Carboxyalkylcobalamins: Effects of Carboxyl Substituents on Base-on / Base-off Equilibria and Mechanochemical Co–C Bond Cleavage*

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Z. Naturforsch. **2008**, *63b,* 143 – 153; received September 9, 2007

A series of primary and secondary carboxyalkylcobalamins with $(CH_2)_nCOOH$ groups ($n = 1-3$), CH_2–$CH(CH_3)COOH$, CH(R)COOH (R = CH_3, C_2H_5, n-C_3H_7), $CH(CH_3)(CH_2)_nCOOH$ (n = 1, 2) and $CH(COOH)CH_2COOH$ attached to cobalt were synthesized or generated *in situ*, mostly by the reaction of vitamin B_{12s} or hydridocobalamin with the respective α- or ω-substituted halocarboxylic acids and reactions with olefinic carboxylic or dicarboxylic acids. Their rates of decomposition and the activation parameters $\Delta G^{o\neq}$, $\Delta H^{o\neq}$ and $\Delta S^{o\neq}$ of Co–C bond cleavage were determined spectrophotometrically in aqueous solutions at different pH values. Carboxyalkylcobalamins are generally more stable in solution than comparable unsubsitituted alkylcobalamins. In secondary 1-carboxyalkylcobalamins this is attributable primarily to the inductive effect and smaller size of the carboxyl group. The presence of the carboxyl group also strengthens the axial interactions of cobalt with the 5,6-dimethylbenzimidazole (DMBZ) ligand, as evidenced through measurements of the pK_a of DMBZ in these organocobalamins. Although also susceptible to spontaneous ('mechanochemical') decomposition, carboxyalkylcobalamins can exist with significantly longer half-lives in their base-on forms than corresponding alkylcobalamins. Short-lived, but detectable spectroscopically in solution, is succinylcobalamin, the secondary dicarboxyalkylcobalamin with a $CH(COOH)CH_2COOH$ group attached to cobalt, a compound of interest as a model of a postulated intermediate in the coenzyme B_{12}-dependent methylmalonyl-CoA-succinyl-CoA mutase reaction.

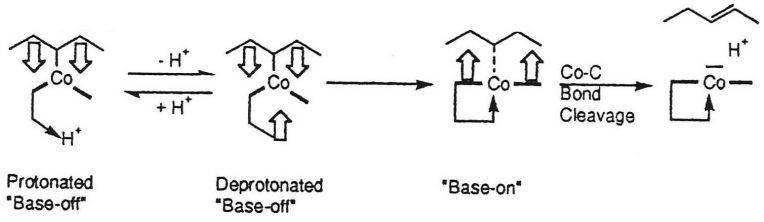
Key words: Carboxyalkylcobalamins, Organocobalamins, Co–C Bond Cleavage Reactions, Vitamin B_{12}

Introduction

Organocobalamins have intriguing properties and have been intensively studied for close to half a century. There is continuing interest in these organometallic compounds as some of them occur, or are assumed to occur, as intermediates of vitamin B_{12}- or coenzyme B_{12}-dependent enzymatic reactions [1 – 3]. Alkylcobalamins are organo-cobalt complexes with a direct Co–C bond and are commonly synthesized by the reaction of alkyl halides with the Co(I) "supernucleophile" [4, 5], vitamin B_{12s}, but initially only primary alkylcobalamins could be obtained by this method. The successful synthesis of isopropyl cobinamide [6], a secondary alkyl cobalt derivative of a corrin lacking a bulky axial base, suggested that the 5,6-dimethylbenzimidazole (DMBZ) ligand coordination was responsible for the instability or inaccessibility of the secondary alkylcobalamins, and this was confirmed through the synthesis of a derivative of cyclohexylcobalamin in which axial coordination of the cobalt atom was prevented through quaternization of the DMBZ imido-nitrogen atom [7]. Work in our laboratory demonstrated that secondary alkylcobalamins can be obtained by performing the alkylation of the cobalt(I) atom in acidic solution, *i. e.* with hydridocobalamin [8 – 10] rather than in alkaline solutions with vitamin B_{12s}. This produces the secondary alkylcobalamins in their more stable protonated base-off forms, which allowed their properties to be investigated. In neutral or alkaline solutions they are generally short-lived because under these conditions the unprotonated DMBZ will tend to attach itself to the cobalt atom. The resulting 'upward' distortion of the corrin ligand

* Paper No. 57 of a series 'Studies on Vitamin B_{12} and Related Compounds'.

