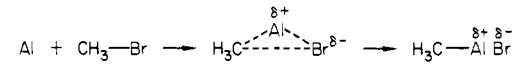
can be rationalized by low IPs for all but higher potential energies due to heat of vaporization differences.[21]

Since low IP does seem to be important, a polar reaction mechanism would seem reasonable, perhaps as follows for Al:



Complete electron transfer may occur during the reaction, and only one Al (or Ga or In) atom is involved. In the case of Mg the reaction must be more complex and Mg clusters may be involved. In the case of transition metals, high IPs, low M-Br bond strengths, and unfilled d orbitals lead to the preference of CH_3Br---M complexation rather than oxidative addition.

Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for the support of this work. Numerous discussions with Dr. R. Hauge of Rice University and G. A. Ozin of the University of Toronto have been very helpful.

Registry No. CH_3Br, 74-83-9; Al, 7429-90-5; Ga, 7440-55-3; In, 7440-74-6; Mg, 7439-95-4; Fe, 7439-89-6; Cu, 7440-50-8; Pb, 7439-92-1; Co, 7440-48-4; Ni, 7440-02-0; Pd, 7440-05-3; Ag, 7440-22-4; Au, 7440-57-5; Tl, 7440-28-0; Ge, 7440-56-4; Sn, 7440-31-5.

(21) Professor P. S. Skell has found a similar reactivity dependency in $M-H_2O$ reactions (28th IUPAC Congress, Aug 16-21, 1981, Vancouver, B.C., Canada, paper IN 002) and has used the term "atom potential" to describe this inherent reactivity.

Mechanism of the Cyanide-Induced Formation of Methyl Acetate from [(Methoxycarbonyl)methyl]cobalamin[1]

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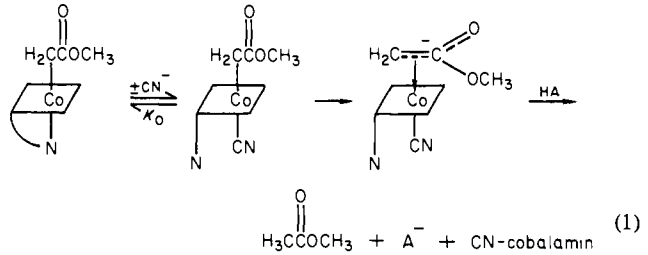
Contribution No. 1389 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254. *Received July 6, 1981*

Abstract: The addition of cyanide to [(methoxycarbonyl)methyl]cobalamin (MeO_2CCH_2Cbl, $MCMB_{12}$) results in the rapid, reversible formation of a cyanide addition compound (K_0 = 0.044 M), followed by cleavage of the carbon-cobalt bond and protonation to give methyl acetate. An analogous, slower reaction occurs with (carboxymethyl)cobalamin (K_0 = 0.77 M). At pH <8 the reaction of $MCMB_{12}$ occurs through specific-acid catalysis, with k_{DOD}/k_{HOH} = 2.7. This inverse isotope effect, the small normal solvent isotope effect of k_{HOH}/k_{DOD} = 1.4 at pH >9, and the absence of general-acid catalysis show that protonation of the leaving carbon atom does not occur in the rate-determining step. There is discrimination against the incorporation of tritium from the solvent into the product, with $[^1H]/[^3H]$ = 4.3-4.9 at pH >9 and $[^1H]/[^3H]$ = 8.6 at pH 1. These isotope effects establish a stepwise reaction mechanism, in which protonation of the intermediate occurs after the rate-determining step. α-Deuterium isotope effects of k_{2H}/k_{2D} = 1.19 ± 0.06 at pH 11.4 and k_{2H}/k_{2D} = 1.11 ± 0.06 at pH 0.9 demonstrate a significant change toward sp^2 hybridization of the α-carbon atom in the rate-determining transition state. Partial inversion of configuration at the α-carbon atom in the methyl acetate produced from (*R*)-$[^2H]MCMB_{12}$ in tritiated water shows that protonation must occur, at least in part, before separation of the enolate from cobalamin and provides evidence that the intermediate is a π complex with a lifetime of $>10^{-6}$ s. The small equilibrium isotope effect of K_{HCN}/K_{DCN} = 2.3 for the dissociation of hydrogen cyanide is attributed to the low bending frequency of the C-H bond.

The ability of adenosylcobalamin and other alkylcobalamins to undergo both cleavage and formation of carbon-cobalt bonds with remarkable ease is probably responsible for the biological activity of these compounds in mediating reactions that cannot be brought about by the simpler chemistry utilized by other coenzymes. This facile carbon-cobalt bond cleavage occurs to give electron-deficient, radical, or carbanionic products, depending on the ability of substituents on carbon to stabilize one or another of these species. The ease with which these different reactions occur reflects the easy accessibility of the three oxidation states of the products, Cob(I)alamin, Cob(II)alamin, and Cob(III)-alamin, that are formed in the three modes of cleavage. The expulsion of leaving groups with a pair of electrons, leaving behind a Cob(III)alamin, is greatly facilitated by the addition to the trans position of cyanide anion or other groups that increase the electron density on cobalt.[2]

The experiments described here were undertaken in order to learn more about the chemistry of heterolytic carbon-cobalt bond cleavage. Adenosylcobalamin and [(methoxycarbonyl)methyl]-cobalamin ($MCMB_{12}$) undergo cyanide-induced fragmentations that give Cob(III)alamin products and appear to proceed through the electron-rich, carbanionic class of mechanism.[3-5] It has been proposed that the cleavage of $MCMB_{12}$, to give methyl acetate as the product, proceeds through a direct, front-side displacement to expel the enolate anion of methyl acetate[4] or through an intermediate adduct in which cyanide ion adds to the α (trans) position, replacing the dimethylbenzimidazole ligand[5] (eq 1, in



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(1) Supported by grants from the National Institutes of Health (Grant GM-21633) to R.H.A. and the National Science Foundation (Grant PCM-77-08369) and National Institutes of Health (Grant GM-20888) to W.P.J. W.W.R. was supported by a training grant from the National Institutes of Health (Grant 5 R01 GM-00212).

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this and subsequent equations the corrin ring of cobalamin is represented by a square and the dimethylbenzimidazole ligand by N). Our results show that the reaction proceeds in at least three steps, with rapid formation of the cyanide adduct at equilibrium (eq 1, first cyanide adduct), followed by cleavage of the C–Co bond to give the enolate or enol of methyl acetate in the rate-determining step, and then protonation to give methyl acetate. The remarkable result is that protonation occurs preferentially on one face of the enolate. This requires that protonation of the enolate occurs before separation from the cobalamin and provides evidence for cleavage of the carbon–cobalt σ bond to give an intermediate π complex with a lifetime of $>10^{-6}$ s. A preliminary communication of this work has appeared.[6]

Experimental Section

Materials. Hydroxocobalamin (OHB_{12}) and cyanocobalamin (CNB_{12}) were kindly given by Glaxo Laboratories and were used without further purification. (*R*)-[^{3}H,^{2}H]Acetic acid was a gift from Dr. J. W. Cornforth. [(*R*)-(Methoxycarbonyl)deuteriomethyl]cobalamin [(*R*)-[^{2}H]$MCMB_{12}$] was prepared by Fenton.[5] ^{1}H NMR analysis showed that (*R*)-[^{2}H]$MCMB_{12}$ contained <10% hydrogen in the pro-*R* position; i.e., it contains <10% (*S*)-[^{2}H]$MCMB_{12}$ or nondeuterated $MCMB_{12}$. (*R*)-[^{2}H]$MCMB_{12}$ was synthesized by displacing the tolylsulfonyl group of (*S*)-methyl [^{2}H](*p*-tolylsulfonyl)glycolate with cob(I)alamin. Assignment of configuration is based on the assumption that this displacement proceeds with inversion of configuration. This assignment is supported by the recent observation that the reaction of *threo*-3,3-dimethyl[1,2-$^2H_2$]butyl tosylate with cob(I)oxime proceeds with inversion to give (*erythro*-3,3-dimethyl[1,2-$^2H_2$]butyl)cobaloxime.[7] All organic reagents used in kinetic studies and potassium cyanide were purified prior to use;[8] other inorganic reagents were used without further purification. Deuterium oxide (Bio-Rad) was distilled in glass. Radioisotopes were purchased from New England Nuclear and acetic acid was purchased from Stohler Chemical. Enzymes were purchased from Boehringer-Mannheim except for malate dehydrogenase, which was purified from baker's yeast by the method of Dixon and Kornberg.[9] Solutions of hydrogen cyanide were prepared shortly before use by neutralizing aqueous potassium cyanide with 1 equiv or a small excess of hydrochloric acid (caution) and were stored in stoppered flasks.

[(Methoxycarbonyl)methyl]cobalamin ($MCMB_{12}$). $MCMB_{12}$ was prepared according to published procedures.[5,10] Corrins were separated from salt by phenol extraction,[10] concentrated, and applied to a 23 × 1.5 cm CM cellulose column in the H^+ form. Alkylcobalamins were eluted with water, while unreacted aquocobalamin was retained. The eluted cobalamin was concentrated and applied to a 20 × 1.5 cm DEAE cellulose column in the OH^- form. $MCMB_{12}$ was eluted with water, while CMB_{12}, a minor impurity, was retained. Purity was established by (1) paper electrophoresis, (2) paper chromatography, which gave single bands with R_f's, relative to methylcobalamin, identical with those previously reported,[10] and (3) the demonstration that the UV and the visible spectra were identical with those reported previously.[5,10] Based on the starting material, $(CN)_2B_{12}$, a yield of 12% was obtained.

