(21) Only two (0.90 and 3.76 ppm) of the four ^{13}C triplets are completely resolved; the other two overlap at ca. 2.3 ppm. However, in a first-order spectrum of this type, the observation of one of the ^{13}C proton triplets characterizes both of them.
(22) B. T. Golding and S. Sakrikar, *J. Chem. Soc., Chem. Commun.*, 1183 (1972).
(23) E. A. Parfenov, T. G. Chervyakova, and A. M. Yurkevich, *J. Gen. Chem. USSR (Engl. Transl.)*, **42**, 2579 (1972).
(24) M. Herberhold, "Metal π-Complexes", Vol. II, Part I, Elsevier, New York, N.Y., 1972.
(25) D. Thusius, *J. Am. Chem. Soc.*, **93**, 2629 (1971).
(26) R. A. Firth, H. A. O. Hill, J. M. Pratt, R. G. Thorp, and R. J. P. Williams, *J. Chem. Soc. A*, 381 (1969).
(27) The percentages of products and starting material in the reaction solution can be estimated from the relative NMR peak areas of dimethylglyoximato methyl protons. These absorptions occur at 2.10, 2.18, and 2.37 ppm (in CH_2Cl_2) for the acetal, the aldehyde, and starting material, respectively.
(28) R. Breslow, R. Fairweather, and J. Keana, *J. Am. Chem. Soc.*, **89**, 2135 (1967).
(29) R. M. Silverstein and C. C. Bassler, "Spectrophotometric Identification or Organic Compounds", 2nd ed, Wiley, New York, N.Y., 1967, p 88.
(30) G. N. Schrauzer and R. J. Windgassen, *J. Am. Chem. Soc.*, **89**, 1999 (1967).
(31) E. S. Gould, "Mechanism and Structure in Organic Chemistry", Holt, Rinehart, and Winston, New York, N.Y., 1959.
(32) H. W. Heine, A. D. Miller, W. H. Barton, and R. W. Greiner, *J. Am. Chem. Soc.*, **75**, 4778 (1953).
(33) This, of course, assumes one can compare first- and second-order rate constants directly.
(34) T. H. Fife and L. K. Jao, *J. Org. Chem.*, **30**, 1492 (1965).
(35) The reaction can be followed by NMR, observing the decrease in the dimethylglyoxime methyl proton absorption at δ 2.37 and the increase at δ 2.09.
(36) By the procedure of H. A. O. Hill and K. G. Morallee, *J. Chem. Soc. A*, 554 (1969).
(37) The most intense band in the near-ultraviolet.
(38) Good results were obtained using Brinkmann cellulose plates (EM plates distributed by Brinkmann Instruments as Celplate-22, without indicator), precoated with microcrystalline cellulose.
(39) All of the cobalamins discussed in this paper can be cleanly separated using the prescribed solvent system. Satisfactory separations were also made with 1-butanol–2-propanol–water (7:6:7) and 2-butanol–methanol–water (55:15:30) both containing 0.5% concentrated aqueous ammonia.
(40) A standard 0.025 M borax–0.1 N HCl buffer was used: "Handbook of Chemistry and Physics", 46th ed, Chemical Rubber Publishing Co., Cleveland, Ohio, 1965, p D-73.
(41) R. B. Silverman, D. Dolphin, T. J. Carty, E. K. Krodel, and R. H. Abeles, *J. Am. Chem. Soc.*, **96**, 7096 (1974).
(42) If the enol of acetaldehyde exists at all, it is less than 1 part in ten million: A. Gero, *J. Org. Chem.*, **19**, 469 (1954).
(43) K. L. Brown and L. L. Ingraham, *J. Am. Chem. Soc.*, **96**, 7681 (1974).
(44) R. M. Lemmon in "Isotopic Carbon", M. Calvin, C. Heidelberger, J. C. Reid, B. M. Tolbert, P. F. Yankwich, Ed., Wiley, New York, N.Y., 1949, p 178.
(45) Purchased from Monsanto having 91% ^{13}C label.
(46) Variation of the procedure by G. A. Ropp, *J. Am. Chem. Soc.*, **72**, 4459 (1950).
(47) The reaction was followed by recording the NMR spectra of aliquots, observing the decrease in the dimethylglyoximato methyl protons' absorption at 2.37 ppm and the increase at 2.10 ppm.
(48) H. S. Hill and L. M. Pidgeon, *J. Am. Chem. Soc.*, **50**, 2718 (1928).
(49) The reaction was followed by NMR, observing the decrease in the dimethylglyoximato methyl protons' absorption at 2.37 ppm and the increase at 2.09 ppm.
(50) D. Dolphin, *Methods Enzymol.*, **18C**, 34 (1971).
(51) The photolysis is complete when all of the red color of an aliquot remains at the origin of a carboxymethylcellulose column after eluting with water.
(52) When cyanocobalamin was used, the product partially decomposed, presumably by cyanide ion dealkylation.
(53) Sodium or potassium carbonate can be substituted as the base.
(54) The reaction was followed, observing the decrease in the optical absorption at 357 nm or by TLC as described in the procedure (R_f 0.56 for the product, 0.22 for hydroxocobalamin).
(55) The reaction was followed by observing the decrease in the optical absorption at 357 nm or by TLC [appearance of a new spot with R_f 0.62, cellulose, *n*-BuOH–EtOH–H_2O (10:3:7) containing 0.5% concentrated aqueous ammonia].
(56) Recrystallization from water (containing Et_3N^{53})–acetone caused slight hydrolysis, giving a trace of formylmethylcobalamin and a small amount of hydroxocobalamin.

Model Studies for Coenzyme B_{12} Dependent Enzyme-Catalyzed Rearrangements. Kinetics and Mechanism of Decomposition of Formylmethylcobalamin and Its Acetals

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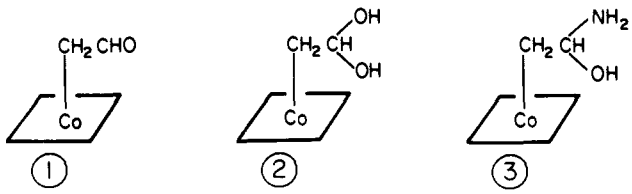
Abstract: Syntheses of formylmethylcobalamin and the kinetics and mechanism of decomposition of the aldehyde and its acetals are reported. Two different pathways were observed for the acid-catalyzed decomposition of the cobalamin acetals: direct cobalt–carbon bond cleavage to B_{12b} and a vinyl ether, and normal acetal hydrolysis to formylmethylcobalamin, followed by a rate-determining fission of the cobalt–carbon bond to B_{12b} and acetaldehyde. The rate law of decomposition of formylmethylcobalamin (A) is $d[A]/dt = -K[A][H_3O^+]$; the acid sensitivity and bimolecularity of the decomposition were rationalized as an initial protonation of the formyl carbonyl followed by a direct cleavage of the cobalt–carbon bond.