0932–0776 / 08 / 0200–0143 $ 06.00



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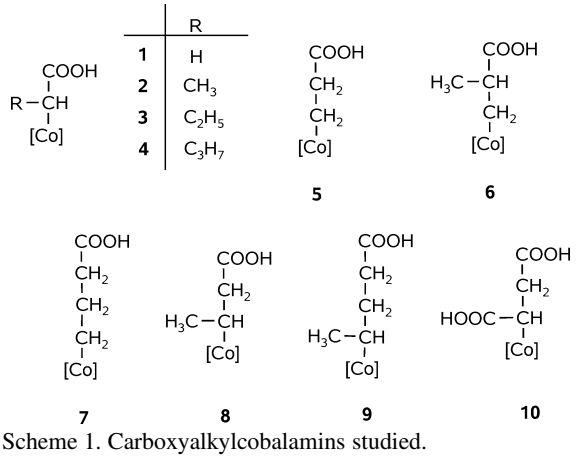

Scheme 1. Carboxyalkylcobalamins studied.

then causes the Co–C bond to cleave according to Eq. 1.

Co–C bond cleavage reactions of this type have been termed 'mechanochemical'. However, until now, information on such reactions was limited largely to unsubstituted secondary alkyl- or cycloalkylcobalamins. Since in coenzyme B_{12}-dependent enzymatic reactions such as the methylmalonyl-CoA-succinyl-CoA mutase reaction the substrates and products are organic acids, we decided to synthesize a number of secondary carboxyalkylcobalamins and to investigate their properties. Specifically, the cobalamins **1** – **10** given in Scheme 1 were prepared or generated *in situ*, and their rates of decomposition were determined under various conditions by spectroscopic means.

Since the carboxyl group is sterically less demanding than an alkyl group, its electron-attracting effect should stabilize the base-on forms. It is thus reasonable to expect α-carboxyalkylcobalamins to be less susceptible to spontaneous Co–C bond cleavage than the corresponding unsubstituted secondary alkylcobalamins. In the carboxyalkylcobalamins, the transmission of the inductive effect of the carboxyl group to the cobalt atom should progressively decrease as the carboxyl group is moved from the α to the ω position. This should weaken the axial Co–N bond and increasingly favor the base-off form. The stability of the Co–C bond in these compounds could also be influenced by interactions between the carboxyl group and the corrin substituents. However, predictions as to the magnitude and direction of these effects are difficult as both attractive and repulsive interactions operate. We also attempted the synthesis of succinylcobalamin, **10**. This secondary dicarboxyalkylcobalamin is of interest as succinyl-CoA-cobalamin is a postulated intermediate in the methylmalonyl-CoA-succinyl-CoA mutase reaction [11]. Previous studies [12] demonstrated that vitamin B_{12s} reduces maleic and fumaric acid and their diethyl esters to succinic acid or its ester, respectively. These reductions have been described as 2 electron processes involving the addition of nucleophilic Co(I) species to the unsaturated acids followed by the protonation of the resulting carbanionic cobalamins, but under the conditions of these experiments the intermediate formation of these compounds could not be demonstrated. We will show herein that **10** has a short half-life but can be detected spectroscopically if generated *in situ*.

In attempting the preparation of the substituted organocobalamins, several unexpected observations were made that should be mentioned at this point. For example, while hydridocobalamin reacts rapidly with cyclohexene to yield cyclohexylcobalamin, *cis*-4-cyclohexene-1,2-dicarboxylic acid failed to react with hydridocobalamin. The formation of secondary carboxyalkylcobalamins was also found to be very sensitive to reaction conditions. Thus, the addition of alkenes to hydridocobalamin follows the Markovnikov rule, but the addition of vinyl carboxylic acid does not. Possible explanations for these findings will be provided. Finally, reactions of several other unsaturated mono- and dicarboxylic acids and their derivatives with hydridocobalamin will be mentioned which failed to yield the expected products.

Experimental Section

Materials

Vitamin B_{12a} (N. F., 82.5 % hydroxocobalamin) was obtained from Merck Sharp & Dohme Research Laboratories, Rahway, N. J. Nitrogen was dried with anhydrous cal-

cium sulfate. Alkylating reagents were high-purity commercial products and distilled as necessary. Racemic mixtures of chiral alkylating agents were used throughout. Buffer solutions were prepared by a published procedure [13]. All other reagents and chemicals were used as received except where indicated. The syntheses of the organocobalamins were performed in Pyrex test tubes of 10 mL capacity except where noted. As cuvettes, 4 mL test tubes were used for the spectroscopic measurements. Very short-lived organocobalamins were synthesized in specially constructed 4 mL capacity pyrex test tubes with side-arms. These test tubes were constructed so that they could also be employed as optical cuvettes.