$MeO_2{}^{14}CCH_2Cbl$ ([^{14}C]$MCMB_{12}$). [^{14}C]Iodoacetic acid, $ICH_2{}^{14}COOH$ (0.76 μmol, 10 μCi), was dissolved in 0.20 mL of ether and diluted with 10 μmol of iodoacetic acid in 0.50 mL of ether. Diazomethane, prepared from Diazald (Aldrich),[11] was added until a yellow color persisted. The ether solution was added directly to a 10-mL solution of cob(I)alamin generated from 0.074 mmol of aquocobalamin. After the reaction had proceeded for 2 min, 0.2 mL of methyl chloroacetate was added. $MCMB_{12}$ was purified and its purity was established as described above. Strip scanning with a Tracer Lab π4 scanner after electrophoresis and chromatography revealed the presence of only one radioactive peak. Based on conversion to $(CN)_2B_{12}$, 8.3 μmol of [^{14}C]-$MCMB_{12}$ (11% from OHB_{12}) of specific activity 0.58 μCi/μmol was isolated.

$MeOCC^2H_2Cbl$ ([$^2H_2$]$MCMB_{12}$). Tetradeuterioacetic acid (2.5 mL, 0.04 mol) was stirred with 0.25 mL of phosphorous tribromide (2.57 mmol) at room temperature for 30 min. Bromine (2.5 mL, 0.45 mol) was added dropwise and the reaction mixture refluxed for 2 h. The reaction mixture was then cooled with an ice bath and bubbled with N_2 to remove bromine. Crystals formed, which showed no protons by NMR. These were dissolved in 4.0 mL of methanol and refluxed for 2 h. The methanol was removed by rotary evaporation at low temperature, and a brown oil was isolated. The ^{1}H NMR spectra in $CDCl_3$ showed a major singlet due to $BrC^2H_2CO_2Me$ at δ 3.78 and a minor singlet at δ 3.42, whose size was increased by the addition of methanol. As no downfield peak was seen, the α carbon was judged to be greater than 98% deuterated. Methyl dideuteriobromoacetate (0.30 mL) was added to cob(I)alamin prepared from 0.074 mmol of aquocobalamin as described for the synthesis of [^{14}C]$MCMB_{12}$. [$^2H_2$]$MCMB_{12}$ was isolated and its purity established by the chromatographic procedures described above. The yield was 8% based on starting cobalamin (5.7 μmol of product).

(Carboxymethyl)cobalamin (CMB_{12}). Starting from Cbl[1] and iodoacetic acid the procedure was analogous to that described for $MCMB_{12}$. Purity was established by the same criteria described for $MCMB_{12}$.

All manipulations involving alkylcobalamins were performed in a darkened room, with the aid of flashlights. Scintillation counting was done with a Packard 3320 scintillation counter, using Bray's[12] solution as the fluor. Concentrations of corrin species were determined from the absorbance at 367 nm of dicyanocobalamin (ϵ_{364} = 30.4 mM^{-1}),[13] which was formed by photolysis in the presence of cyanide (>10 min, 5 cm from a tensor lamp). The purity of all alkylcobalamins was established by (1) electrophoresis on Whatman 3-mm paper at 10 V/cm, in 0.50 M ammonium hydroxide,[10] (2) descending chromatography on Whatman 3-mm paper with water-saturated *sec*-butyl alcohol,[10] and (3) comparison of the ultraviolet and visible spectra with published spectra.[2,5,10]

Determination of the Chirality of Protonation of the α Carbon of Methyl Acetate. (1) Reaction of (*R*)-[^{2}H]$MCMB_{12}$ with Cyanide. (*R*)-[^{2}H]$MCMB_{12}$ (1.01 μmol in 1.5 mL) was lyophilized and then dissolved in 1.0 mL of a pH 11.3 reaction mixture, 2.0 M in potassium chloride, 0.50 M in potassium cyanide, and containing 445 mCi of [^{3}H]H_2O. The reaction was run for 90 min at 30 °C, then 0.70 mL of 2.0 M sodium hydroxide was added to hydrolyze the methyl acetate, and after 1 min the pH was adjusted to pH 8.5 (indicator paper) with 2 N sulfuric acid. Sodium [^{14}C]acetate (0.20 μCi in 25 mmol) was added and the acetate was isolated by silicic acid chromatography.[14] Column fractions containing acetate were pooled and titrated with base to pH 5.5, the solvent was removed by rotary evaporation, and the residue was taken up in 1.0 mL of water and counted; the total recovery, based on ^{14}C, was 41%.

(2) Conversion of Acetate to Acetyl-CoA. The acetate of the previous section was evaporated to dryness under reduced pressure and added to 10 μL of potassium chloride (10 μmol), 100 μL of trisodium phosphoenolpyruvate (15 μmol), 50 μL of ATP (16 μmol), 30 μL of magnesium chloride (30 μmol), 500 μL of Tris-HCl, pH 8.0 (25 μmol), 50 μL of glycerol, 15 μL of pyruvate kinase (22 units), 50 μL of acetate kinase (17 units), and 10 μL of phosphotransacetylase (10 units). The pH was adjusted to 7.5 with 1.0 M sodium hydroxide. The reaction was started by adding 10.4 mg of trilithium coenzyme A (13 μmol) and run at room temperature. At 20-min intervals, 0.5-μL aliquots were assayed for acetyl-CoA.[15] After 65 min the reaction was stopped by the addition of 2.0 mL of acetone, and the reaction mixture was evaporated to dryness under reduced pressure. Unreacted coenzyme A was oxidized with potassium ferricyanide and the pH was adjusted to 3.5 with 1.0 M hydrochloric acid (indicator paper). Acetyl-CoA was isolated by ascending chromatography on Whatman 3-mm paper with 66/1/33 (v/v/v) isobutyric acid/reagent grade ammonium hydroxide/water. Acetyl-CoA was located by UV absorption, eluted with water, and washed with ether. The solution was then taken to 1.0 mL, and samples were counted and frozen. Based on ^{14}C the yield from acetate was 30%.

(3) Conversion of Acetyl-CoA to (2*S*)-Malate. The frozen solution of acetyl-CoA was thawed and added to 5.0 mL of 0.1 M sodium pyrophosphate (pH 8.1), 0.50 mL of 0.1 M magnesium chloride, 0.10 mL of 0.1 M NaEDTA (pH 8.0), and 2.0 mL of malate synthetase (0.87 units). The reaction was started by adding 0.50 mL of 60 mM sodium glyoxylate and incubated at room temperature. At 20-min intervals 100-μL aliquots were assayed for malate.[16] The reaction was complete in 1 h and the

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solution was heated at 95 °C for 3 min. (2S)-Malic acid (0.5 mmol) and 0.5 mL of 1 M sodium hydroxide were added, and the solution was applied to a 1.5 × 20 cm Dowex AG1-X8 formate column. The column was washed with 30 mL of water and then eluted with 1 M formic acid. The fractions containing malic acid were located,[16] pooled, and concentrated. To ensure total removal of formic acid, malate was evaporated to dryness under reduced pressure. This process was repeated 3 times. The malate was then dissolved in water and neutralized, the solution was brought to a final volume of 5.0 mL, and aliquots were counted. Based on ^{14}C the yield from acetyl-CoA was 80%.

(4) Equilibration of Malate with Fumarase. To 2.0 mL (0.16 mmol) of sodium malate was added 0.20 mL of 0.5 M potassium phosphate, and the pH was adjusted to pH 7.0 with 1 M potassium hydroxide. After 20 μL of fumarase (14 units) was added the solution was incubated at 37 °C for 4 h. The reaction was stopped by heating at 95 °C for 3 min, and the solution was applied to a 0.8 × 18 cm Dowex AG1-X8 formate column. The column was eluted as described in the previous section. Malic acid was isolated as before, a solution was taken to 2.00 mL, and aliquots were removed and counted.

Kinetics. Kinetic and equilibrium measurements were performed at 30 °C and ionic strength 1.8 M, maintained with potassium chloride. Kinetic runs were performed in 3.0-mL quartz cuvettes with Teflon stoppers. Experiments with [(methoxycarbonyl)methyl]cobalamin ($MCMB_{12}$) were followed at 367 nm (pH >7), 361 nm (pH 2–7), or 359 nm (pH <2); experiments with (carboxymethyl)cobalamin (CMB_{12}) were followed at 590 nm. All experiments were performed under pseudo-first-order conditions with a large excess of cyanide. Pseudo-first-order rate constants were determined either from plots of $\ln |A_\infty - A_t|$ against time, for reactions that were followed for 3–4 half-times, or by dividing the total absorbance change by the initial rate of absorbance change, for reactions that were followed to <5% completion. In both cases the absorbance at infinite time was determined by photolysis of the reaction mixture. This procedure was shown to produce a spectrum identical with that produced by the nonphotolytic reaction at infinite time. Determination of pH was carried out at room temperature with a Radiometer 26 pH meter with a B electrode and calibrated with standard buffers or with 0.10 M hydrochloric acid at pH 1.10.[17] The pH values of highly radioactive solutions were determined by using identical reaction mixtures without radioactive material.