Formylmethylcobalamin (**1**), as its hydrated (**2**) or ammoniated (**3**) form, has been suggested as an intermediate in

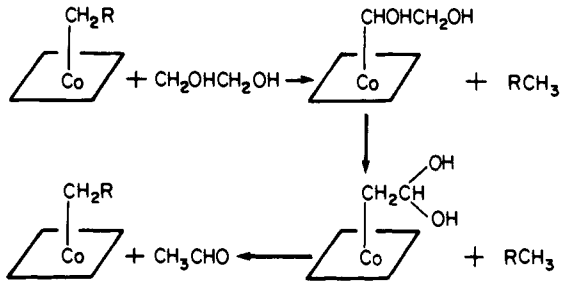


① ② ③

the enzymic conversion of ethylene glycol to acetaldehyde by dioldehydrase[2] and of ethanolamine to acetaldehyde by ethanolamine ammonia lyase.[3] Both of these enzymic reactions are vitamin B_{12} coenzyme dependent, and Abeles[2] has proposed the minimal mechanism described by the sequence outlined in Scheme I for the dioldehydrase reaction.

When acetaldehyde and ammonium ions were incubated with ethanolamine ammonia lyase and coenzyme B_{12}, hydrogen was transferred to acetaldehyde from the C-5′ of the coenzyme and the cobalt–carbon bond of the coenzyme dissociated. Omission of ammonium ion caused an acceleration of the cobalt–carbon bond dissociation, but no hydrogen was transferred from the coenzyme to acetaldehyde under these conditions.[3] Interpretation of the observations in the absence

Scheme I



of ammonium ion suggested the intermediacy of formylmethylcobalamin (**1**), which was believed to have a labile cobalt-carbon bond. When ethanolamine ammonia lyase and coenzyme B_{12} were treated with ethylene glycol, a reaction which according to Scheme I should also produce formylmethylcobalamin, dissociation of the cobalt-carbon bond occurred and 5′-deoxyadenosine, hydroxocobalamin, and acetaldehyde were isolated.[4] Since formylmethylcobalamin was not observed during these catalytic processes, it was suggested[3] that it was inherently unstable.

Schrauzer,[5] on the other hand, favors a completely different mechanism for the dioldehydrase reaction, and claims to have observed that formylmethylcobalamin is as stable as any organocorrin,[6] and suggests that if it were an intermediate in the enzymic reaction it should be readily isolable.

During our initial studies[7,8] we prepared formylmethylcobalamin by the hydrolysis of 1,3-dioxa-2-cyclopentylmethylcobalamin (**4**)[9] and observed that formylmethylcobalamin was readily hydrolyzed, even in basic medium, to hydroxocobalamin.

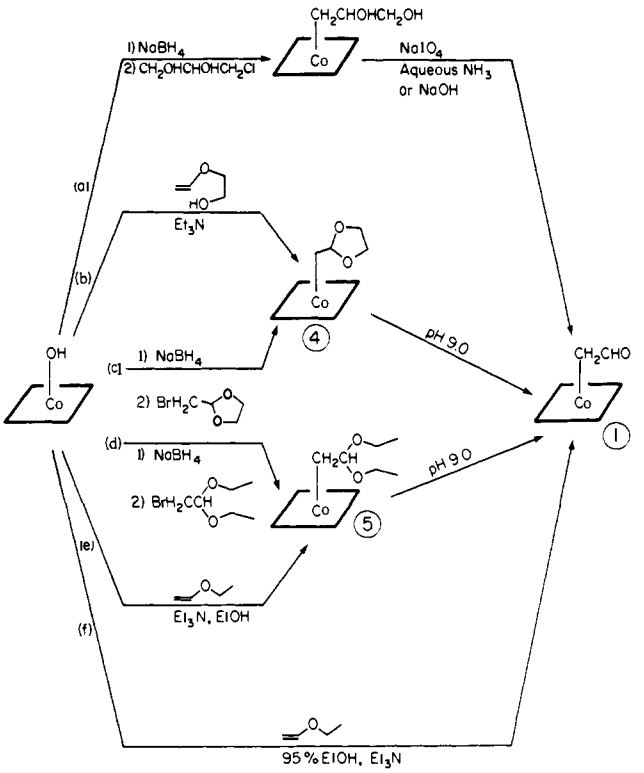
We have studied, therefore, the kinetics of the acid-catalyzed decomposition of formylmethylcobalamin in order to determine its relevance as a possible intermediate in the dioldehydrase and ethanolamine ammonia lyase reactions. It has further been stated[6] that hydrolysis of either 1,3-dioxa-2-cyclopentylmethyl- or 2,2-diethoxyethylcobalamin (**4** and **5**) gives hydroxocobalamin directly with no intermediate formation of formylmethylcobalamin. These observations were contrary to ours and so, in addition, we have examined the kinetics and mechanism of hydrolysis of the acetals of formylmethylcobalamin.

Results and Discussion

The routes for the synthesis of formylmethylcobalamin, used for the kinetic studies, are outlined in Scheme II. Numerous attempts to prepare formylmethylcobalamin by the procedures of Schrauzer et al.[6] failed, in our hands, to give any cobalt-alkylated B_{12} products.

2,3-Dihydroxypropylcobalamin. The synthesis of 2,3-dihydroxypropylcobalamin and its oxidation with sodium metaperiodate were developed by Abeles and Carty.[8,10] The production of formylmethylcobalamin during the oxidation reaction was monitored by the release of formaldehyde from the diol using a modification of earlier procedures.[10,11] Because of the inherent lability of formylmethylcobalamin, phenol extraction procedures could not be employed to remove salts. Bio-Gel P-2[12] gel filtration with 0.5 N aqueous ammonia was found, however, to be an efficient method for purification of this cobalamin.[10] In general, gel filtration provides an efficient technique for the separation of molecules according to size by elution through porous beads. However, it is not intended to be a chromatographic procedure for materials of similar molecular size, since all molecules larger than the largest pores of the beads elute with the void volume. Unexpectedly though, hydroxocobalamin is held up by Bio-Gel P-2, whereas the alkylcobalamins are eluted. Concentration of the purified

Scheme II. Syntheses of Formylmethylcobalamin



formylmethylcobalamin solution, under reduced pressure, removed the ammonia and gave a neutral solution.

