, these workers must have formed hydridocobalamin since their spectrum of the "reduced vitamin B_{12}" is closer to that of hydridocobalamin than B_{12r}. Beaven and Johnson (1955), showed that hydridocobalamin was obtained on further reduction of B_{12r} with chromous acetate and thus clarified the relation between the reduction products obtained by Diehl's group and by Boos *et al.* Schindler (1951), found that B_{12r} was produced by the action of bisulfite or dithionite ions on the original vitamin, while treatment with zinc in NH_4Cl solution gave a *hellblau* product, which we assume was hydridocobalamin. Recent synthetic work in the area of cobalamin coenzymes by Smith *et al.* (1962) and Mueller and Mueller (1962) has shown that hydridocobalamin is a valuable intermediate in the synthesis of compounds which contain a carbon-cobalt bond. Chemical reduction of cyanocobalamin or hydroxocobalamin by means of zinc or sodium borohydride is ordinarily used to produce hydridocobalamin.

In the present work we shall show that hydridocobalamin is the final product in the titration of cyanocobalamin with chromous chloride in alkaline medium. Furthermore, the evidence is strong that hydridocobalamin is the product of electrolysis of cyanocobalamin at the dropping mercury electrode. This fact has led to the successful application of controlled potential electrolysis at a mercury pool cathode as a means of producing hydridocobalamin. Finally we shall present data on the rate of oxidation of hydridocobalamin by water in solutions of varying *p*H.

RESULTS AND DISCUSSION

Formation of Hydridocobalamin.—When one equivalent of chromous ion is added to cyanocobalamin in

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TABLE I
RESULTS OF AMPEROMETRIC TITRATIONS
All samples titrated in 0.1 M EDTA buffer at *p*H 9.6 with 0.1000 M $CrCl_2$.

Sample No.	B_{12} Sample Wt (corr.) (mg)	$CrCl_2$ Consumed At 1st e.p. (meq)	$CrCl_2$ Consumed At 2nd e.p. (meq)	Equivalent Wt of B_{12}, Calcd at 1st e.p.	Equivalent Wt of B_{12}, Calcd at 2nd e.p.
42	1.79	0.0158	0.0323	1133	554
43	5.97	0.0479	0.112	1247	533
44	4.46	0.0335	0.0800	1331	557

0.1 M EDTA at *p*H 9.6, the polarographic wave of B_{12} is replaced by that of B_{12r} and the solution acquires the orange-brown color of the latter compound. This reaction, carried out as an amperometric titration, was used by Boos *et al.* (1953) to determine the equivalent weight of vitamin B_{12}. The addition of a second equivalent of chromous ion to the solution eliminates the B_{12r} wave, gives rise to an anodic wave with $E_{1/2}$ = −0.87 v vs. saturated calomel electrode, and changes the color of the solution from orange to green. It is this green product which has been designated hydridocobalamin.

Table I shows data for amperometric titrations in which cyanocobalamin was successively reduced to B_{12r} and hydridocobalamin. Deviation of the equivalent weight calculated from the first equivalence point from the accepted value of 1300 is attributable to uncertainty in determining the point of intersection of the two branches of the titration curve, which intersect at almost a straight angle. The fact that more reagent was used in titrating B_{12r} to hydridocobalamin than was used in titrating cyanocobalamin to B_{12r} is explained by the reaction of hydridocobalamin with water, to be discussed below, a side reaction which requires the consumption of additional reagent and leads to an equivalent weight which is surely too low.

The same final reaction product, the green hydridocobalamin, as shown by identical absorption spectra and polarographic waves, has been obtained by electrolyzing the original cyanocobalamin solution at a mercury pool cathode, at a constant potential of −1.4 v vs. saturated calomel electrode.

Because the polarographic wave of cyanocobalamin completely disappears on the addition of two equivalents of reductant per mole of vitamin, we can conclude that the reduction of cyanocobalamin at the dropping mercury electrode is a two-electron process. Barring the possibility of any nonelectrolytic step which may occur in the large scale electrolysis or chemical reduction and not in the polarographic experiment, e.g., a rearrangement, we may identify the product of reduction at the dropping mercury electrode as hydridocobalamin also. Coulometric evidence supporting this conclusion is given below.