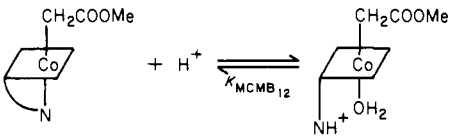
Solvent deuterium isotope effects were determined from parallel experiments in water and 97% deuterium oxide under conditions in which the rates are pH independent. The solvent deuterium isotope effect on the ionization of hydrogen cyanide was measured as described previously.[18] A value of $\Delta pK = 0.36$ was obtained from the difference between the pK_a of hydrogen cyanide (buffers) and *p*-bromophenol (indicator) in water and in deuterium oxide, which was determined spectroscopically, and the value of $\Delta pK = 0.59$ for *p*-bromophenol.[19] The isotope effect was also determined from the measured pH values of buffer solutions of known acid–base ratio and an observed pH meter correction for 97% deuterium oxide of 0.36 ± 0.01 (average of 12 measurements with dilute hydrochloric acid); this value differs slightly from the value of 0.39 calculated from the data of Salomaa et al.[20] Values of $\Delta pK = 0.35$ for hydrogen cyanide and $\Delta pK = 0.57$ for *p*-bromophenol were obtained by this procedure.

The tritium selection isotope effect at pH 11.3 was determined by mixing 2.67 μmol of $MCMB_{12}$ in 500 μL of water, 0.80 mmol of potassium chloride, 200 μL of 5.0 M potassium cyanide, 200 μL of [^3H]H_2O (195 mCi), 5 μL of sodium [^{14}C]acetate in ethanol (0.50 μCi), and water to give a total volume of 1.0 mL. After incubation at 30 °C for 180 min (5 half-times) 0.10 mL of 3.5 M potassium hydroxide was added to hydrolyze methyl acetate and after 1 min the pH was adjusted to 8.5 (indicator paper) with 1 M hydrochloric acid. After addition of carrier sodium acetate (25 mmol), the sample was shell frozen and distilled bulb-to-bulb in a closed system under reduced pressure. The acetate was separated from the residue by silicic acid chromatography,[14] using 3% (v/v) butanol/chloroform. The tritium selection isotope effect at pH 1.0 was determined by mixing 1.94 μmol of $MCMB_{12}$ in 500 μL of water, 200 μL of [^3H]H_2O (212 mCi), 100 μL of 1 M hydrochloric acid, 100 μL of 0.30 M potassium cyanide, 5 μL of sodium [^{14}C]acetate in ethanol (0.35 μCi), and water to give a total volume of 1.0 mL. After incubation at 30 °C for 15 min (>6 half-times) 0.25 mL of 3.5 M potassium hydroxide was added and after 1 min the pH was adjusted to 8.5 with hydrochloric acid. The acetate was isolated as described above.

The predominant product of the cyanide-induced cleavage of $MCMB_{12}$ was shown to be methyl acetate rather than acetate by ether extraction of reaction mixtures initially containing $MCMB_{12}$ labeled with ^{14}C in the methylene carbon of the acetyl group. After 3 half-times 1.0 mmol of sodium acetate, 0.1 mL of methyl acetate, water to 10 mL, and phosphate buffer to give pH 7 were added to the reaction mixture, which was then extracted 5 times with 10 mL of ether. This procedure gave 95–98% extraction of methyl acetate and <2% extraction of sodium acetate. The radioactivity of the aqueous phase was corrected for unreacted [^{14}C]$MCMB_{12}$, which is not extracted into ether.

Results

Properties of $MCMB_{12}$ and CMB_{12}. The ionization of [(methoxycarbonyl)methyl]cobalamin ($MCMB_{12}$), which involves dissociation and protonation of the dimethylbenzimidazole group (eq 2), was found to occur with $pK_a = 2.6$ at 30 °C in 1.8 M

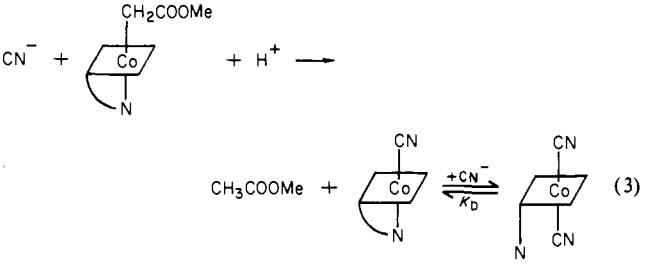


(2)

potassium chloride by spectrophotometric titration at 525 nm. This value is larger than a previously reported value of $pK_a = 2.25$[4] because of the high ionic strength and weak binding of chloride ion to cobalt in the base-off species.[21] (Carboxymethyl)cobalamin (CMB_{12}) has a similar pK_a of 2.20[22] and was found to undergo dissociation of the carboxylic acid group with $pK_a = 7.1$ at 30 °C, ionic strength 1.8 M (KCl) (determined by spectrophotometric titration at 368, 480, and 560 nm). pK_a values of 7.15 and 7.2 have been reported previously[10,22] and were shown to represent ionization of the carboxymethyl group by ^{13}C NMR.[22] The pK_a of (aminoethyl)cobalamin was found to be 10.45 ± 0.05 by titration with base and by spectrophotometric titration at 370, 500, and 550 nm.

There is no detectable ester hydrolysis or cleavage of the carbon–cobalt bond upon incubation of $MCMB_{12}$ at pH 13 in the absence of light and cyanide for 20 h at 30 °C as shown by electrophoresis. This gives an upper limit of $k_{OH} = <2 \times 10^{-5}$ M^{-1} s^{-1} for alkaline hydrolysis of the ester bond. The spectrum of $MCMB_{12}$ in 4.0 M hydrochloric acid is identical with that of protonated $MCMB_{12}$ at pH 1.0 and showed no evidence for cleavage of the carbon–cobalt bond after 5 h at 30 °C.

Cyanide-Induced Carbon–Cobalt Bond Cleavage. In the presence of cyanide, $MCMB_{12}$ undergoes cleavage to give the final products shown in eq 3; CMB_{12} undergoes an analogous reaction. The



(3)

corrin products are an equilibrium mixture of cyanocobalamin and dicyanocobalamin that are related by a dissociation constant of $K_D = 2 \times 10^{-4}$ M;[23] they were identified by comparison of their ultraviolet spectra with the spectra of authentic compounds. At pH values of <2 the end points of the cleavage reaction show a slow drift that is caused by the cyanide-catalyzed isomerization of the initially formed α-cyanocobalamin to an equilibrium mixture of α- and β-cyanocobalamins.[23] Stable end points were obtained by following the kinetics at 359 nm, an isosbestic point for the isomerization. The other product of the reaction was found to

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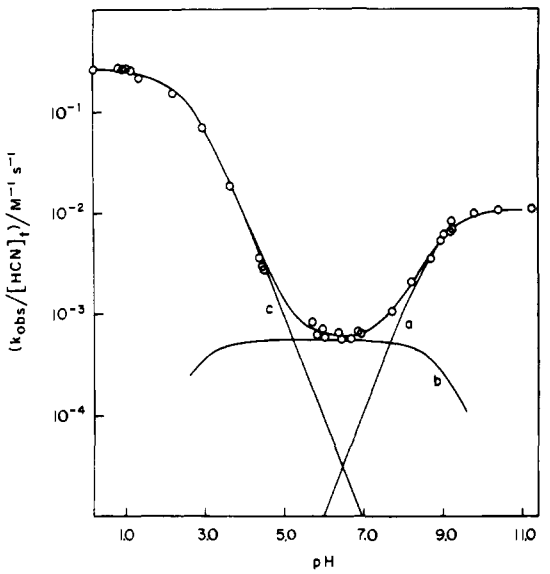


Figure 1. Dependence on pH of the observed second-order rate constants (based on the total concentration of cyanide) for the reaction of $MCMB_{12}$ with cyanide at 30 °C and ionic strength 1.8 M (KCl). The lines a, b, and c are calculated for the contributions to the observed rate constants of k_A, k_B, and k_C, respectively, based on the rate constants in Table I.

Table I. Rate Constants for the Reaction of $MCMB_{12}$ with Cyanide[a]

term	solvent	pH or pD	$[CN^-]/M^b$	runs	value
$k_A/M^{-1}\ s^{-1}$	H_2O	10.4	0.002–0.010	7	1.06×10^{-2}
	D_2O^c	11.2	0.002–0.010	4	0.75×10^{-2}
k_1/s^{-1}	H_2O	11.3	0.008–1.0	10	4.65×10^{-4} [d]
	H_2O	11.4	1.0	8	4.4×10^{-4} [e]
	D_2O^c	11.6	1.0	6	3.1×10^{-4} [e]
$k_B/M^{-2}\ s^{-1}$	H_2O	5.8–7.0	0.02–0.10	10	5.2×10^5
	D_2O^c	6.2–6.9	0.10	5	14.2×10^5
$k_C/M^{-2}\ s^{-1}$	H_2O	0.9–1.7	0.02–0.10	15	2.3×10^8 [f]
	D_2O^c	0.5–1.0	0.03	10	6.1×10^8 [f]

[a] 30 °C and ionic strength 1.8 M (KCl). The rate constants are defined in eq 4 and 6. [b] Total cyanide concentration. [c] 97% deuterium oxide, 3% water. [d] From Figure 2. [e] Observed rate constant at 1.0 M [KCN]. [f] $k_C' = k_C K_{HCN} = 0.24\ M^{-1}\ s^{-1}$ in H_2O and $0.28\ M^{-1}\ s^{-1}$ in D_2O.

be methyl acetate rather than acetate (>85% at pH 4.6 and >95% at pH 9.0) by extraction into ether of products from the cleavage of ^{14}C-labeled $MCMB_{12}$.