1,3-Dioxa-2-cyclopentylmethylcobalamin. The synthesis of the cyclic acetal cobalamin is discussed in a previous paper.[9] Hydrolyses of the acetal with standard buffer solutions at pH 7.0 (KH_2PO_4-NaOH, 0.05 M), 8.0 (KH_2PO_4-NaOH, 0.1 M), 9.0 (borax (0.025 M)-HCl, 0.1 M) and 10.2 (0.5% concentrated aqueous ammonia v/v) were followed by TLC.[13,14] In all cases varying amounts of starting material, formylmethylcobalamin, and hydroxocobalamin (R_f 0.56, 0.51, and 0.22, respectively) were observed, and the lower the pH the more rapid the hydrolysis. Visual inspection of the spot intensities showed that the maximum concentration of aldehyde was obtained at pH 9.0 after 48 h, when there appeared a 1:1:2 ratio of acetal, aldehyde, and hydroxocobalamin. At pH 7.0 the solution contained more than 50% B_{12b}, and within a day the alkylated cobalamins had completely decomposed. At pH 8.0 about one-third B_{12b} and at pH 9.0 one-fourth B_{12b} had been formed after 1 day; after 48 h the former solution contained three-fourths and the latter one-half B_{12b}. In all of these solutions the concentration of formylmethylcobalamin varied from 25% to a trace. The pH 10.2 solution showed a small amount of B_{12b} and the rest starting material after 2 days.

Large-scale preparations were carried out on 2-mg lots which were chromatographed on 20 cm^2 cellulose plates.[14] In order to minimize decomposition of the separated aldehyde, the cellulose band containing this material was transferred, while still wet with basic eluent, directly into 0.5 N aqueous ammonia. Further purification was accomplished on Bio-Gel P-2.

Direct Synthesis of Formylmethylcobalamin. The most convenient method of synthesis of formylmethylcobalamin was by the addition of ethyl vinyl ether to hydroxocobalamin,[9] except in 95% ethanol instead of absolute ethanol. After standing at room temperature for 6 days, TLC on cellulose[13,14] showed two spots (R_f 0.62 and 0.51), the acetal and the aldehyde in the ratio of 1:4. Separation on Brinkmann cellulose as described above gave formylmethylcobalamin which needed no further purification.

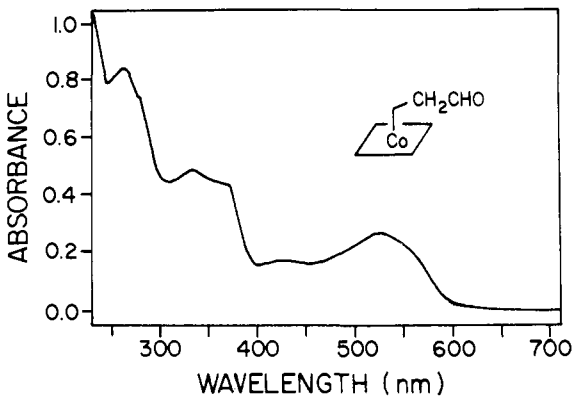


Figure 1. Optical absorption spectrum (H_2O) of formylmethylcobalamin.

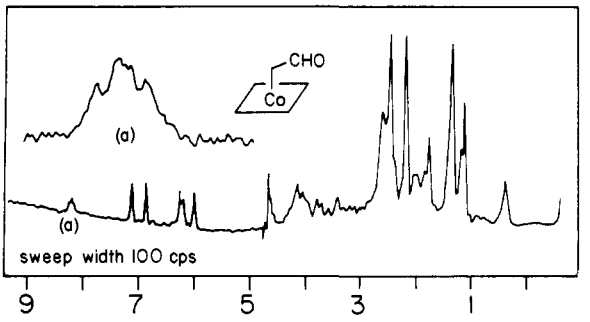


Figure 2. ^1H NMR spectrum (100 MHz) of formylmethylcobalamin in D_2O.

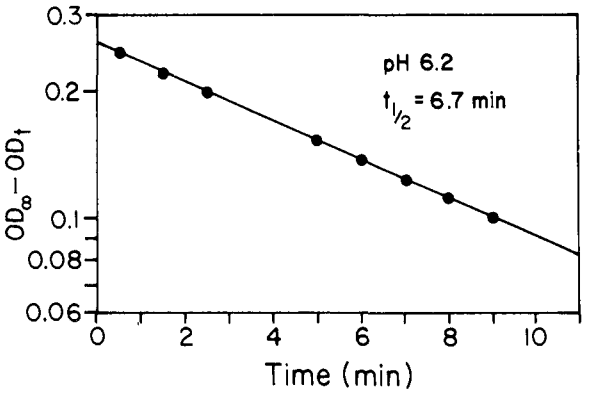


Figure 3. Semilogarithmic plot of $OD_\infty - OD_t$ vs. time for the decomposition at pH 6.2 of formylmethylcobalamin prepared by hydrolysis of 1,3-dioxa-2-cyclopentylmethylcobalamin.

Table I. Half-Times of Decomposition of Formylmethylcobalamin

	$t_{1/2}$, min	
pH	From oxidation of 2,3-dihydroxypropylcobalamin	From hydrolysis of 1,3-dioxa-2-cyclopentylmethylcobalamin
5.3	1.1	1.1
5.8		3.1
6.2	6.7	6.7
6.5	12.2	12.1
6.8	21.9	21.6

Properties of Formylmethylcobalamin. All of the methods of synthesis of formylmethylcobalamin shown in Scheme II produced the same compound when compared by TLC properties, optical spectral data (Figure 1), and rates of acid decomposition. The NMR spectra of **1** (Figure 2) made by routes a and f (Scheme II) were identical.