Methods and instrumentation

De-aeration was accomplished by flushing nitrogen through the rubber-stoppered test tubes by inlet and outlet needles. Visible absorption spectra of the solutions were recorded with a Beckman DU-5O spectrometer which was equipped with a thermostated cell holder and interfaced with an IBM PC. The spectra were obtained and stored through the use of the Beckman "Data Capture" program. Photodealkylations of the organocobalamins were performed at a 15-cm distance from a 150-W flood lamp.

Characterization of carboxyalkylcobalamins

Stable organocobalamins were isolated by phenol extraction [14] and their purity was established by thin-layer chromatography (TLC) on Bakerflex cellulose with *n*-butanol : acetic acid : water (10 : 3 : 7) as the ascending phase. Unstable organocobalamins were characterized by visible spectroscopy, half-life, and photodegradation in solutions of varying pH.

Preparation of carboxyalkylcobalamins

Six different methods of synthesis, designated A–F, were employed for the synthesis of the carboxyalkylcobalamins, as shown in Table 1. Methods D–F were modifications of Method C and provided us with a route by which alkylcobalamins with short life times could be synthesized.

A. With vitamin B_{12s} in alkaline solution

This method is applicable for all primary carboxyalkylcobalamins and for 1-carboxyethylcobalamin. Substituted alkylcobalamins were obtained by the addition of the alkylating agent to an alkaline acetone solution of vitamin B_{12s} [15]. For the preparation of carboxymethylcobalamin, chloroacetic acid was dissolved in a minimal amount of water under a stream of nitrogen, and a few drops of the concentrated solution were injected into the solution of vitamin B_{12s}.

Table 1. Synthetic methods for carboxyalkylcobalamins employed.

No.	Carboxyalkyl group	Method[a]	Alkylating acid derivative
1	carboxymethyl	A	chloroacetic
2	1-carboxyethyl	A	2-bromopropionic
3	1-carboxypropyl	C	2-bromobutyric
4	1-carboxybutyl[b]	D	2-bromovaleric
5	2-carboxyethyl	A, B	3-bromopropionic
		B	acrylic
6	2-carboxypropyl	B	methacrylic
7	3-carboxypropyl	A, B	4-bromobutyric
8	2-carboxy-1-methylethyl	B	3-chlorobutyric
		B	vinylacetic
9	3-carboxy-1-methylpropyl	B	4-pentenoic
10	succinyl[b]	E	maleic
		F	bromosuccinic

[a] Refers to method in Experimental Section; [b] identified by visible spectroscopy in reaction solution under nitrogen.

B. With hydridocobalamin (vitamin B_{12s} in glacial acid)

This method is applicable for all secondary, α- to ω-carboxyalkylcobalamins and all primary carboxyalkylcobalamins except carboxymethylcobalamin. Concentrated solutions of the halogenated carboxylic acids in glacial acetic acid were added under nitrogen to a solution of hydridocobalamin. The latter was prepared in a 10 mL test tube by the reduction typically of 25 mg of hydroxocobalamin dissolved in 3 mL of glacial acetic acid with 100 mg of oven-dried metallic zinc. After completion of the reactions, as indicated by the color change of the solutions from green to yellow, the supernatant solutions were decanted from the zinc and two drops of concentrated phosphoric acid were added to facilitate the product's precipitation upon ether addition.

C. With vitamin B_{12s} in aqueous buffer

Vitamin B_{12a} (25 mg) was dissolved in 3 mL of a pH = 2.0 buffered solution in a 10 mL test tube. To the solution, 100 mg of oven-dried zinc dust was added, and the tube was serum capped and de-aerated. The tube was then shaken (to effect the reduction to vitamin B_{12s}) and a few drops of the halocarboxylic acid were injected to produce the carboxyaIkylcobalamins.

D. Short-lived carboxyalkylcobalamins

The appropriate amount of vitamin B_{12a} was dissolved in 3 mL of pH = 2.0 buffered solution in a 4 mL test tube to produce a solution with the absorbance $A_{h\nu}$ of 3.0 at 355 nm. A spatula tip of zinc dust was added to the solution. The tube was capped, deaerated, and shaken until the production of B_{12s} was complete. Approximately 4 drops of the halocarboxylic acid were injected into the tube, which was then again shaken to complete the reaction. The absorption spectra of the organocobalamins generated were recorded in the centrifuged reaction solutions.