The dependence on pH of the observed second-order rate constants, based on total cyanide concentration, shows three pH-independent regions (Figure 1). This behavior is described by the three terms of the rate law of eq 4. Above pH 8 an unca-

$$v = k_A[MCMB_{12}][CN^-] + k_B[MCMB_{12}][CN^-][H^+] + k_C[MCMB_{12}H^+][CN^-][H^+] \quad (4)$$

talyzed reaction of $MCMB_{12}$ and cyanide anion occurs with the rate constant k_A. Near neutral pH the predominant pathway is an acid-catalyzed reaction of $MCMB_{12}$ and cyanide anion, which is described by the rate constant k_B. Below pH 5 the reaction involves acid catalysis of the reaction of cyanide anion with the protonated, base-off species of $MCMB_{12}$ and can be described by the rate constant k_C or by the kinetically equivalent rate constant k_C' (eq 5), which is related to k_C by the dissociation

$$k_C[MCMB_{12}H^+][H^+][CN^-] = k_C'[MCMB_{12}H^+][HCN] \quad (5)$$

constant of hydrogen cyanide. The lines a, b, and c in Figure 1 show the contributions of the different terms in eq 4 to the observed rate constants and are based on the rate and equilibrium constants in Tables I and II.

No general-acid or -base catalysis was detected in buffers of trifluoroethanol (pH 11.24, up to 0.9 M), cacodylic acid (pH 6.05, up to 0.8 M), or acetic acid (pH 4.50, up to 1.3 M). The reaction

Table II. Equilibrium Constants for the Reactions of $MCMB_{12}$ and Cyanide[a]

term	solvent	determinations	value/M	pK_a
K_0	H_2O	10	4.4×10^{-2}	
	D_2O^b	8	4.3×10^{-2}	
$K_{MCMB_{12}}$	H_2O	13	2.5×10^{-3}	2.6
K_{HCN}^c	H_2O	25	1.05×10^{-9}	8.98[d]
	D_2O^b	14	0.46×10^{-9}	9.34[d]

[a] 30 °C and ionic strength 1.8 M (KCl). The equilibrium constants are defined in eq 1 and 8, unless noted. [b] 97% deuterium oxide. [c] Dissociation constant for HCN. [d] ±0.02.

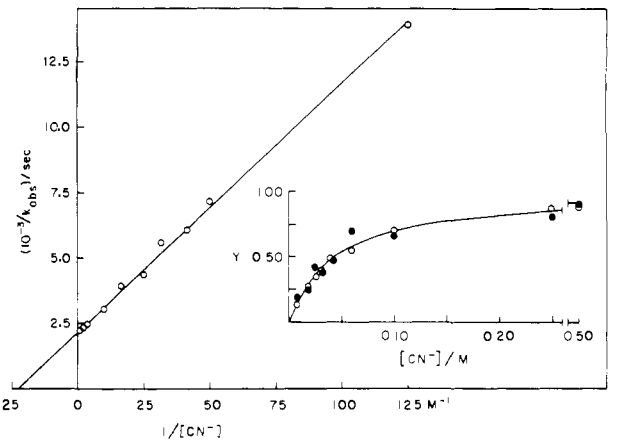
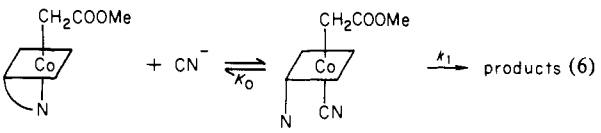


Figure 2. Double-reciprocal plot of the observed rate constants for the reaction of cyanide anion with $MCMB_{12}$ at pH 11.27 at varying cyanide concentrations at 30 °C and ionic strength 1.8 M (KCl). Inset: Dependence on cyanide concentration of the ratio k_{obsd}/k_1 (○) and the ratio $\Delta A/\Delta A_{max}$ (●), where $\Delta A = A_{obsd} - A_{MCMB_{12}}$ and $\Delta A_{max} = A_{MCMB_{12}\text{-}CN} - A_{MCMB_{12}}$; A_{obsd} is the initial absorbance after the addition of cyanide. The solid line is calculated from the equation $Y = [CN^-]/([CN^-] + K_0)$ with $K_0 = 0.044$ M.

is inhibited by the amines piperidine (50% at 0.76 M) and *N,N*-dimethylglycine. This inhibition may be caused by complexation with the amine, but no change in the spectrum of $MCMB_{12}$ was observed in the presence of 1.0 M piperidine.

At concentrations of hydrochloric acid greater than 1 M the rate of cleavage was found to decrease. The values of log k in the presence of 0.1 M hydrogen cyanide follow H_0[24] with a slope of 0.9 and there is a rate decrease by a factor of 50 at $H_0 = -2.7$. The absorption spectrum of $MCMB_{12}$ in 4 M hydrochloric acid does not show significant changes, such as are observed upon ionization of CMB_{12} and (aminoethyl)cobalamin, and binding of chloride ion is expected to give only a small inhibition under these conditions (the association constant for aquocobalamin and chloride ion is 1.3 M^{-1} and binding to an alkylcobalamin is expected to be weaker;[21] furthermore, an increase in the concentration of chloride ion from 1.8 to 4 M could not cause a rate decrease by a factor of 50).

At high pH values there is a leveling of the observed rate constants and a change in the spectrum of the reaction mixture, extrapolated to zero time, with increasing concentration of cyanide anion. This is caused by accumulation of the α-cyano addition compound (eq 6). A plot of $1/k_{obsd}$ against $1/[CN^-]$ (Figure



2) gives a limiting first-order rate constant of $k_1 = 4.65 \times 10^{-4}\ s^{-1}$ at high cyanide concentrations, a second-order rate constant

(24) Paul, M. A.; Long, F. A. *Chem. Rev.* **1957**, *57*, 1–45.

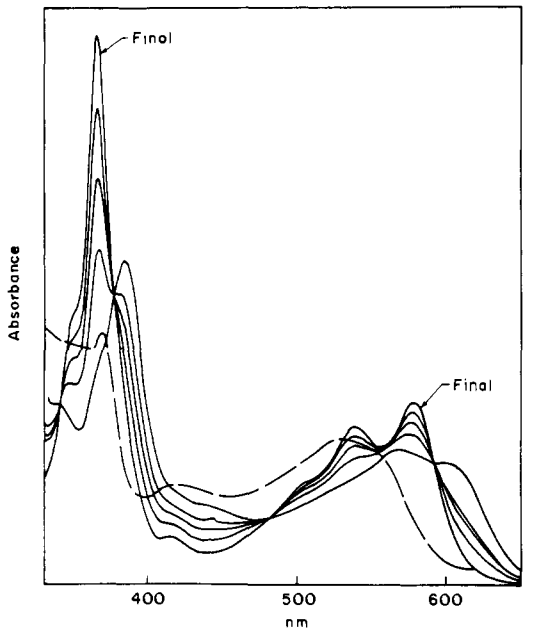


Figure 3. Spectra of 0.038 mM $MCMB_{12}$ in the absence (dashed line, pH 8) and the presence (solid lines) of 0.5 M potassium cyanide at pH 11.3, 30 °C, and ionic strength 1.8 M (KCl). The spectra were taken 180, 600, 1500, 3600, and 11 340 s after the addition of cyanide to $MCMB_{12}$.

Table III. Solvent Deuterium Isotope Effects for the Reaction of $MCMB_{12}$ with Cyanide[a]

term	pH	[cyanide]/M[b]	runs	k_{H_2O}/k_{D_2O} or K_{H_2O}/K_{D_2O}[c]
$k_A/M^{-1}\ s^{-1}$	10.4, 11.3[d]	0.002–0.010	11	1.4 ± 0.16
k_1/s^{-1}	11.4	0.98	12	1.44 ± 0.08
K_0/M			17	1.02 ± 0.20
$k_B/M^{-2}\ s^{-1}$	6.2–6.7	0.10	20	0.37 ± 0.04[e]
$k_C/M^{-2}\ s^{-1}$	0.9–1.35	0.05	10	0.37 ± 0.01
K_{HCN}/M	8–10		28	2.29 ± 0.1

[a] 30 °C, ionic strength 1.8 M (KCl); data from Tables I and II. [b] Total concentration of cyanide. [c] In 97% deuterium oxide. [d] Runs in deuterium oxide at pD = 11.3. [e] The observed rate constants have not been corrected for the contributions from k_A and k_C; see text.

of $k_A = 1.06 \times 10^{-2}\ M^{-1}\ s^{-1}$ at low cyanide concentration, and a dissociation constant of $K_0 = 0.044$ M. The same dissociation constant describes the change in absorbance extrapolated to zero time (inset, Figure 2); this change was found to be complete by the time of the first measurement a few seconds after the addition of cyanide. Figure 3 shows the spectra of a reaction mixture containing 0.5 M cyanide at pH 11.3 at different times. The same behavior was observed with the carboxylate compound, CMB_{12}, at pH 11.5, with values of $k_1 = 1.8 \times 10^{-5}\ s^{-1}$ and $K_0' = 0.77$ M. The second-order rate constant of $2.3 \times 10^{-5}\ M^{-1}\ s^{-1}$ at low cyanide concentration agrees with an extrapolation of data obtained by Fenton.[5] The products of this reaction are acetic acid[10] and an equilibrium mixture of cyanocobalamin and dicyanocobalamin, as determined spectrophotometrically.