The optical spectrum (Figure 1) of formylmethylcobalamin is that of a typical alkylated B_{12} derivative. Unlike the formylmethylcobalamin reported by Schrauzer[6] which has an NMR spectrum containing a triplet at 9.01 ppm, the 100-MHz ^1H NMR (Figure 2) of our formylmethylcobalamin has a broad triplet (doublet of doublets) at 8.22 ppm (relative area, 1 proton). This absorption decreases slowly on standing in pH 7.3 buffer, as the formylmethylcobalamin decomposes. The prochiral protons on the cobalt-bound carbon of coenzyme B_{12}[15] and other alkylcobalamins and cobinamides[16] are nonequivalent for both base-on and base-off configurations. Consequently, a doublet of doublets rather than a triplet is expected for the NMR absorption of the aldehyde proton. Furthermore, the methyl protons of the ethyl ligand in ethylcobalamin (in D_2O) are shifted upfield by 1.52 ppm[17] relative to the methyl protons of 1-butanol (in D_2O).[18] As in the case of the infrared carbonyl-stretching frequency of formylmethyl(pyridine)cobaloxime, which is considerably lower than that of a normal aldehyde absorption,[9] presumably as a result of d-orbital interaction of the cobalt atom with the carbonyl group, a similar interaction appears to affect the NMR resonance of the aldehyde proton. Since the resonance of this proton in acetaldehyde (in D_2O) occurs at 9.97 ppm,[19] an upfield shift to 8.22 ppm in formylmethylcobalamin (in D_2O) is consistent with the above observations.

Carty[10] observed that formylmethylcobalamin was photolabile, decomposing aerobically and anaerobically to hydroxocobalamin and acetaldehyde. Addition of dilute acid decomposed formylmethylcobalamin to aquocobalamin. The dicyano complex was formed when sodium cyanide was added to either of the above two decomposition products.

The kinetics for the acid decomposition of formylmethylcobalamin made by each of the synthetic routes (Scheme II) were identical. Decomposition was followed by observing the increase in optical absorption at 350 nm, the γ band[21] of aquocobalamin, at pH 3.6, 4.2, 5.3, 5.8, 6.2, 6.5, and 6.8. In each case an aqueous solution of formylmethylcobalamin at neutral pH was diluted tenfold in 0.1 M buffer and the increase in OD at 350 nm was recorded vs. time; the end point was determined as the OD given by the solution at each pH after photolysis. Pseudo-first-order kinetics were observed at each pH. At pH 3.6 and 4.2 decomposition was complete within 3 min of mixing. Semilogarithmic plots of $OD_\infty - OD_t$ vs. time for the acid decomposition of formylmethylcobalamin at the other pH values were linear. A representative plot is shown in Figure 3. Half-times ($t_{1/2}$) of decomposition of formylmethylcobalamin, prepared by two of the routes, at the pH values studied are summarized in Table I. The observed pseudo-first-order rate constants (k_{obsd}), calculated from the half-times using the relationship $k_{obsd} = \ln 2/t_{1/2}$, are shown in Table II.

Thus, the rate law of decomposition is $d[\text{formylmethylcobalamin}]/dt = -k[\text{formylmethylcobalamin}][H_3O^+]$. The second-order rate constant was determined from a plot of the observed pseudo-first-order rate constants against the hydrogen-ion concentration. One of these plots is shown in Figure 4. The second-order rate constant, which is the slope in a plot such as Figure 4, is 2020 M^{-1} s^{-1} for formylmethylcobalamin prepared by oxidation of 2,3-dihydroxypropylcobalamin and 2060 M^{-1} s^{-1} for that prepared by hydrolysis of the cyclic acetal cobalamin. The acid sensitivity and bimolecularity of the decompositions can be readily rationalized by the mechanism shown in Scheme III.

The results show that formylmethylcobalamin is acid labile, and this may be responsible for the inability, so far, to observe the compound during the coenzyme B_{12}-dependent dioldehydrase reaction.

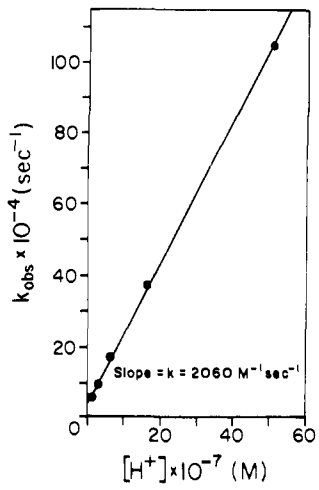


Figure 4. Observed pseudo-first-order rate constants vs. the hydrogen-ion concentration for the decomposition of formylmethylcobalamin, prepared by hydrolysis of 1,3-dioxa-2-cyclopentylmethylcobalamin.

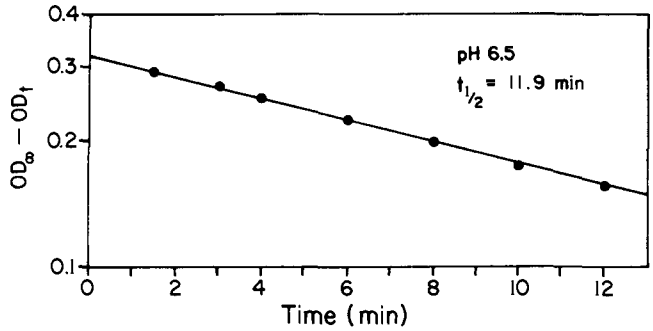
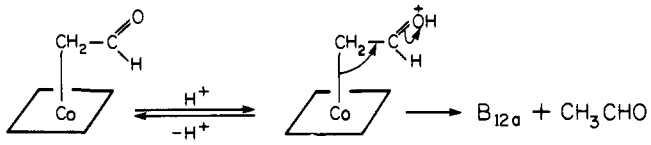


Figure 5. Semilogarithmic plot of $OD_\infty - OD_t$ vs. time for the decomposition at pH 6.5 of 1,3-dioxa-2-cyclopentylmethylcobalamin prepared by the olefin method.

Scheme III. Proposed Mechanism for the Acid-Catalyzed Decomposition of Formylmethylcobalamin



Decomposition of Acetal Cobalamins. The kinetics of the acid decomposition of 1,3-dioxa-2-cyclopentylmethylcobalamin and of 2,2-diethoxyethylcobalamin were studied under the same conditions as those for formylmethylcobalamin.