E. Succinylcobalamin, ***10***

Excess bromosuccinic acid was placed in the side arm connected to a 4 mL test tube. The correct amount of vitamin B_{12a} was dissolved in 3 mL of pH = 2.0 buffered solution to make a solution with the $A_{h\nu}$ of 3.0 at 355 nm. This solution was transferred to the test tube. A spatula tip of zinc dust was added to the solution. The tube was capped, de-aerated, and carefully shaken to produce B_{12s} without disturbing the acid in the side arm. The zinc was allowed to settle to the bottom of the test tube, the bromosuccinic acid was mixed into the B_{12s} solution by tapping, and the spectrum was recorded.

F. Succinylcobalamin, ***10****, alternate synthesis*

Method E was employed using maleic acid instead of bromosuccinic acid as the alkylating agent, and acetic acid in place of the aqueous pH = 2.0 buffered solution as the solvent.

Kinetic measurements

Depending on the half-life of the organocobalamin, two different measurement methods (A and B) were employed to determine the spontaneous dealkylation rate. All measurements of the half-lives were performed under aerobic conditions. Solution pH values were checked before and after the experiment to ensure that no change in the pH had occurred during the experiment.

A. Cobalamins with half-lives $>$ *5 min*

The isolated solid carboxyalkylcobalamin or aliquots of the supernatant carboxyalkylcobalamin solution were added to specified buffer solutions in foil-wrapped 4 mL test tubes. A portion of each solution was photolyzed immediately, and the spectrum was recorded to obtain the end point of the reaction and the A_i at 355 nm. At appropriate time intervals, visible spectra were recorded of the unphotolyzed solutions, and the $A_{t(exp)}$ at 355 nm were measured at different time points. After approximately two half lives, these solutions were photolyzed; the visible spectra were recorded, and their A_∞ at 355 nm were obtained. The observed absorbances $A_{h\nu}$ were compared to A_i to determine the amount of corrin decomposition that occurred during the spontaneous dealkylation reaction:

$$A_{t(355)} - A_{h\nu(355)} = R \cdot t \quad (2)$$

$$A_{t(exp)} + R \cdot t = A_t \quad (3)$$

Corrections in the absorbances were made if corrin decomposition was observed by the following equations where R is the rate of corrin decomposition. Sharp isosbestic points were observed, indicating that no corrin decomposition had occurred. The first order rate constants of dealkylation were obtained from the least-squares slope of $\ln(A_{h\nu} - A_t)$ *vs.* time plots, where A_t is the absorbance at 355 nm at time t. For rates at temperatures $>$ 20 °C, the test tubes were stored in a heat block between spectral scans.

B. Half-lives $<$ 5 *min*

Isolated solid carboxyalkylcobalamins or aliquots of the supernatant carboxyalkylcobalamin solutions were added to specified buffer solutions in 4 mL test tubes. The test tubes were shaken and rapidly placed in the spectrometer. Absorbances were measured at 355 nm at appropriate time-intervals by pre-programming the spectrometer. After approximately two half lives, each solution was photolyzed to obtain the end point. The first order rate constants were determined from the least-squares slope of $\ln(A_{h\nu(355)} - A_t)$ *vs.* time plots, where A_t is the absorbance at 355 nm at time t. For rates at temperatures other than r. t., the cell holder and buffers were previously equilibrated at the required temperature.

pK_a Determinations

Solutions of the primary carboxyalkylcobalamins were prepared in water. Aliquots (0.5 mL) were added to three 4 mL test tubes that contained 3.0 mL of pH = 1.5, 3, and 7 buffer solutions, respectively. Visible spectra were recorded at pH = 1.5 to obtain the maximum absorbance reading for the base-off species at 465 nm and the minimum absorbance (baseline) reading at 525 nm. Spectra were also recorded at pH = 7.0 to obtain the maximum absorbance reading for the base-on species at 525 nm and the minimum absorbance reading at 465 nm. Spectra were then recorded for the pH = 3.0 samples, measuring the absorbance at 465 and 525 nm. All samples were then photolyzed; the absorbance was measured at 355 nm to determine the possible differences in concentration between the three corresponding samples. Absorbances were corrected for concentration error when necessary. The pK_a was calculated from Eqs. 4 – 7:

$$K_a = [\text{base-on}]/[\text{base-off}] \times [H^+] \quad (4)$$

$$[\text{base-on}] = \{A_{525nm}(pH = 3) - A_{525nm}(pH = 1.5)\} / A_{525nm}(pH = 7) \quad (5)$$

$$[\text{base-off}] = \{A_{465nm}(pH = 3) - A_{465nm}(pH = 7)\} / A_{465nm}(pH = 1.5) \quad (6)$$

$$K_a = 1/[H^+], \text{ where } [\text{base-on}] = [\text{base-off}] \quad (7)$$

Results and Discussion

The methods employed for the synthesis of the carboxyalkylcobalamins are summarized in Table 1. Table 2 lists optical absorption spectral data of cobalamins **1** – **10**. Table 3 compares the observed pK_a of

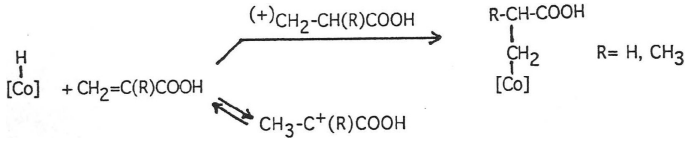

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Table 2. Spectral data for carboxyalkylcobalamins.