Polarographic Characteristics of Hydridocobalamin.—The anodic polarographic wave of hydridocobalamin has a half-wave potential of −0.87 v vs. saturated calomel electrode in the *p*H range 7.0–9.7, regardless of the buffer constituents. In a solution of cyanocobalamin which has been completely reduced to hydridocobalamin the corrected height of this wave is 0.51 times the height of the original vitamin reduction wave. This is strong evidence that hydridocobalamin is oxidized at the dropping mercury electrode in a one-electron step.

Controlled potential coulometry (Lingane, 1958) was employed to confirm the *n* value for the electrode process. The electrolysis on a mercury pool cathode of 9.15 mg of cyanocobalamin in 0.1 M EDTA at *p*H 9.6 at −1.4 v vs saturated calomel electrode required 1.52 coulombs to convert the original vitamin completely to hydridocobalamin. If the molecular weight of the vitamin is taken as 1300, this leads to an *n* value of 2.2 equiv/mole. This slightly high value, which is equivalent to a current efficiency for the reduction of less than 100%, is consistent with our finding that hydridocobalamin reduces water and is thereby reoxidized to B_{12r}. Thus, the deviation from 2 is in the expected positive direction for a side reaction which consumes electricity.

At the conclusion of the reduction the cathode potential was changed to −0.6 v vs. saturated calomel electrode, whereupon 0.52 coulomb was required to oxidize hydridocobalamin to B_{12r}, the latter easily identifiable by its color and its polarographic wave. The oxidation yields an *n* value of 0.76 equiv/mole. The low *n* value indicates that some hydridocobalamin was being oxidized in a nonelectrolytic step during the electrooxidation. The side reaction expected for a reductant as powerful as hydridocobalamin is its oxidation by hydrogen ion or water to form B_{12r} and hydrogen. The presence of this side reaction was confirmed by detection of hydrogen in the gas in equilibrium with the final electrolysis product solution. An appropriate correction of the *n* value for this side reaction can be made by referring to experiments reported herein, which show that during the time required for the electrooxidation, which was 30 minutes, 10% of the hydridocobalamin originally present in a solution of *p*H 9.6 decomposed. The *n* value can thus be considered to be greater than 0.8, and in agreement with the evidence of the polarographic diffusion currents, which show a one-electron oxidation of hydridocobalamin to B_{12r}.

Since the half-wave potential for the oxidation of hydridocobalamin is 0.08 v more positive than that for the reduction of B_{12r} (Jaselskis and Diehl, 1954), the process, though chemically reversible, is polarographically irreversible.

Reaction of Hydridocobalamin with H_2O.—Solutions of hydridocobalamin spontaneously reverted to B_{12r}, even in the absence of air, as shown clearly by the change in color of the solution. Results of experiments in which solutions of hydridocobalamin were allowed to stand for varying lengths of time are shown as the first four entries in Table II. The number of moles of hydridocobalamin disappearing within the time period, determined by the decrease in its diffusion current, is twice the number of moles of hydrogen found, within the accuracy of determination of the latter. An exception is the experiment done at *p*H 7.0, probably because the current efficiency for the reduction of cyanocobalamin was less than 100% at this *p*H. Thus, appreciable hydrogen would have been formed by cyclic reduction of the vitamin and reaction of hydridocobalamin with water even before the vitamin sample was fully reduced. In the last two experiments listed in Table I, where the solution was electrooxidized,

TABLE II
HYDROGEN ANALYSIS AFTER DECOMPOSITION OF HYDRIDOCOBALAMIN

pH	Time of Standing (hours)	Hydrido-cobalamin Decomposed (μmoles)	H_2 Found (μmoles)	Hydrido-cobalamin Lost minus H_2 Found (μequiv)
9.6	1	1.12	0.56	0.0
9.6	1	1.49	0.70	0.09
9.6	$2^1/_2$	2.64	1.13	0.38
7.0	$1^3/_4$	4.04	2.95	−1.86
9.6	$^3/_4$[a]	7.70	0.60	
9.6	$^2/_3$[a]	7.04	0.47	

[a] Simultaneously electrooxidized at −0.6 v vs. saturated calomel electrode.

the abnormally small yield of hydrogen is to be expected, since the electrooxidation removes hydridocobalamin from the system but generates no hydrogen.