1,3-Dioxa-2-cyclopentylmethylcobalamin. Semilogarithmic plots of $OD_\infty - OD_t$ vs. time for the decomposition of 1,3-dioxa-2-cyclopentylmethylcobalamin, prepared by the oxidative addition of bromoethylidene ethylene glycol to B_{12s} and by the olefin addition to hydroxocobalamin (routes c and b, Scheme I), were linear. A representative plot is shown in Figure 5. Half-times ($t_{1/2}$) of decomposition at the pH values studied are nearly identical with those found for formylmethylcobalamin (Table I) and are summarized in Table III. The observed pseudo-first-order rate constants (k_{obsd}) at the hydrogen-ion concentrations studied are shown in Table IV. The rate law of decomposition is $d[\text{cobalamin}]/dt = -k[\text{cobalamin}][H_3O^+]$. A representative plot of observed pseudo-first-order rate constants vs. the hydrogen-ion concentration is shown in Figure 6. The second-order rate constant is 2020 M^{-1} s^{-1} for the cyclic acetal cobalamin prepared by oxidative addition to B_{12s} and 2120 M^{-1} s^{-1} for that prepared by the olefin method.

Table II. Observed Pseudo-First-Order Rate Constants for the Decomposition of Formylmethylcobalamin

	$k_{obsd} \times 10^{-4}$, s^{-1}	
[H$^+$] × 10^{-7} M	From oxidation of 2,3-dihydroxypropylcobalamin	From hydrolysis of 1,3-dioxa-2-cyclopentylmethylcobalamin
50.10	105	105
15.80		37.3
6.31	17.2	17.2
3.16	9.5	9.5
1.58	5.3	5.4

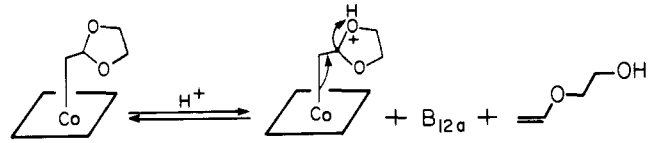
Table III. Half-Times of Decomposition of 1,3-Dioxa-2-cyclopentylmethylcobalamin at Various pH Values

	$t_{1/2}$, min	
pH	Oxidative addition to B_{12s}	Olefin method
5.3	1.1	1.0
5.8	3.3	3.0
6.2	7.0	6.7
6.5	12.2	11.9
6.8	21.8	21.2

Table IV. Observed Pseudo-First-Order Rate Constants for Decomposition of 1,3-Dioxa-2-cyclopentylmethylcobalamin at Various Hydrogen-Ion Concentrations

	$k_{obsd} \times 10^{-4}$, s^{-1}	
[H$^+$] × 10^{-7} M	Oxidative addition to B_{12s}	Olefin method
50.10	105	115
15.80	35.0	38.5
6.31	16.5	17.2
3.16	9.5	9.7
1.58	5.3	5.5

Scheme IV



Mechanistic Implications. These data show that 1,3-dioxa-2-cyclopentylmethylcobalamin (**4**) hydrolyzes with essentially the same rate constant as formylmethylcobalamin (**1**) and can be explained in two ways. Either the acetal hydrolyzes in a fast step to formylmethylcobalamin, which then undergoes a rate-determining cobalt-carbon bond cleavage, or the rate of direct cobalt-carbon bond cleavage of the acetal is coincidentally identical with that for the decomposition of the aldehyde.

If the mechanism involves the two-step decomposition, there should be an accumulation of aldehyde. This was shown to be the case when the hydrolysis of the cyclic acetal cobalamin in buffers of varying pH was followed by TLC (vide supra). At pH 9.0 a maximum of about 25% aldehyde was observed; at lower pH, about 10% aldehyde was seen. As the pH was increased, decomposition of formylmethylcobalamin became slower, and since it is the rate-limiting step, a larger steady state concentration of this cobalamin was observed.

If the mechanism proceeds via direct cobalt-carbon bond cleavage of the acetal then B_{12b}, 2-hydroxyethyl vinyl ether, would be produced (Scheme IV) instead of B_{12b}, acetaldehyde

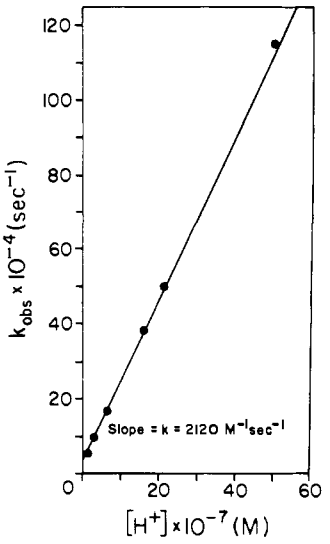


Figure 6. Observed pseudo-first-order rate constants vs. hydrogen-ion concentration for the decomposition of 1,3-dioxa-2-cyclopentylmethylcobalamin prepared by the olefin method.

Table V. Half-Times of Decomposition of 2,2-Diethoxyethylcobalamin at Various pH Values

	$t_{1/2}$, min	
pH	Oxidative addition to B_{12s}	Olefin method
6.2		0.42
6.5		0.75
6.8	1.4	1.5
7.0		2.3
7.3	3.8	3.9
7.7	9.6	9.8
8.0	21.3	21.4

Table VI. Observed Pseudo-First-Order Rate Constants for the Rapid Decomposition of 2,2-Diethoxyethylcobalamin at Various Hydrogen-Ion Concentrations

	$k_{obsd} \times 10^{-4}$, s^{-1}	
[H$^+$] $\times 10^{-8}$ M	Oxidative addition to B_{12s}	Olefin method
63.1		275
31.6		154
15.8	80.1	77.0
10.0		50.2
5.0	30.4	29.6
2.0	11.5	11.8
1.0	5.4	5.4

and ethylene glycol, as expected from the two-step mechanism.

When, however, 1,3-dioxa-2-cyclopentylmethylcobalamin was decomposed in phosphate buffer at pH 6.5 or 6.8, 2-hydroxyethyl vinyl ether was also detected, suggesting that this acetal decomposes both by direct cobalt-carbon bond cleavage and by hydrolysis to the aldehyde followed by cobalt-carbon cleavage.