No.	Carboxyalkyl group	Maxima (nm) Base-on[a]	Maxima (nm) Base-off[b]
1	carboxymethyl	520, 420, 375, 335	460, 425, 370
2	1-carboxyethyl	520, 405, 375, 340	460, 420, 360
3	1-carboxypropyl[c]	—	455(s), 420, 340
4	1-carboxybutyl[c]	—	455(5), 420, 340
5	2-carboxyethyl	520, 375, 340	460, 375
6	2-carboxypropyl	520, 375, 345	460, 380
7	3-carboxypropyl	515, 375, 340	460, 380
8	2-carboxy-1-methylethyl[d]	—	485(s), 450, 385
9	3-carboxy-1-methylpropyl	—	485(5), 445, 385
10	succinyl[c]	—	455(s), 420, 340

[a] pH = 2 buffer solution; [b] pH = 7 buffer solution; [c] glacial acetic acid solution under nitrogen; [d] partial base-on character at pH = 7.0.

Table 3. Observed pK_a values of different alkyl- and carboxyalkylcobalamins.

No.	Carboxyalkyl group	pK_a[a]	Comparable alkyl group	pK_a
1	carboxymethyl	2.20	ethyl	3.87[b]
2	1-carboxyethyl	3.70	isopropyl	—[c]
5	2-carboxyethyl	3.25	*n*-propyl	3.84[d]
6	2-carboxypropyl	3.20	isobutyl	4.20[d]
7	3-carboxypropyl	3.64	*n*-butyl	3.93[e]

[a] Calculated by equations described in the Experimental Section; [b] ref. [16]; [c] the base-on form is not observable in neutral solution; [d] ref. [8]; [e] ref. [17].

the carboxyalkylcobalamins with corresponding alkylcobalamins. Table 4 gives the observed first-order rates of decomposition of 2-carboxyethylcobalamin, **5**, in solutions of different acidities, and Table 5 the observed $t_{1/2}$ values of selected carboxyalkylcobalamins and related compounds. Tables 6 and 7 summarize the rates and activation parameters of aerobic thermal decomposition of carboxyalkylcobalamins in pH = 2.0 and pH = 7.0 buffered solutions. Absorption spectra of cobalamins 2, 5, 6 at different pH values are given in Figs. 1, 2.

Primary carboxyalkylcobalamins

The primary carboxyalkylcobalamins were obtained by the methods given in Table 2. Most of these cobalamins could also be prepared by the addition of ω-halocarboxylic acids to hydridocobalamin in glacial acetic acid. Unexpectedly, however, the reaction of chloroacetic acid with hydridocobalamin yielded only vitamin B_{12r} and H_2. On reaction of vitamin B_{12s} with 3-bromopropionic acid 2-carboxyethylcobalamin, **5**, was obtained, as expected. However, the same compound and not the isomeric 1-carboxyethylcobalamin formed on reaction of hydridocobalamin with acrylic acid. Similarly, the reaction of methacrylic acid with hydridocobalamin produced the primary 2-methyl-2-carboxyethylcobalamin according to Eq. 8.

According to the Markovnikov rules, methacrylic acid should react with hydridocobalamin to yield 1-carboxy-1-methyl-ethylcobalamin. Its failure to form could be attributable to a steric effect since tertiary organocobalamins are generally unstable. However, in

Table 4. Observed first-order rates of decomposition of 2-carboxyethylcobalamin, **5**, in solutions of different acidities.

Acidity[a]	$k_{observed}$ (sec^{-1})[a]	Half-life[b]
1 M H_3PO_4	5.4×10^{-6}	1.5 d
pH = 2.0	2.0×10^{-6}	4.0 d
pH = 3.0	2.2×10^{-6}	3.6 d
pH = 4.0	8.4×10^{-7}	9.5 d
pH = 5.0	2.3×10^{-7}	35 d
pH = 6.0	7.7×10^{-7}	10.4 d
pH = 7.0	7.5×10^{-7}	10.7 d
pH = 10.0	1.8×10^{-5}	11.0 h
1 M NaOH	3.9×10^{-4}	30 min

[a] Aerobic decomposition in solution at 20 °C; [b] ±10 %.