On the basis of these experiments we postulate an over-all homogeneous chemical reaction between hydridocobalamin and water which yields B_{12r} and hydrogen. We have studied the rate of this reaction as a function of *p*H by following the decrease of the anodic diffusion current of hydridocobalamin in various buffer solutions. At all *p*H values studied the rate of disappearance of hydridocobalamin was first order. The data from representative decomposition experiments are shown in Figure 1. Rate constants, defined by equation (1),

$$-\frac{d(\mathrm{HCo})}{dt} = k'(\mathrm{HCo}) \qquad (1)$$

where (HCo) indicates hydridocobalamin concentration, are plotted vs. *p*H in Figure 2. The slope of this plot indicates that k' is *p*H dependent, and for the *p*H interval studied, $k' = k\,(\mathrm{H}^+)^q$. The experimental value of k is $10^{0.38}$, when concentrations are expressed in moles per liter and time in minutes. The slope of the best straight line connecting the points of Figure 2 yields a value for q of 0.31. We cannot presently

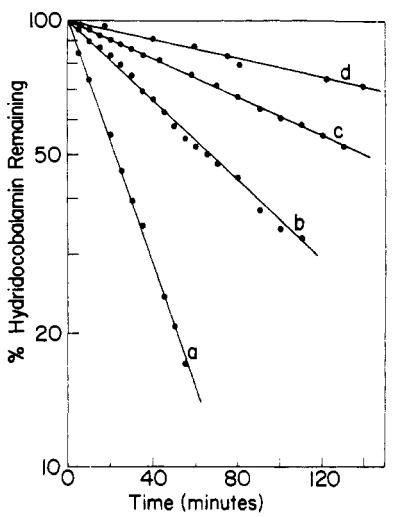

FIG. 1.—Decomposition of hydridocobalamin in aqueous solution.

Curve	B_{12} taken	Initial *p*H	Final *p*H	Initial B_{12} conc'n	−log k'
a	11.60 mg	6.86	6.98	1.81 mM	1.51
b	9.56	7.88	8.01	1.50	1.99
c	11.42	8.83	9.01	1.78	2.31
d	10.33	9.85	9.93	1.61	2.63

All volumes were 5.0 ml; temperature, 25°.

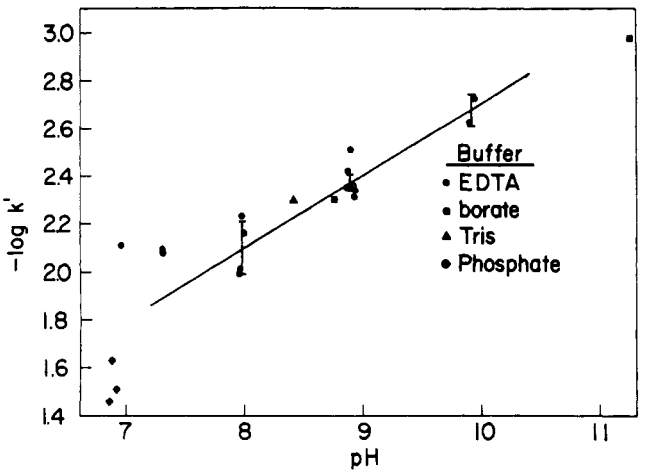

FIG. 2.—Decomposition rate as a function of *p*H. Vertical lines indicate ± S (standard deviation) of log k' from mean at *p*H 7.98, 8.88, and 9.91.

give a mechanism for the reduction of hydrogen ion or water by hydridocobalamin which accounts for this dependence of rate on *p*H. However, the direction of the deviation of q from unity suggests that some of the hydrogen ion entering into the reaction may come from within the hydridocobalamin molecule itself.

While the redox stoichiometry and the polarographic results reported here are consistent either with the existence of cobalt in the +1 oxidation state or with +3 cobalt and a hydridic hydrogen within the green reduction product, evidence is accumulating that the material contains a cobalt-hydrogen bond: thus, the name hydridocobalamin. The synthetic work of Smith *et al.* (1962), Smith and Mervyn (1963), and Mueller and Mueller (1962) is indicative of this, and the discovery by King and Winfield, (1961), of $[Co(III)(CN)_5H]^{\equiv}$ supports the possibility that an analogous compound may exist among the reduction products of cyanocobalamin. The chemical properties of hydridocobalamin are the subject of continuing work in these laboratories.