2,2-Diethoxyethylcobalamin. The kinetics for the hydrolysis of 2,2-diethoxyethylcobalamin were also measured and it was found that this cobalamin decomposed more rapidly than the cyclic acetal. Half-times for decomposition at the pH values studied are summarized in Table V. Semilogarithmic plots of $OD_\infty - OD_t$ vs. time for the diethyl acetal cobalamin prepared by oxidative addition of bromoacetal to B_{12s} and by the olefin method[9] were linear for the first half-life; then an upward

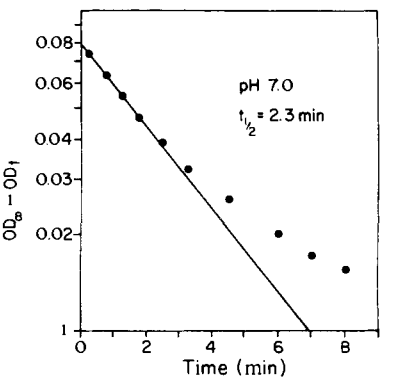


Figure 7. Semilogarithmic plot of $OD_\infty - OD_t$ vs. time for the decomposition at pH 7.0 of 2,2-diethoxyethylcobalamin prepared by the olefin method.

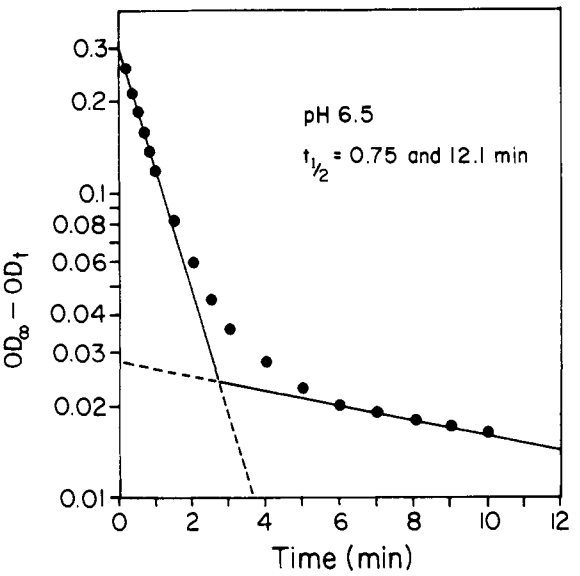


Figure 8. Semilogarithmic plot of $OD_\infty - OD_t$ vs. time for the decomposition at pH 6.5 of 2,2-diethoxyethylcobalamin prepared by the olefin method.

deviation (i.e., the reaction proceeded more slowly than expected) occurred (see Figure 7).

Thus, it appears that these hydrolyses do not follow pseudo-first-order kinetics. However, if the kinetics are then followed for more than 10 half-lives, this initial rapid first-order decomposition converts to the slower first-order kinetics observed for the decomposition of formylmethylcobalamin. An example of this is shown in Figure 8. The half-times observed after 10 half-lives of decomposition of 2,2-diethoxyethylcobalamin were identical with the half-times for decomposition of formylmethylcobalamin at the corresponding pH. Therefore, it appears that the diethyl acetal cobalamin also decomposes by two different first-order pathways. The rapid decomposition route gives the observed first-order rate constants shown in Table VI for the various hydrogen-ion concentrations studied. The rate law for this decomposition is $d[\text{cobalamin}]/dt = -k_1[\text{cobalamin}][H_3O^+]$. A representative plot of k_{obsd} vs. $[H_3O^+]$ for 2,2-diethoxyethylcobalamin is shown in Figure 9. The second-order rate constant is 51 500 M^{-1} s^{-1} for the rapid decomposition of the cobalamin prepared by oxidative addition and 48 000 M^{-1} s^{-1} for that prepared by the olefin route.

The rate constants reported by Schrauzer et al.[6] for 2,2-diethoxyethyl- and 1,3-dioxa-2-cyclopentylmethylcobalamin are 2050 and 6.0 s^{-1},[22] respectively.[23]

The hydrolysis of the diethyl acetal cobalamin at various pH's was followed by TLC, as in the case of the cyclic acetal

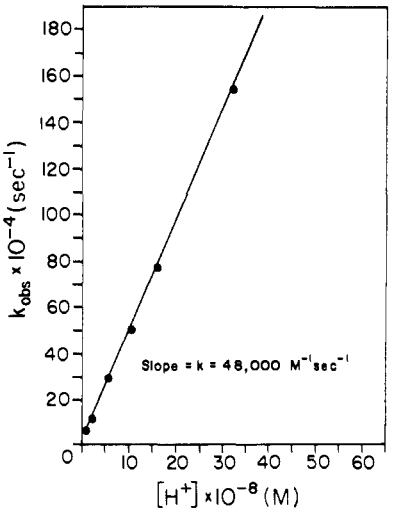
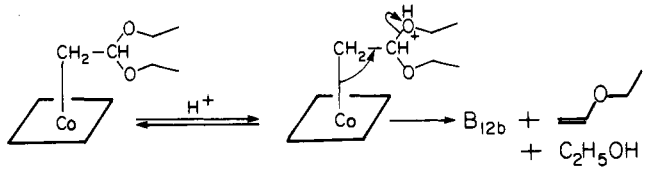


Figure 9. Observed pseudo-first-order rate constants vs. hydrogen-ion concentration for the rapid decomposition of 2,2-diethoxyethylcobalamin prepared by the olefin method.

cobalamin. Decomposition was more rapid than for the cyclic acetal and only a small amount of formylmethylcobalamin was observed. Since hydrolysis of the aldehyde cobalamin is much slower than direct hydrolysis of the acetal (as evidenced by the kinetic data), the small amount of formylmethylcobalamin observed must indicate that only a small amount is formed. The rapid decomposition of 2,2-diethoxyethylcobalamin, then, probably arises from direct Co-C bond heterolysis. If this should occur, the organic ligand would be eliminated to give ethyl vinyl ether, as shown in Scheme V. GLC of the reaction mixture revealed the presence of ethyl vinyl ether. Acetaldehyde and ethanol were also detected, but under the reaction conditions, ethyl vinyl ether slowly hydrolyses to these two components. Schrauzer reported[6] that acid hydrolysis of 2,2-diethoxyethylcobalamin produced acetaldehyde, ethanol, and acetal (the diethyl acetal of acetaldehyde). No mention of ethyl vinyl ether was made. Since the driving force for the cobalt-carbon bond cleavage must arise from loss of the β-leaving group, it is difficult to imagine why that bond should break heterolytically to form acetal. No acetal was detected in the above reaction.