Table 5. Half-life comparisons[a] of alkyl- and carboxyalkylcobalamins in aqueous buffer solutions of different pH.

	pH = 2	pH = 5.0	pH = 7.0
Primary alkyl groups			
methyl	indefinite[b]	indefinite[b]	indefinite[b]
ethyl			6.0 months[b]
carboxymethyl	2.3 d[c]		10.3 h[c]
n-propyl			4 – 5 months[b]
2-carboxyethyl	4.2 d	35 d	10.9 d
n-butyl			6.0 months[b]
2-carboxypropyl	7.4 d	15 d	1.5 d
3-carboxypropyl	6.9 d	17 d	23 d
Secondary alkyl groups			
isopropyl	21.0 h	4.4 min	2.8 min[b]
1-carboxyethyl	23 d	months	19 d
2-butyl	8.1 h		1.5 min[b]
2-carboxy-1-methylethyl	2.1 d	14.9 min	12.0 min
2-pentyl	9.6 h		2.1 min[b]
3-carboxy-1-methylpropyl	2.5 d	7.9 d	6.2 min
1-carboxypropyl	34 min		< 20 s
3-pentyl	33 min		3.2 s[b]

[a] From measured aerobic decomposition rates in the dark at ambient temperature, error on $t_{1/2}$ = 10 %; [b] ref. [8]; [c] measured in buffer solution at 56 °C.

Table 6. Rates and activation parameters for aerobic thermal decompositions of primary carboxyalkylcobalamins in pH = 2.0 and 7.0 buffered solutions[a].

No.	carboxyalkyl group	pH	T (°C)	k_{obsd} (sec^{-1})	$\Delta H^{\circ\ddagger}$ (kcal mol^{-1})[b]	$\Delta S^{\circ\ddagger}$ (cal mol^{-1} K^{-1})[b]	$\Delta G^{\circ\ddagger}$ (kcal mol^{-1})[c]
1	carboxymethyl	2	46	1.2×10^{-6}			
		2	56	3.6×10^{-6}	20.6 ± 0.2	-21.2 ± 1.0	26.9 ± 0.3
		7	46	3.6×10^{-6}			
		7	56	1.8×10^{-5}	31.4 ± 0.2	14.8 ± 0.9	26.9 ± 0.4
5	2-carboxyethyl	2	20	1.2×10^{-6}			
		2	48	1.2×10^{-5}			
		2	70	4.6×10^{-5}	14.1 ± 0.3	-37.4 ± 0.1	25.3 ± 0.2
		7	20	1.8×10^{-6}			
		7	42	2.9×10^{-6}			
		7	62	6.3×10^{-6}	11.2 ± 0.3	-48.7 ± 0.1	25.8 ± 0.2
6	2-carboxypropyl	2	20	1.1×10^{-6}			
		2	48	1.9×10^{-5}			
		2	70	7.7×10^{-5}	16.5 ± 0.1	-29.2 ± 0.2	25.2 ± 0.7
		7	20	5.4×10^{-6}			
		7	48	8.9×10^{-6}			
		7	70	6.0×10^{-5}	9.7 ± 0.7	-51.0 ± 5	24.9 ± 2
7	3-carboxypropyl	2	20	1.2×10^{-6}			
		2	48	1.3×10^{-5}			
		2	70	8.0×10^{-5}	16.2 ± 0.1	-30.3 ± 0.1	25.3 ± 0.1
		7	20	3.4×10^{-7}			
		7	48	2.2×10^{-6}			
		7	70	1.2×10^{-5}	13.3 ± 0.1	-42.9 ± 1.0	26.1 ± 1.0

[a] Rates measured in 0.10 M sodium phosphate; [b] uncertainties correspond to standard deviations of the slopes and intercepts of $1/\ln(k_{obsd}/T)$ *vs.* $(1/T)$; [c] uncertainties are the sums of those arising from the standard deviations of the enthalpies and entropies for cobalt-carbon cleavage.

Table 7. Rates and activation parameters for aerobic thermal decomposition of secondary carboxyalkylcobalamins in pH = 2.0 and 7.0 buffered solutions[a].