EXPERIMENTAL

Chemicals.—The crystalline cyanocobalamin (Nutritional Biochemicals Corp., Cleveland, O.) was assayed spectrophotometrically according to the method of Cords and Ratycz (1959). Corrected weights reported herein are based on analysis of the sample used in this work, which was 89% cyanocobalamin. The 0.1 M EDTA solution was prepared from the disodium salt of EDTA, and the *p*H was adjusted to the desired value by the addition of 0.1 M NaOH prior to final dilution. The 0.1 M chromous titrant solution in 0.1 M HCl was prepared by the method of Lingane and Pecsok (1948). Its concentration was checked by potentiometric titration of a standard $CuSO_4$ solution. The boric acid–borate buffer solutions were prepared by adding 9 M NaOH to 0.05 M H_3BO_3 to give the desired *p*H. The tris(hydroxymethyl)aminomethane buffer was prepared by dissolving the solid base (Sigma Chemical Co.) and adding 1 M HCl to give the desired *p*H. National Bureau of Standards 0.025 M phosphate buffer was used for standardization of the glass electrode as well as for a buffer medium for reactions conducted at *p*H 6.86. Tank nitrogen was freed of the last traces of oxygen by passage over hot copper and bubbling through 0.1 M chromous solution in 1.0 M HCl. Argon was not purified, but a correction

was made for its small hydrogen impurity by running a blank determination.

Amperometric Titrations.—Approximately 5 mg of cyanocobalamin was accurately weighed and placed in a conventional H-type polarographic cell with a small Teflon-covered stirring bar. Ten ml of EDTA buffer solution was then pipetted into the cell, and it was closed tightly with a rubber stopper containing a dropping mercury electrode, a mercury bubbler gas outlet, and the microburet tip. The solution was deaerated 15 minutes with purified nitrogen, and the gas inlet tube was closed. A complete polarographic wave was recorded on a Leeds and Northrup Electrochemograph, Type E, at the beginning and after the addition of each increment of 0.1 M chromous solution. The solution was stirred after each addition of titrant by agitation of the magnetic stirring bar. Titrant was added from a 0.25-cc tuberculin syringe driven by a microdrive mechanism (Model SB-2, Micrometric Inst. Co., Cleveland, O.). A three-way stopcock, connected to the syringe, the reagent storage flask, and the delivery tube, allowed the reagent to be prepared, stored, and delivered in the absence of air. The end-point was determined by plotting the current, corrected for residual current, measured at −1.35 v vs. saturated calomel electrode vs. the volume of chromous solution. The data fell on straight lines which intersected at the first equivalence point; the second equivalence point was taken as the point where the second branch crossed the zero-current axis.

For quantitative analysis of solutions of cyanocobalamin, amperometric titration to B_{12r} is more satisfactory than the two-electron reduction. The first equivalence point can be found by measuring currents at −1.35 v vs. saturated calomel electrode and correcting them for currents at −0.6 v. The sum of diffusion currents at −1.35 v and −0.6 v remains constant between the two equivalence points and gives a titration curve on which the first equivalence point can be precisely found.

Electrolysis.—Conventional controlled potential electrolysis technique (Lingane, 1958), with manual adjustment of the applied voltage, was employed. The cell was a small polarographic cell (E. H. Sargent Co., S-29370, modified by addition of a side compartment) in which a mercury pool about 5 cm^2 in area could be placed. A coil of platinum wire dipping into saturated KCl in the side arm of the cell served as the other working electrode, separated by an agar-KCl plug from the cathode compartment. The cell current was drawn from a 6-v storage battery and was controlled by a 10-ohm, 50-w variable resistor connected as a potential divider. The potential of the mercury electrode vs. a separate saturated calomel electrode was observed on a Kiethley Model 610 electrometer. Current in the circuit was measured on a Laboratory Standard Milliammeter (Sensitive Research Instrument Co., Mt. Vernon, N.Y.).