Scheme V. Proposed Mechanism for the Chiral Cobalt Carbon Cleavage of 2,2-Diethoxyethylcobalamin



The observation that the kinetics of direct cobalt-carbon cleavage of 1,3-dioxa-2-cyclopentylmethylcobalamin are the same as those for cobalt-carbon bond cleavage of formylmethylcobalamin can be rationalized. Fife and Jao[24] found that the diethylacetal of benzaldehyde hydrolyzed 28 times faster than the corresponding cyclic acetal, and we have observed that the diethyl acetal cobalamin hydrolyzes 25 times faster than formylmethylcobalamin or the cyclic acetal cobalamin.

Mechanistic Implications. The data presented suggest two different pathways for acid decomposition of the cobalamin acetals: direct cobalt-carbon bond cleavage to B_{12b} and the vinyl ether and normal acetal hydrolysis to formylmethylcobalamin, followed by rate-determining fission of the Co-C bond to B_{12b} and acetaldehyde (Scheme VI).[25]

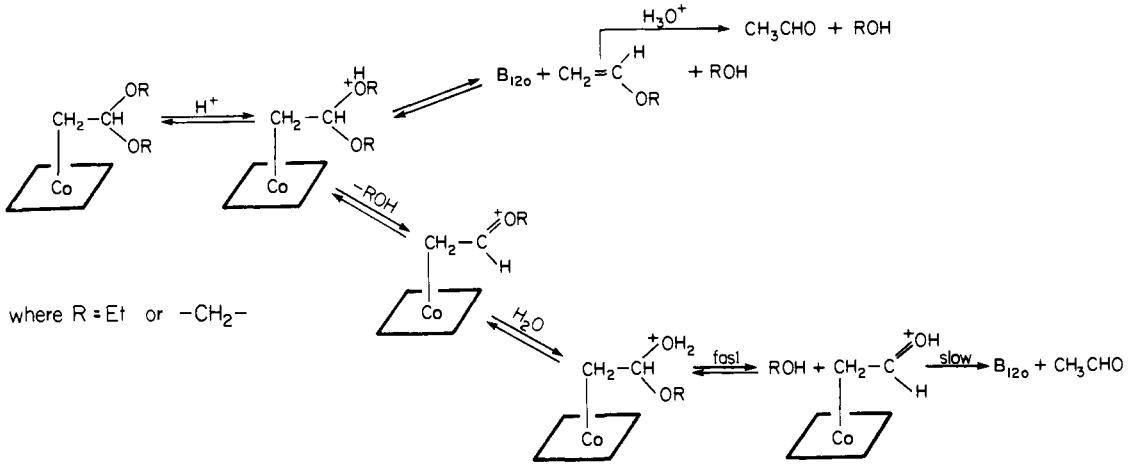
Experimental Section

2,3-Dihydroxypropylcobalamin.[26] To a deaerated solution of hydroxocobalamin (50 mg) in water (5 ml) containing a catalytic amount of cobalt nitrate (<1 mg) was syringed a solution of sodium borohydride in water [100 μl of a 10% (2.64 M) solution]. After 30 min 3-chloro-1,2-propanediol (20 μl) was added to the brown solution and after stirring for 45 min, the excess borohydride was quenched by the addition of acetone (0.1 ml). The red solution was purified by phenol extraction,[27] concentrated to 0.5 ml, and chromatographed in the dark (preparative layer chromatography; silica gel; 100:99:1 1-propanol-water-concentrated aqueous ammonia). A broad band (R_f 0.29–0.52) was eluted with the chromatographic solution, which afforded 38 mg of 2,3-dihydroxypropylcobalamin: λ^{H_2O} 264 nm (ε 21 800), 281 (19 700), 288 sh (18 600), 303 (13 300), 317 (13 500), 338 sh (12 600), 359 sh (11 600), 375 (11 100), 434 sh (4840), 492 sh (7540), 522 (8790).

Formylmethylcobalamin. a. Oxidation of 2,3-Dihydroxypropylcobalamin.[26] To a solution of 2,3-dihydroxypropylcobalamin (4 mg) in 0.5 N aqueous ammonia (0.5 ml) was added an aqueous solution of 0.25 M sodium metaperiodate (1.0 ml). After 20 min the red solution was purified by gel filtration on a column (2.3 × 62 cm) of Bio-Gel P-2 (200–400 mesh)[12] preequilibrated with 0.5 N aqueous ammonia. The first red component was collected and the ammonia removed by concentration in vacuo below room temperature to one-tenth the original volume.[28] The cobalamin was isolated as a solid by evaporation of the solvent or by precipitation with acetone (acetone is often acidic and should be neutralized with aqueous ammonia).

b. Hydrolysis of 1,3-Dioxa-2-cyclopentylmethylcobalamin. 1,3-Dioxa-2-cyclopentylmethylcobalamin (2 mg) was added to each of six 0.5-dram vials, dissolved in pH 9.0 buffer[29] (2 drops), and allowed to stand capped in the dark for 48 h. The contents of each vial were chromatographed on a separate Brinkmann cellulose TLC plate [20 cm^2, 1-butanol–ethanol–water (10:3:7) containing 0.5% concentrated

Scheme VI. Proposed Mechanism for the Acid-Catalyzed Decomposition of the Cobalamin Acetals



aqueous ammonia]. The second band (R_f 0.51) of each plate was transferred into 0.5 N aqueous ammonia (4 ml),[30] stirred, and filtered by suction. The pink filtrate was concentrated in vacuo, while keeping the temperature below 25°, to 0.5 ml[28] and purified on Bio-Gel P-2, as described above.

c. Reaction of Hydroxocobalamin and Ethyl Vinyl Ether. To a deaerated solution of hydroxocobalamin (16 mg) in 95% ethanol (4 ml) was added triethylamine (a drop) and then ethyl vinyl ether (0.12 ml). The red solution was allowed to stand in the dark for 6 days. After concentration under reduced pressure at room temperature to 0.5 ml, 1 drop of 0.5 N aqueous ammonia was added to stabilize the cobalamins. Chromatography on eight separate Brinkmann cellulose TLC plates [20 cm^2, 1-butanol–ethanol–water (10:3:7) containing 0.5% concentrated aqueous ammonia] separated the mixture into three components. The major band (R_f 0.51) of each plate was scraped[30] into 0.5 N aqueous NH_3 (4 ml), stirred, and filtered by suction. The red filtrate was concentrated in vacuo, while keeping the temperature below 25°, to one-tenth the original volume.[28]

All three procedures gave a product with the identical optical (see Figure 1) and NMR spectra (see Figure 2): λ^{H_2O} 262 nm (ϵ 26 300), 278 (23 300), 288 sh (20 000), 334 (15 100), 370 (13 900), 430 (5420), 496 sh (6540), 526 (8370), 550 sh (7120); ^1H NMR see Figure 2.