No.	carboxyalkyl group	pH	T (°C)	k_{obsd} (sec^{-1})	$\Delta H^{\circ\ddagger}$ (kcal mol^{-1})[b]	$\Delta S^{\circ\ddagger}$ (cal mol^{-1} K^{-1})[b]	$\Delta G^{\circ\ddagger}$ (kcal mol^{-1})[c]
2	1-carboxyethyl[d]	2	20	4.0×10^{-7}			
		2	48	2.1×10^{-6}			
		2	70	1.0×10^{-5}	13.0 ± 0.1	-44 ± 1.0	26.0 ± 0.6
		7	20	4.2×10^{-7}			
		7	48	2.2×10^{-6}			
		7	70	0.9×10^{-6}	10.4 ± 0.1	$-52 \pm 0/2$	25.9 ± 0.7
3	1-carboxypropyl[e]	2	20	3.4×10^{-4}			
		2	30	1.7×10^{-3}			
		2	41	1.1×10^{-2}	30.9 ± 0.1	29.2 ± 0.2	22.2 ± 0.1
8	2-carboxy-1-methylethyl	2	20	3.8×10^{-6}			
		2	48	1.5×10^{-4}			
		2	70	9.9×10^{-4}	21.7 ± 0.1	-8.9 ± 0.2	24.4 ± 0.1
		7	20	9.6×10^{-4}			
		7	31	3.1×10^{-3}			
		7	48	6.0×10^{-3}	11.9 ± 0.1	-31.1 ± 1.0	21.2 ± 0.3
9	3-carboxy-1-methylpropyl	2	20	3.3×10^{-6}			
		2	48	2.3×10^{-4}			
		2	70	8.0×10^{-4}	21.9 ± 0.2	-8.5 ± 2.0	24.4 ± 0.6
		7	20	1.9×10^{-3}			
		7	36	5.3×10^{-3}			
		7	48	2.3×10^{-2}	15.8 ± 0.1	-17.2 ± 0.3	21.0 ± 0.1

[a] Rates measured in 0.10 M sodium phosphate; [b] uncertainties correspond to standard deviations of the slopes and intercepts of $1/\ln(k_{obsd}/T)$ *vs.* $(1/T)$; [c] uncertainties are the sums of those arising from the standard deviations of the enthalpies and entropies for cobalt-carbon cleavage; [d] rates could not be obtained at higher temperatures as corrin decomposition occurs faster than cobalt-carbon cleavage; [e] rate at pH = 7 not measured as too fast with instrumentation employed.

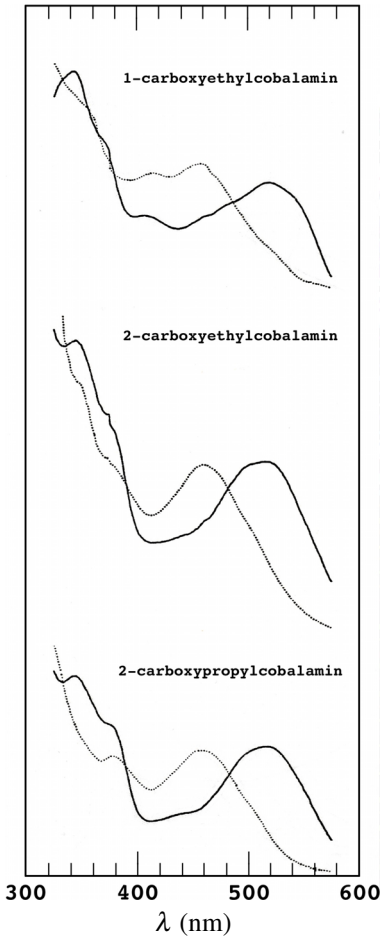


Fig. 1. Absorption spectra of 1-carboxyethylcobalamin, **2**, 2-carboxyethylcobalamin, **5**, and 2-carboxyethylcobalamin, **6**, in pH = 2 (---) and pH = 7 buffer (—) solutions at 20 °C.

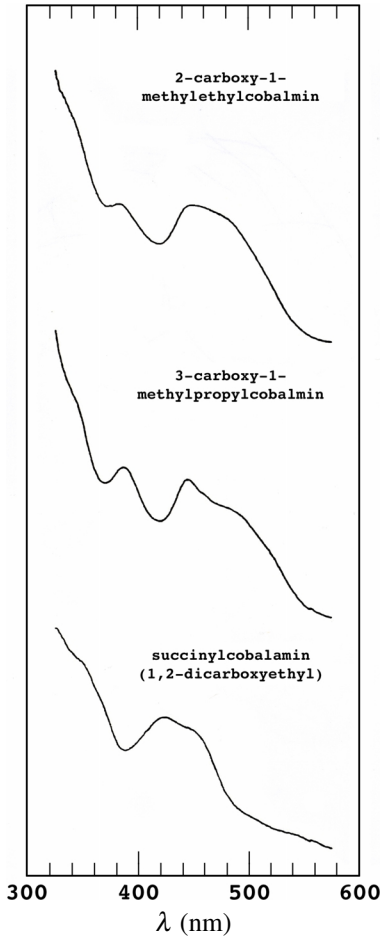


Fig. 2. Absorption spectra of 2-carboxy-1-methylethylcobalamin, **8**, 3-carboxy-1-methylpropylcobalamin, **9**, and of succinylcobalamin, **10**, in glacial acetic acid at 20 °C.