Solvent deuterium isotope effects were determined in the three plateau regions of the pH–rate profile and are reported in Table III. The isotope effect on k_B was determined at pH (pD) = 6.2–6.7, where the k_B term accounts for >90% of the observed reaction. The isotope effects are small at high pH but are inverse at intermediate and low pH, with $k_{D_2O}/k_{H_2O} = 2.7$.

The isotope effects for rate constants at pH <9 required the determination of the equilibrium isotope effect for the ionization of hydrogen cyanide, which was found to be $K_{HCN}/K_{DCN} = 2.3$. This value was obtained from measurements of the pH (pD) of buffer solutions of known, identical acid–base ratios and from spectrophotometric comparison of the pK_a values of *p*-bromophenol and hydrogen cyanide in water and deuterium oxide solutions, which gave good agreement. It differs from a reported value of $K_{HCN}/K_{DCN} = 1.2$ that was based on measurements of the observed pH of solutions of KCN in H_2O and D_2O.[25] At pH <8 the observed rate constants (k_B and k_C) show only a small isotope effect of $k_{D_2O}/k_{H_2O} = 1.2$ because of compensation of this equilibrium isotope effect and the inverse isotope effects on k_B and k_C. The isotope effect on the dissociation constant of the dimethylbenzimidazole group was not determined but does not affect the rate constants at low pH values, at which this group is entirely in the base-off, protonated form, or at intermediate pH values, at which the concentration of the protonated base-off species is not significant.

Table IV. Secondary Deuterium Isotope Effects for the Reaction of $MCMB_{12}$ with Cyanide[a]

term	pH	[cyanide]/M[b]	runs	$k(MCMB_{12})/k([^2H]MCMB_{12})$[c]
k_1/s^{-1}	11.39	0.98	8	1.19 ± 0.065
$k_C/M^{-2}\ s^{-1}$	00.91	0.05	12	1.11 ± 0.06
	4 M HCl	0.10	6	1.21 ± 0.03[d]

[a] At 30 °C and ionic strength 1.8 M (KCl). [b] Total cyanide concentration. [c] Observed rate constant ratio for the di-H and di-^2H compounds. [d] Ionic strength = 4.1 M.

Table V. Solvent $[^3H]H_2O$ Selection Isotope Effect for Discrimination against ^3H Incorporation into Methyl Acetate[a]

pH	[cyanide]/M[b]	spec act of H_2O/(μCi of ^3H/μmol of H)	spec act of MA[c]/(μCi of ^3H/μmol of MA)	selection isotope effect[d]
11.3	1.0	1.77	0.36	4.9
11.3	0.50	1.70	0.40	4.3
9.0	0.10	2.17	0.49	4.4
1.0	0.03	1.92	0.22	8.7

[a] 30 °C and ionic strength 1.8 M (KCl). [b] Total cyanide concentration. [c] Formed from $MCMB_{12}$; see Experimental Section. [d] Ratio of the specific activity of the solvent to the specific activity of methyl acetate formed from $MCMB_{12}$.

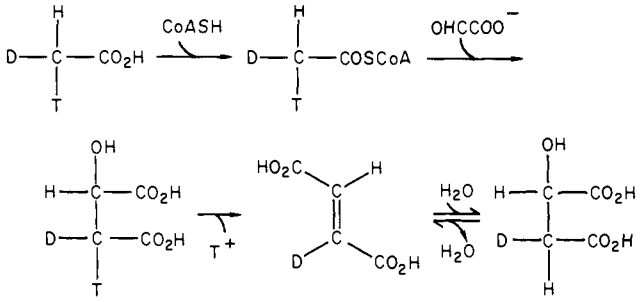
Secondary α-deuterium isotope effects for the reaction of $MCMB_{12}$ and $[(\alpha\text{-}^2H)_2]MCMB_{12}$ at pH 11.4 and 0.98 M potassium cyanide (k_1), at pH 0.91 (k_C), and in 4 M hydrochloric acid are reported in Table IV. These isotope effects were determined from multiple kinetic runs with the two compounds under identical conditions.

The discrimination isotope effects for incorporation of tritium into the methyl acetate product from solvent are 4.3–4.9 at high pH and 8.7 at pH 1.0 (Table V). These normal isotope effects are in contrast to the small or inverse solvent deuterium isotope effects on the rate constants under the same conditions.

Stereochemistry. The cleavage of the carbon–cobalt bond of $MCMB_{12}$ by cyanide formally involves the replacement of the carbon–cobalt bond by a carbon–hydrogen bond. $MCMB_{12}$, in which the pro-*R* hydrogen of the carboxymethyl group was replaced by deuterium, (*R*)-$[^2H]MCMB_{12}$, was allowed to react with CN^- in $[^3H]H_2O$. The resulting methyl $[^3H,^2H]$acetate was converted to $[^3H,^2H]$acetic acid and the chirality of this acetic acid was determined. The determination of chirality involves transformation of acetate to malate and fumarate through the series of enzymic reactions shown in Scheme I.[26,27] As indicated in Scheme A acetic acid is converted to malic acid. The latter is then equilibrated with fumaric acid in the presence of fumarase. Malic acid obtained from (*R*)-$[^3H,^2H]$acetic acid retains tritium during the equilibration process, while malic acid obtained from (*S*)-$[^3H,^2H]$acetic acid loses tritium. In practice, the tritium loss

(25) Dunford, H. B.; Hewson, W. P. *J. Phys. Chem.* **1979**, *83*, 3307.
(26) Cornforth, J. W.; Redmond, J. W.; Eggerer, H.; Buckel, W.; Gutschow, C. *Nature (London)* **1969**, *221*, 1212–1213.
(27) Luthy, J.; Retey, J.; Arigoni, D. *Nature (London)* **1969**, *221*, 1213–1215.

Scheme I



or retention is not 100%. The actual amount of loss or retention depends upon the intramolecular isotope effects which occur in the condensation between acetyl-CoA and glyoxylic acid.

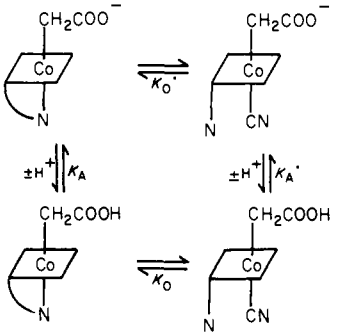
The results obtained when acetic acid derived from (*R*)-[^{2}H]MCMB$_{12}$ was subjected to the procedure outlined above are shown in Table VI. In Table VI results from two independent experiments with (*R*)-[^{2}H]MCMB$_{12}$ are shown. Malate derived from (*R*)-[^{2}H]MCMB$_{12}$ retains 60–64% of its tritium. The table also includes several control experiments. When (*R*)-[^{3}H,^{2}H]acetic acid is subjected to the analysis outlined above, 88% of the tritium is retained in malate after equilibration in the presence of fumarase. A similar value has been obtained previously.[27] However, the value is higher than would be expected for the intramolecular deuterium isotope effect of 3.5 reported for the condensation of acetyl-CoA and glyoxylic acid.[28,29] We have no explanation for this discrepancy. With nonisotopic MCMB$_{12}$ 47% of the tritium is retained in malate. This is as expected since the [α-^{3}H]acetic acid formed under these conditions is achiral.

If methyl acetate were derived from (*R*)-[^{2}H]MCMB$_{12}$ by nonstereoselective protonation, then approximately 50% of the initial ^{3}H would be retained in malate. Since 62% of the ^{3}H was retained, some stereospecific protonation occurred. If (*R*)-[^{2}H]MCMB$_{12}$ were chirally pure and if the protonation were completely stereospecific, 88% retention of ^{3}H in malate is expected. The observation that 62% of tritium is retained in malate is consistent with the production of 32% (*R*)-[^{2}H,^{3}H]acetic acid and 68% (*RS*)-[^{2}H,^{3}H]acetic acid. This conclusion is only valid if the starting material consisted entirely of (*R*)-[^{2}H]MCMB$_{12}$. Contamination by nonchiral MCMB$_{12}$ would raise the percentage (*S*)-[^{2}H,^{3}H]acetic acid produced in the reaction of (*R*)-[^{2}H]-MCMB$_{12}$ with CN$^-$. NMR analysis[5] of (*R*)-[^{2}H]MCMB$_{12}$ showed no detectable hydrogen in the pro-*R* position. Therefore the pro-*R* position contained less than 10% hydrogen; i.e., the compound contained less than 10% nondeuterated or (*S*)-[^{2}H]-MCMB$_{12}$. If one assumes the maximum amount of contamination by (*S*)-[^{2}H]MCMB$_{12}$, then maximally 40% of (*R*)-[^{2}H,^{3}H]acetate would have been formed.[30] It is also important to consider whether 100% chiral methyl acetate could be consistent with the observed retention of 62% tritium in malate. It can be calculated that this requires that the starting MCMB$_{12}$ is contaminated with either 34% *S* isomer or 68% nondeuterated MCMB$_{12}$. This amount of contamination is not consistent with the NMR data. Two pathways for methyl acetate formation must therefore exist: one proceeding with stereospecific, and the other with nonstereospecific carbon–hydrogen bond formation. The data also establish that carbon–hydrogen bond formation proceeds with inversion of configuration.