Kinetics of the Decomposition of Formylmethylcobalamin. An aqueous solution of formylmethylcobalamin at neutral pH was diluted tenfold in 0.1 M buffer[31] at 25°. Decomposition was followed (at 25 ± 0.1°) by observing the increase in optical absorption at 350 nm with time for pH 3.6, 4.2, 5.3, 5.8, 6.2, 6.5, and 6.8. The end point was determined by photolysis with a 100-W light bulb held 1 cm away for 1 min.

GLC Analysis of the Decomposition of 1,3-Dioxa-2-cyclopentylmethylcobalamin. 1,3-Dioxa-2-cyclopentylmethylcobalamin[9] (2 mg) was allowed to decompose in pH 6.5 buffer[31] (1 drop) for 30 min or pH 6.8 buffer[31] for 2 h. A 5-μl aliquot was analyzed by GLC using a flame ionization detector (6 ft, 5% Carbowax 20 M; 80–100 W; 65°; 10 cm/18 s) and two major products were observed: acetaldehyde (t_r 2.0 min) and 2-hydroxyethyl vinyl ether (t_r 21.0 min).

GLC Analysis of the Decomposition of 2,2-Diethoxyethylcobalamin. 2,2-Diethoxyethylcobalamin (1.5 mg) was allowed to decompose in pH 6.8 buffer (1 drop) for 30 min. A 10-μl aliquot was analyzed by using a thermal conductivity detector (20 ft, 10% β,β-oxydipropionitrile; 80–100 WHP; 50°; 10 cm/14.5 s) and three products were observed: ethyl vinyl ether (t_r 3.8 min), acetaldehyde (t_r 7.2 min), and ethanol (t_r 19.2 min).

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References and Notes

(1) (a) National Institutes of Health Predoctoral Trainee, 1971–1974; Harvard University; (b) The University of British Columbia.
(2) R. H. Abeles, *Adv. Chem., Ser.*, **No. 100**, 346 (1971).
(3) T. J. Carty, B. M. Babior, and R. H. Abeles, *J. Biol. Chem.*, **246**, 6313 (1971).
(4) B. M. Babior, *J. Biol. Chem.*, **245**, 1755 (1970).
(5) G. N. Schrauzer and J. W. Silbert, *J. Am. Chem. Soc.*, **92**, 1022 (1970).
(6) G. N. Schrauzer, W. J. Michaely, and R. J. Holland, *J. Am. Chem. Soc.*, **95**, 2024 (1973).
(7) R. B. Silverman and D. Dolphin, *J. Am. Chem. Soc.*, **96**, 7094 (1974).
(8) R. B. Silverman, D. Dolphin, T. J. Carty, E. K. Krodel, and R. H. Abeles, *J. Am. Chem. Soc.*, **96**, 7096 (1974).
(9) R. B. Silverman and D. Dolphin, *J. Am. Chem. Soc.*, preceding paper in this issue.
(10) T. J. Carty, Ph.D. Thesis, Brandeis University, Waltham, Mass., 1973.
(11) E. R. Frisell, L. A. Meech, and C. G. MacKenzie, *J. Biol. Chem.*, **207**, 709 (1954).
(12) Bio-Rad Laboratories brand of polyacrylamide gel beads.
(13) 1-Butanol–ethanol–water (10:3:7) containing 0.5% concentrated aqueous ammonia.
(14) Good results were obtained using EM plates distributed by Brinkmann as Celplate-22, which are precoated with microcrystalline cellulose without indicator.
(15) J. D. Brodie and M. Poe, *Biochemistry*, **11**, 2534 (1972).
(16) J. D. Brodie and M. Poe, *Biochemistry*, **10**, 914 (1971).
(17) H. A. O. Hill, B. E. Mann, J. M. Pratt, and R. J. P. Williams, *J. Chem. Soc. A*, 564 (1968).
(18) In order to make a comparison of chemical shifts, the compound used as a reference must be soluble in the same solvent as the compound with which it is being compared. Since the effect of deshielding caused by proximity of a more electronegative group (e.g., OH) on a proton more than two saturated carbon atoms away is slight,[19] the resonance of the methyl protons in 1-butanol should approximate that of methyl protons in *n*-butane.
(19) J. R. Dyer, "Applications of Absorption Spectroscopy of Organic Compounds", Prentice-Hall, Englewood Cliffs, N.J., 1965, p 83.
(20) W. P. Jencks, "Catalysis in Chemistry and Enzymology", McGraw-Hill, New York, N.Y., 1969.
(21) The most intense band in the near-ultraviolet.
(22) The first-order dimensions reported[6] for the second-order rate constants are clearly in error.
(23) In the decomposition of formylmethylcobalamin, we observed that the half-life could vary by a factor of 2 by the addition of minute amounts of $NaIO_3$ or $NaIO_4$.
(24) T. H. Fife and L. K. Jao, *J. Org. Chem.*, **30**, 1492 (1965).
(25) The general mechanism for acetal hydrolysis, as shown in Scheme VI, is well documented: C. K. Ingold, "Structure and Mechanism in Organic Chemistry", 2nd ed, Cornell University Press, Ithaca, N.Y., 1969, p 447.
(26) Modification of the procedure of Carty.[10]
(27) D. Dolphin, *Methods Enzymol.*, **18C**, 34 (1971).
(28) Decomposition of the product occurs as soon as the ammonia is removed.
(29) A standard 0.025 M borax–0.1 M HCl buffer was used: "Handbook of Chemistry and Physics", 46th ed, Chemical Rubber Publishing Co., Cleveland, Ohio, 1965, p D-73.
(30) The bands were transferred while still wet in order to maintain a basic environment.
(31) Acetate buffers, prepared by mixing 0.1 M solutions of sodium acetate and acetic acid, were used for runs with pH <6; phosphate buffers, prepared by mixing 0.1 M solutions of mono- and dibasic sodium phosphate, were used for runs with pH >6.