A weighed cyanocobalamin sample, a stirring bar, and 5.00 ml of supporting electrolyte (buffer solution) were placed in the cell containing the mercury pool, and the cell was closed tightly with a rubber stopper fitted with a dropping mercury electrode. After purging with nitrogen for 15 minutes and closing off the gas inlet and outlet, the circuit was closed, and the applied voltage was continually adjusted so that the cathode potential remained at −1.4 v vs. saturated calomel electrode, a potential selected because it was on the diffusion current plateau of the cyanocobalamin wave. The solution was stirred during the electrolysis, and the progress of the reduction was followed polarographically. (Electrolysis was halted while polarograms were run; a saturated calomel electrode which shared the anode compartment with the working anode served as the nonpolarizable electrode.) The number of coulombs passed in an electrolysis was determined from a plot of current vs. time by estimating the area under the curve.

Electrooxidation of hydridocobalamin was performed on solutions which had been exhaustively electrolyzed as described above. When a pure hydridocobalamin solution had been prepared, as proved by the complete disappearance of the B_{12} wave and the appearance of the hydridocobalamin wave, the potential of the mercury pool was changed to −0.6 v vs. saturated calomel electrode, whereupon the mercury pool functioned as anode. This potential was maintained as above, and the electrolysis was continued until the anodic wave of hydridocobalamin had been replaced by the cathodic wave of B_{12r}.

Absorption Spectra.—A 1-cm quartz spectrophotometric cell was fitted with a rubber stopper, cemented into place, and an entrance and an exit tube were inserted through the stopper. The cell was placed in the nitrogen line immediately before the polarographic cell, and the two cells were deaerated simultaneously. After deaeration, the cyanocobalamin was reduced to the desired product either by chromous reagent or electrolysis. The direction of nitrogen flow was then reversed, and some of the product solution was forced into the spectrophotometric cell. The cell was closed by clamping shut short lengths of gum rubber tubing connected to the entrance and exit tubes. The cell was sufficiently tight to prevent air-oxidation during the time required for recording the spectrum. The spectrum was recorded on a Cary Model 14 spectrophotometer, with water as the reference solution. Spectra were corrected for absorption of chromic ion in cases where the solution was prepared with chromous reagent.

Absorption maxima, in mμ, and absorbancies (given in parentheses) of 1% solutions in a 1-cm cell were as follows: cyanocobalamin in 0.1 M EDTA at *p*H 8.85, 361 (210), 550 (65); vitamin B_{12r} in 0.1 M EDTA at *p*H 8.85, 312 (190), 405 (52), 473 (67); hydridocobalamin in 0.1 M EDTA at *p*H 9.6, 280 (250), 286 (250), 383 (234), 455 (22), 553 (25).

Rate of Decomposition Measurements.—Hydridocobalamin was formed by controlled potential electroreduction as described above. After reduction was complete, the electrolysis circuit was disconnected, and the solution in the sealed polarographic cell was stirred intermittently with the magnetic stirrer. Decomposition was followed by measuring the height of the anodic wave at −0.6 v vs. saturated calomel electrode at 5- to 15-minute intervals for about 2 hours.

Hydrogen Analysis.—The cyanocobalamin sample was reduced to hydridocobalamin by controlled potential electrolysis as described above, except that argon was used instead of nitrogen for sample deaeration. After complete reduction and measurement of the diffusion current at −0.6 v, the sample was allowed to stand a certain length of time, and the decrease in the diffusion current was observed. The polarographic cell was then opened to an evacuated 50-ml gas-sample bulb, in order to collect the gas from above the solution.

The gas samples were analyzed on a Consolidated Engineering Co. mass spectrometer, Model 21-620, after freezing out water and reducing the partial pressure of argon by immersion of the sample bulb in liquid nitrogen. An instrument blank was run before and after each sample, and a blank on an electrolysis without cyanocobalamin was run for comparison. Calibration of the method for detecting and estimating

hydrogen was effected by constant-current electrolysis of 0.1 N H_2SO_4 in the same electrolysis cell for a measured time interval and treatment of the resulting gas samples exactly as described above.