Discussion

Cyanide-Induced Carbon–Cobalt Cleavage. The addition of cyanide anion to the α-position of MCMB$_{12}$, replacing the nitrogen atom of dimethylbenzimidazole (eq 1), provides the driving force for expulsion of the enolate anion of methyl acetate from cobalt as a result of the large, electron-donating trans effect of cyanide anion.[2,31,32] The addition of cyanide occurs in a rapid equilibrium step with a dissociation constant of K_0 = 0.044 M (Figure 2). This equilibrium constant is in the expected range for the addition of cyanide to alkylcobalamins; the binding is stronger than with methyl- and vinylcobalamin and weaker than with the acetylene and cyanide derivatives.[33] The assignment of structure for these addition compounds is based on the visible spectra of cyanocobalamin and cyanocobalamide adducts[33] and the decreased stretching frequency of the C≡N bond with increasing electron donation by the trans alkyl group;[34] MCMB$_{12}$ shows the characteristic red shift upon substitution of the benzimidazole ligand by cyanide[2] (Figure 3).

The large transmission of electron density through the cobalt atom upon the binding of cyanide, as a consequence of the trans effect, is illustrated by the increase of $>10^4$ in the equilibrium constant for the dissociation of CN$^-$ from dicyanocobalamin (to give β-aquo-α-cyanocobalamin) compared with cyanocobalamin.[23,33,34] The same effect causes an increase in the pK_a of the carboxymethyl group of CMB$_{12}$ and a correspondingly weaker binding of cyanide to CMB$_{12}$ anion than to MCMB$_{12}$. The negative charge of the carboxylate group of CMB$_{12}$ results in an 18-fold increase in the equilibrium constant, to K_0' = 0.77 M, for the dissociation of cyanide from the addition compound for CMB$_{12}$ compared with MCMB$_{12}$. There is a corresponding increase of 1.3 units in the pK_a of the carboxyl group of CMB$_{12}$, to pK_A' = 8.4, as a result of the increased electron density upon the binding of cyanide, if it is assumed that K_0 is the same for the acid and its methyl ester. These mutual destabilizations are described by the relationship $K_0'/K_0 = K_A/K_A'$ for the equilibrium constants of eq 7.



(7)

The simplest mechanism for the cyanide-induced formation of methyl acetate from MCMB$_{12}$ is cleavage of the carbon–cobalt bond to expel the carbanion of methyl acetate into the solvent, followed by protonation of the carbanion in a fast step. However, this mechanism cannot be correct because the protonation shows stereoselectivity and the planar carbanions that are formed from esters would give nonstereoselective protonation if they were released free into solution.[35] The stereoselectivity of the methyl acetate product formed from chiral (*R*)-[^{2}H]MCMB$_{12}$ in tritiated water (Table VI) shows that a large fraction of the reaction proceeds with protonation before the carbanion has separated from the cobalamin, giving inversion of configuration. The observation of 62% retention of tritium in the C-3 pro-*S* hydrogen of malate

(28) Lenz, H.; Eggerer, H. *Eur. J. Biochem.* **1976**, *65*, 237–246.
(29) Lenz, H.; Wunderwald, P.; Buschmeier, V.; Eggerer, H. *Hoppe-Seyler's Z. Physiol. Chem.* **1971**, *352*, 517–519.
(30) (a) $^3H_{max}$ = maximal fraction of ^{3}H retained in malate for 100% stereospecific protonation for various mixtures of isotopic species of MCMB$_{12}$. The $^3H_{max}$ for pure chiral (*R*)-[^{2}H]MCMB$_{12}$ is taken as 0.88 based on the results obtained with (*R*)-[^{3}H,^{2}H]acetic acid (Table I). $^3H_{max}$ = $0.88F_{(R)\text{-}[^2H]MCMB_{12}} + 0.5F_{MCMB_{12}} + 0.12F_{(S)\text{-}[^2H]MCMB_{12}}$; *F* = fraction of each MCMB$_{12}$ species. (b) F_c = fraction of stereospecifically protonated acetic acid derived from methyl acetate. F_{RA} = fraction of acetic acid derived from racemic [α-^{3}H,^{2}H]-methyl acetate. $F_c = 2F_{(R)\text{-}[^3H,^2H]HAc} - 1$, $F_{RA} = 1 - F_c$, $F_{(R)\text{-}[^3H,^2H]HAc} = (0.62 + {}^3H_{max} - 1)/2{}^3H_{max} - 1$.
(31) Poon, C. K. *Coord. Chem. Rev.* **1973**, *10*, 1–35.
(32) Hayward, G. C.; Hill, H. A. O.; Pratt, J. M.; Williams, R. J. P. *J. Chem. Soc. A* **1971**, 196–200.
(33) Hayward, G. C.; Hill, H. A. O.; Pratt, J. M.; Vanston, N. J.; Williams, R. J. P. *J. Chem. Soc.* **1965**, 6485–6493.
(34) Firth, R. A.; Hill, H. A. O.; Pratt, J. M.; Thorp, R. G.; Williams, R. J. P. *J. Chem. Soc. A* **1968**, 2428–2433.
(35) Cram, D. J.; Rickborn, B.; Kingsbury, C. A.; Haberfield, P. *J. Am. Chem. Soc.* **1961**, *83*, 3678–3687.

after enzymatic analysis requires that protonation occur partly by inversion and partly by a pathway that does not give stereospecific product; it is consistent with approximately 40% stereospecific protonation before separation and 60% nonstereospecific protonation, which may occur after separation of the enolate anion from cobalamin at pH 11.3. These results suggest that the reaction proceeds in two steps after the initial addition of cyanide, with the formation of a carbanion intermediate that can undergo protonation either before or after its release into solution. Inspection of space-filling molecular models indicates that it is unlikely that protonation can occur with retention before cleavage of the carbon–cobalt bond, because of steric hindrance from the corrin, but this possibility cannot be excluded rigorously.

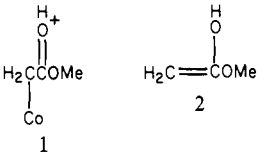
The differences between the kinetic solvent deuterium isotope effects and the discrimination tritium isotope effects for product formation establish that the reaction proceeds in two steps, with rate-determining cleavage of the carbon–cobalt bond to form an intermediate in the first step and protonation of the intermediate in the second step (eq 1). The small solvent isotope effects of $k_{H_2O}/k_{D_2O} = 1.4$ for k_A and k_1 at high pH and the inverse isotope effects of $k_{H_2O}/k_{D_2O} = 0.37$ for k_B and k_C at intermediate and low pH (Table III) are inconsistent with protonation of carbon in the rate-determining step; the small isotope effect for k_A and k_1 may reflect hydrogen bonding by solvent to the developing negative charge on the oxygen atom of the enolate anion in the transition state of the first step. The solvent discrimination isotope effects of $k_{1H}/k_{3H} = 4.3$–4.9 at high pH and $k_{1H}/k_{3H} = 8.7$ at low pH (Table V) show that there is a normal isotope effect for the protonation of carbon in the final step and that the intermediate has a sufficient lifetime to discriminate among different solvent molecules before it is protonated.

The absence of buffer catalysis confirms that protonation of carbon does not occur in the rate-determining step. Buffer catalysis of proton transfer to carbon in this kind of reaction is easily detectable in aqueous solution[36-39] and trifluoroethanol has been shown to be an effective catalyst for the protonation of bis(methoxyethyl)ketene acetal,[40] which is a model for the enol of an acetate ester.

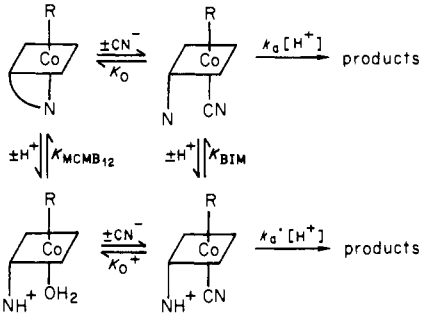
The stepwise mechanism for cyanide-induced cleavage of $MCMB_{12}$ may be contrasted with the concerted electrophilic displacements by mercuric ion on alkylcobalamins and alkylcobaloximes, in which the electrophilic reagent provides the driving force for cleavage of the carbon–cobalt bond. These reactions proceed by an S_E2 mechanism with inversion of configuration at carbon and no evidence for an intermediate.[41-45] The acid-catalyzed cleavage of the carbon–mercury bond of organomercurials proceeds by a similar S_E2 displacement mechanism but with predominant retention of configuration.[46,47]

The secondary α-deuterium isotope effects of $k_{2H}/k_{2D} = 1.19$ for k_1 and 1.11 for k_C (Table IV) show that there is significant carbon–cobalt bond cleavage and rehybridization toward sp^2 carbon of the leaving CH_2COOMe group in the transition state of the rate-determining step. These isotope effects correspond to isotope effects of $k_H/k_D = 1.05$–1.09 (per deuterium) that are somewhat smaller than the isotope effects near $k_H/k_D = 1.15$ that have been observed for carbanion formation with carbon–hydrogen bond cleavage.[48] However, the fraction of rehybridization that has taken place in the transition state for carbon–cobalt cleavage cannot be estimated at this time because the fractionation factor for deuterium in the starting $MCMB_{12}$ is not known.

The inverse isotope effect of $k_{D_2O}/k_{H_2O} = 2.7$ for k_B and k_C shows that the reactions at intermediate and low pH values involve specific-acid catalysis of carbon–cobalt bond cleavage in the rate-determining step, after formation of the cyanide addition compounds of $MCMB_{12}$ and the protonated, base-off species of $MCMB_{12}$ at intermediate and low pH, respectively. This isotope effect is intermediate between the values of $k_{D_2O}/k_{H_2O} = 2.3$ and 3.4 that are calculated from the fractionation factors for equilibrium addition of a proton to form the O-protonated ester **1** and the ester enol **2**, respectively.[49] (Formylmethyl)cobalamin undergoes a similar acid-catalyzed cleavage to acetaldehyde in the absence of cyanide, presumably through the more stable enol of acetaldehyde.[50]



The identical solvent isotope effects for k_B and k_C suggest that carbon–cobalt cleavage at intermediate and low pH proceeds by the same mechanism and that the rate constants should differ only by an amount corresponding to the more favorable equilibrium constant for addition of cyanide to the protonated, base-off species than to $MCMB_{12}$ itself. Assuming that the protonation state of the base (which is well removed from the reaction center in both addition compounds) does not affect the rate of carbon–cobalt bond cleavage ($k_a = k_a'$, eq 8), it follows from eq 8–10 that the



(8)

$$k_B = k_a/K_0 \quad (9)$$

$$k_C = k_a'/K_0^+ \quad (10)$$

observed rate constants k_C and k_B should differ by $K_{MCMB_{12}}/K_{BIM}$, the ratio of the dissociation constants of protonated dimethylbenzimidazole with and without reclosure of the benzimidazole–cobalt bond. Taking $pK_{BIM} = 5.0$, the same as for dissociation of the protonated dimethylbenzimidazole group of dicyanocobalamin,[23] the calculated value of log (k_C/k_B) is 2.4, which agrees satisfactorily with the observed value of log $(k_C/k_B) = 2.6$. This result confirms the conclusion that the rapid rate of the acid-catalyzed reaction at low pH (k_C) is a consequence of the favorable equilibrium constant for the addition of cyanide to the protonated, base-off species of $MCMB_{12}$. A similar argument shows that the k_B term represents specific-acid catalysis (eq 8 and

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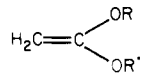
9) and not uncatalyzed carbon–cobalt bond cleavage after the addition of cyanide to the protonated, base-off species. According to the latter interpretation the k_B term would be described in an alternative, kinetically equivalent form (eq 11) with $k_B' = k_B K_{MCMB_{12}}$ and the ratio k_B'/k_A would be equal to $K_0/K_0^+ = 250$, the advantage for adding cyanide to the protonated, base-off form; in fact, the ratio k_B'/k_A is 1.2×10^5.

$$k_B[MCMB_{12}][CN^-][H^+] = k_B'[MCMB_{12}H^+][CN^-] \quad (11)$$

Nature of the Intermediate. The intermediate is formed by carbon–cobalt cleavage with a small or inverse solvent deuterium isotope effect and is protonated to give methyl acetate with a normal primary isotope effect and at least partial inversion of configuration at carbon. The stereospecific protonation requires that protonation must occur before the carbanion rotates or leaves the asymmetric environment of the cobalamin because a carbanion α to a carbonyl group is planar and would be protonated non-stereospecifically in free solution.[35]

The possibility that a free carbanion is protonated stereospecifically because it is so unstable that it is protonated extremely rapidly, before it can rotate or diffuse away from the cobalamin, can be excluded by the known rate constants for related proton transfer reactions. The rate constant for protonation can be calculated from the rate constant of 0.09 s^{-1} for proton transfer from water to diethylketene acetal[39] (**3**, R = R′ = Et), which is a model for the enol of methyl acetate (**3**, R = H, R′ = Me), and



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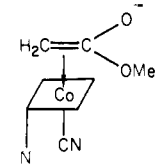
a factor of $\leq 10^7$ for the faster protonation of an enolate anion than of an enol, which is calculated from the rate constants for catalysis of acetone enolization by acetic acid and acetate ion.[51-53] It has been shown that substitution of an alkyl group for the hydroxylic proton of an enol has only a small or no effect on the rate constant for protonation.[54] The factor of 10^7 for the difference between enolate and enol is an upper limit because (1) the oxyanion provides less driving force for protonation in the enolate of methyl acetate than in the enolate of acetone because of electron withdrawal by the OR′ group and (2) the additional electron donation by resonance from the OR′ group in the protonation step is expected to diminish the importance of electron donation from the oxyanion. This gives a rate constant of $\leq 10^6$ s^{-1} for the protonation of the enolate ion of methyl acetate by water, which is much less than the rate constant of $\sim 10^{10}$ s^{-1} for the diffusional separation of an encounter pair in water. Furthermore, the observation of a primary ^3H isotope effect shows that the protonation is not diffusion controlled.

The rate constant of $\leq 10^6$ s^{-1} for protonation and the observed stereoselectivity require that the rate constant for dissociation must also be $\leq 10^6$ s^{-1}; i.e., there must still be significant bonding of the enolate to cobalt even though the initial carbon–cobalt bond of $MCMB_{12}$ has already been cleaved in the rate-determining step. We conclude that the immediate product of carbon–cobalt bond cleavage is a π complex of the enolate and Co^{III}, **4**, and that



4

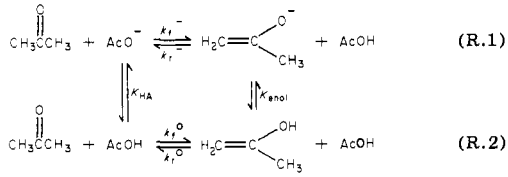
protonation of the enolate occurs in large part before it separates from the corrin. The existence of a symmetrical π complex has been demonstrated previously in the cobaloxime series using isotopically labeled substrates by showing that the two carbon atoms of the ethylene group of (2-acetoxyethyl)(pyridine)cobaloxime become equivalent in the course of solvolysis in methanol to give the methyl ether.[55,56] The observation of equal amounts of (methoxyethyl)(pyridine)cobaloxime labeled in the 1 and 2 carbon atoms of the ethyl group[55] shows that the Co^{III} π complex can collapse back to a σ complex upon nucleophilic attack by methanol. In contrast, the enolate π complex of cyanocobalamin

Table VI. Chirality Determination of Acetate Samples

starting material	product	^{14}C/dpm[a]	^3H/dpm[a]	$[^3H]/[^{14}C]$	% ^3H retained in malate
$MCMB_{12}$	acetate	5.02×10^5	2.14×10^6	4.26	
	acetyl-CoA	2.56×10^5	7.92×10^5	3.09	
	malate	1.85×10^5	3.82×10^5	2.06	
	malate[b]	0.38×10^5	0.37×10^5	0.97	47
(*R*)-[^{3}H,^{2}H]-acetate	acetate	6.17×10^5	3.09×10^6	5.01	
	acetyl-CoA	3.97×10^5	1.67×10^6	4.21	
	malate	3.33×10^5	1.22×10^6	3.66	
	malate[b]	7.30×10^4	2.35×10^5	3.22	88
(*R*)-[^{2}H]-$MCMB_{12}$	acetate	1.94×10^5	2.26×10^5	1.16	
	acetyl-CoA	6.80×10^4	7.1×10^4	1.04	
	malate	5.1×10^4	4.5×10^4	0.88	
	malate[b,c]	1.16×10^4	6.4×10^3	0.55	62
	malate[b,c]	9.5×10^3	5.0×10^3	0.53	60
(*R*)-[^{2}H]-$MCMB_{12}$	acetate	1.80×10^5	4.48×10^5	2.49	
	acetyl-CoA	2.1×10^3	4.6×10^4	2.19	
	malate	9.1×10^3	1.7×10^4	1.87	
	malate[b]	2.5×10^3	3.0×10^3	1.20	64

[a] Total dpm in the sample. [b] After equilibration with fumarase. [c] Two separate equilibrations of malate. Reaction conditions: $[MCMB_{12}] = 1 \times 10^{-3}$ M, [KCN] = 0.5 M, [KCl] = 2.0 M, pH 11.3; in 1 mL of $[^3H]H_2O$ (445 mCi). Reaction was allowed to proceed 90 min at 30 °C. The resulting methyl acetate was used for chirality determination as described in the Experimental Section.