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Mercaptan Involvement in Dissociation and Reconstitution of Hemerythrin*

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The oxygen-carrying pigment hemerythrin can be dissociated into eight subunits (merohemerythrins) by the addition of a mercurial such as salyrganic acid. Other mercaptan-blocking reagents are also effective. The native macromolecule can be reconstituted from subunits if the mercurial is removed with cysteine ethyl ester and the iron is reduced to the ferrous state. Similarities and differences in macromolecular constitution between hemerythrin and hemoglobin are discussed.

The oxygen-carrying pigment hemerythrin contains eight oxygen-binding sites per particle of 107,000 mw (Klotz and Keresztes-Nagy, 1962, 1963). It has also been established that a hemerythrin macromolecule can be dissociated into eight subunits (merohemerythrins) by a variety of chemical treatments: exposure to low or high *p*H; treatment with a detergent, sodium dodecyl sulfate; reaction with succinic anhydride. These chemical effects show that in hemerythrin the subunits are held together by noncovalent bonds. It might be possible, therefore, to reverse the disaggregation process and convert merohemerythrin back to hemerythrin. The chemical treatments previously used, however, are relatively strong and generally irreversible, and hence an alternative method of dissociation is essential if reaggregation is to be achieved.

Earlier studies (Klotz *et al.*, 1957) have shown the presence of sulfhydryl groups in hemerythrin. Recalling the discovery of Madsen and Cori (1956) that phosphorylase can be dissociated by blocking of sulfhydryl groups we felt a similar approach might be effective with hemerythrin. Such indeed has proved to be true; the pigment can be dissociated with a mercurial (as well as other mercaptan reagents) and reconstituted upon removal of bound mercurial.

Experimental Procedures and Results

Materials.—Crystalline oxyhemerythrin and methemerythrin were prepared, by procedures described previously (Klotz *et al.*, 1957; Klotz and Keresztes-Nagy, 1963), from the coelomic fluid of the marine worm *Golfingia gouldii* (also known as *Phascolosoma gouldii*).

Salyrganic acid was purchased from Winthrop Laboratories and was warranted by the manufacturer to be 97–103% pure. *p*-Chloromercuribenzoic acid was purchased from Nutritional Biochemicals Corp., *p*-chloromercurisulfonic acid from Sigma Chemical Co., neohydrin from Lakeside Laboratories, *N*-ethylmaleimide from Mann Research Laboratories, and oxidized glutathione from Schwarz Bioresearch. The azomercurial 4-(*p*-dimethylaminobenzeneazo)phenylmercuric acetate was prepared by procedures described previously (Horowitz and Klotz, 1956). All other reagents were obtained from general commercial sources.

Sulfhydryl Content of Hemerythrin.—Previous analyses for mercaptan groups in oxyhemerythrin (Klotz *et al.*, 1957) using an amperometric silver titration (Benesch *et al.*, 1955) indicated an SH-Fe ratio of approximately 1. Since there are 2 Fe atoms per subunit (merohemerythrin) this result implies 2 SH groups in each subunit. Since then evidence has accumulated that silver titrations are not specific for mercaptan groups in hemerythrin. Using the colorimetric monofunctional mercurial titration developed recently (Klotz and Carver, 1961), we find that oxyhemerythrin in 8 M urea, after equilibration with a mercurial for 0.5–1.0 hour, shows an SH-Fe ratio of 0.42. This result indicates 0.84, or approximately 1, SH group per subunit of merohemerythrin (with 2 Fe). Amino acid analyses (by Mr. W. R. Groskopf) also give approximately one cysteic acid residue in merohemerythrin. It seems clear, therefore, that there is only 1 SH in each subunit, or 8 SH in the native hemerythrin macromolecule of 107,000 mw.

Dissociation of Hemerythrin.—Merohemerythrin subunits were obtained by the following procedure. Crystalline oxyhemerythrin was dissolved in 0.1 M sodium cacodylate buffer at *p*H 5.9, the buffer solution containing also 0.15 M sodium chloride. The protein concentration, measured and expressed in terms of iron content, was made 6.0×10^{-4} M. This corresponds to 3×10^{-4} M mercaptan. To a series of 2-ml portions of this solution were added increasing quantities of 3×10^{-3} M salyrganic acid, a mercurial, so as to pro-

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