(51) The ratio of the rate constants for protonation of the enol and enolate ion of acetone by acetic acid, k_r^-/k_r^0 (eq R.1 and R.2),



(R.1)

(R.2)

may be calculated from eq R.3 and from the known rate constants for the forward reactions, $k_f^- = 15 \times 10^{-6}$ and $k_f^0 = 5 \times 10^{-6}$ M^{-1} min^{-1} (Bell, R. P. "The Proton in Chemistry", 2nd ed.; Cornell University Press: Ithaca, N.Y., 1973; p 150), and the dissociation constants of the hydroxyl group of the enol, K_{enol}, and of acetic acid, $K_{HA} = 10^{-4.7}$ M. If the value of pK_{enol} is assumed

$$\frac{k_r^-}{k_r^0} = \frac{K_{HA}}{K_{enol}} \frac{k_f^-}{k_f^0} \quad (R.3)$$

to be 11.3, the same as that for the enol of cyclohexanone (Bell, R. P.; Smith, P. W. *J. Chem. Soc. B* **1966**, 241) the k_r^-/k_r^0 ratio is 1.2×10^7. An analogous calculation for protonation by water gives $k_r^-/k_r^0 = 10^6$.

(52) Protonation of the enolate ion at pH 11.3 is expected to occur with proton donation from water and not from the small amount of hydrogen cyanide that is present at this pH. The rate constants for abstraction of the proton α to the carbonyl group of 4-(4-nitrophenoxy)-2-butanone by cyanide and hydroxide ions are 1.08×10^{-3} and 8.33 M^{-1} s^{-1}, respectively,[53] which means that 97% of the reaction at pH 11.3 in the presence of 0.5 M cyanide anion will be with hydroxide ion. The reverse reactions, which involve protonation of the enolate by hydrogen cyanide and water, must occur with the same ratio under the same conditions from the principle of detailed balance, so that water is the principal proton donor at pH 11.3. The same conclusion can be reached for the protonation of acetone enolate from the value of $\beta = 0.88$ for acetone enolization, the rate constants for catalysis of enolization by acetate and hydroxide ions, and the 40-fold negative deviation of cyanide ion from the Brønsted line for base catalysis of enolization by oxygen bases.[53]

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is formed from the σ complex in an irreversible step and undergoes electrophilic attack by an acid. Solvolysis of the (2-acetoxypropyl)cobaloxime with retention of configuration is also consistent with an intermediate π complex.[56] It has been suggested, but not proved, that a number of other reactions of cobaloximes proceed through intermediate π complexes, and there is evidence that π complexes can be formed in a different class of reactions from the addition of electron-rich Co^I complexes to electron-deficient olefins.[57-62] A π complex is formed from cyanocobalt(I) and diethyl malate that is resistant to hydrolysis and undergoes isomerization to the diethyl fumarate complex, presumably through an unstable σ-bonded carbanion intermediate that undergoes rotation but is not protonated.[59]

The present work shows that the π complex of cyanocobalamin and the enolate of methyl acetate has a significant lifetime. If the rate constant for formation of such a complex from the enolate and cobalamin is close to that for the addition of cyanide ion to α-aquo-β-cyanocobalamin[23] of $k = 8.4 \times 10^4\ M^{-1}\ s^{-1}$ (the rates of ligand exchange reactions of this kind show only a small dependence on the nature of the entering ligand[31]), the rate constant of $<10^6\ s^{-1}$ for dissociation of the complex gives an equilibrium constant of $\gtrsim 0.1\ M^{-1}$ for formation of the complex. This indicates that the stability of the π complex is comparable to or greater than that of the aquo complex. In fact, the stability is probably much greater than this because the rate of protonation of the π complex **4** is expected to be slower than that of a free enolate ion because of the withdrawal of π electrons by cobalt in the complex. Slower protonation also means that the rate constant for dissociation of the enolate from the π complex is considerably less than $10^6\ s^{-1}$.

The nonstereospecific protonation of approximately half of the product could occur in solution after dissociation of the π complex or after isomerization of the π complex to a σ complex bonded through the enolate oxygen atom.[63]

Other Matters. The remarkably weak acidity of the cobalt-bound acetic acid moiety of CMB_{12}, which has $pK_a = 7.1$, can be explained by electron donation from cobalt,[64] by poor solvation of the carboxylate anion in the nonaqueous environment of the corrin ring on one side or, more likely, by both of these factors. The low acidity cannot be explained by hydrogen bonding to amide groups on the corrin ring[22] because the pK_a of (carboxymethyl)cobaloxime, which has no amide groups, is also 7.14.[65] The normal pK_a of 10.5 for the protonated amino group of (aminoethyl)cobalamin, which has an opposite charge but a similar distance to the ionizing group, suggests that electron donation through σ bonds by an inductive effect is not sufficient by itself to account for the low acidity. Inspection of space-filling molecular models suggests that the protonated amino group is more accessible for solvation by water than the carboxylate anion. It may be possible for cobalt to stabilize the carboxylic acid by electron donation to an empty π orbital even in the σ complex.[64]

The low rate of alkaline hydrolysis of the ester group of $MCMB_{12}$, which is slower than that of methyl acetate[66] by a factor of $>7 \times 10^3$, probably reflects a somewhat similar destabilization of the anionic transition state and tetrahedral addition intermediate by the corrin ring through poor solvation and probably nonbonded interactions; it may also involve stabilization by electron donation from cobalt to a π orbital of the ester group.[64] There is precedent for the former effect in the 100-fold decrease in the rate of alkaline hydrolysis of benzocaine when it forms a complex with caffeine.[67]

The addition of cyanide to $MCMB_{12}$ would have to occur with a second-order rate constant of $>2.4 \times 10^8\ M^{-1}\ s^{-1}$ in order to account for the observed rate of cyanide-induced cleavage at pH <1.0 if cyanide anion were the species that adds to the α position of $MCMB_{12}$. Since this is much larger than the rate constant of $\sim 10^3\ M^{-1}\ s^{-1}$ that has been observed for the addition of other ligands to cobalamins,[31] the addition of cyanide at low pH almost certainly occurs through a mechanism involving addition of HCN to form the N-liganded species, followed by loss of a proton and rotation to the C-liganded species. This mechanism has been demonstrated for the addition of cyanide to aquocobalamin and to cyanocobalamin at low pH.[23] The cleavage reaction at low pH cannot proceed through the uncatalyzed cleavage of an HCN–$MCMB_{12}H^+$ complex (which would be kinetically equivalent to acid-catalyzed cleavage of the NC–$MCMB_{12}H^+$ complex) because the observed first-order rate constants at low pH ($0.024\ s^{-1}$ at 0.1 M [HCN]) are larger than the observed first-order rate constant, k_1, for the uncatalyzed cleavage of the CN^- addition compound at high pH ($k_1 = 4.7 \times 10^{-4}\ s^{-1}$), which is expected to be at least as fast as the cleavage of an HCN addition compound.

The reason for the inhibition of the cleavage reaction at acid concentrations above 1 M is not known. This kinetic behavior is not caused by a change to a different, acid-inhibited rate-determining step, such as rotation of N-liganded to C-liganded cyanide after loss of a proton from N-liganded HCN, because the secondary α-deuterium isotope effect of 1.21 in 4 M hydrochloric acid suggests that carbon–cobalt bond cleavage is still rate determining (Table IV). The inhibition may be caused by a difference in the acidity functions for specific-acid catalysis of carbon–cobalt cleavage and for the loss of a proton from HCN or by protonation of $MCMB_{12}H^+$ that gives little or no change in its spectrum.

The small solvent deuterium isotope effect for the dissociation of hydrogen cyanide, $K_{HCN}/K_{DCN} = 2.3$ ($\Delta \log K = 0.36$), may be accounted for in large part by the relatively low C–H bending frequency of 760 cm^{-1} in hydrogen cyanide. The isotope effect for the ionization of hydrogen cyanide is expected to be smaller than for ionization of an oxygen acid by $\Delta pK \cong 0.31$ units, based on the different zero-point energies of the frequencies for hydrogen-bonded ROH (3330, 1370 cm^{-1}), ROD (2476, 950 cm^{-1}), HCN (3232, 2092, 760 cm^{-1}), and DCN (2588, 1909, 608 cm^{-1}).[68] Equilibrium constants of 0.75–0.80 for the formation of DCN + CH_4 from HCN + CH_3D, based on calculations and spectroscopic data, also support a small isotope effect for ionization.[69]

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(63) Although the asymmetry of the corrin ring might give some degree of stereoselectivity for protonation of such an oxygen-bonded σ complex, this would not be expected to give stereoselectivity in the product because the σ complex should exist as a mixture of equal parts of the two geometric isomers. Rotation of the C–C bond of the ester before and (probably) after synthesis of $MCMB_{12}$ is expected to give equal amounts of the *E* and *Z* isomers of the ester in $MCMB_{12}$ and of any such σ complex. It is unlikely that an oxygen-bonded σ complex is formed directly from $MCMB_{12}$ by intramolecular rearrangement through a four-membered ring. Inspection of space-filling molecular models suggests that such a reaction is sterically difficult or impossible and the π orbital of the enol product is orthogonal to the carbon–cobalt σ bond of $MCMB_{12}$. Furthermore, such a direct rearrangement should not be subject to acid catalysis, but the ratio of the rate constants for the acid-catalyzed and uncatalyzed reactions is $k_B/k_A = 5 \times 10^7\ M^{-1}$ (Table I), which is even larger than the corresponding ratio of $k_H/k_w = 6 \times 10^4\ M^{-1}$ for the enolization of acetone (cf. ref 51). If a different mechanism were significant for the uncatalyzed rearrangement of $MCMB_{12}$, k_A would be expected to be larger than expected for a normal enolization, and the ratio k_B/k_A would be correspondingly smaller.
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