the case of acrylic acid, steric effects must be discounted since the reaction of ethylacrylate with hydridocobalamin has previously been shown to yield the secondary 1-(carbethoxy)ethylcobalamin in accord with the Markovnikov rules. However, according to Eq. 8 the analogous reaction of acrylic acid with hydridocobalamin would require a carbonium ion to be generated adjacent to its carboxyl group, which is highly unfavorable.

Secondary carboxyalkylcobalamins

Carboxyethylcobalamin, **2**, the first member of the series of 1-carboxyalkylcobalamins studied, was prepared by reacting α-bromopropionic acid with vitamin B_{12s} in alkaline solution. Most of the secondary carboxyalkyl cobalamins were produced by the reaction of short-chain α-halocarboxylic acids with hydridocobalamin in glacial acetic acid pH = 2 buffered aqueous solution or in glacial acetic acid (Table 2). However, when higher α-halogenated organic acids were added to hydridocobalamin in glacial acetic acid, only vitamin B_{12r} precipitated, and no organocobalamin was produced, as in the reaction with chloroacetic acid. Under the same conditions, only vitamin B_{12r} was also observed upon addition of crotonic acid to hydridocobalamin, even though 1-carboxypropylcobalamin formed rapidly and quantitatively in the reaction of 2-bromobutyric acid with hydridocobalamin at pH = 3.0. Furthermore, only vitamin B_{12r} was detected in the reactions of 2-bromovaleric, 2 bromohexanoic, or α-bromophenylacetic acid with vitamin B_{12s} at pH = 2.

The addition of 3-hexenoic acid or 2,4-hexadienoic acid to hydridocobalamin likewise produced only vitamin B_{12r}. These bulky substrates or their protonated derivatives apparently cannot reach the cobalamin cobalt atom for Co–C bond formation. As to the life-times of the secondary carboxyalkylcobalamins, they were found to depend on the alkyl chain length in α-position relative to cobalt just as in the case of the secondary alkylcobalamins. For example, while 1-carboxypropylcobalamin, **3**, could still be isolated, 1-carboxybutylcobalamin was only detectable spectroscopically in the reacting solution, and the 1-carboxypentyl- and 1- and 2-(propyl)ethylcobalamin was no longer observable. Similarly, 3-carboxy- (1-methyl)propylcobalamin, **9**, could still be synthesized, but 3-carboxy-(1-ethyl)propylcobalamin could not be obtained, nor was it detectable spectroscopically in reacting solutions. Thus, the maximum length of the alkyl chain that is allowed for a stable 1-carboxyalkylcobalamin appears to be limited to three carbon atoms; the chain length limit for a 2-carboxyalkyl cobalamin is two carbon atoms, and the chain length limit for a 3-carboxyalkyl derivative is one carbon atom. As the position of the carboxy group increases in distance from cobalt, the alkyl group chain length must decrease to produce a "stable" secondary carboxyalkylcobalamin. The same trend of destabilization by longer alkyl chains is seen for the unsubstituted secondary alkylcobalamins in the series of 2-butyl-, 3-pentyl- and 3-hexyl-cobalamin [8]. These structural limitations obviously apply only to the vitamin B_{12} derivatives in solution; in enzymes, they could possibly be overcome through distortions of the corrin by its attachment to the apoprotein.

Dicarboxyalkylcobalamins

The synthesis of several dicarboxyalkylcobalamins was attempted through the reactions of the Co(I)-derivatives of vitamin B_{12} with maleic, bromosuccinic, fumaric, *cis*-4-cyclohexene-1,2-dicarboxylic, glutaconic; *trans*-hydromuconic, itaconic, citraconic, *trans,trans*–muconic, and mesaconic acid. Maleic acid and bromosuccinic acid reacted with hydridocobalamin in glacial acetic acid and pH = 2.0 buffered aqueous solution, respectively, to form succinylcobalamin, **10**, based on spectroscopic evidence (see Fig. 2), but fumaric acid failed to react with hydridocobalamin. This suggests that there is a steric interference between the corrin and the *trans* carboxyl groups, preventing the close approach of the fumaric acid to the corrin ring. Solvent-dependent electrostatic repulsion effects may also be operative, as fumaric acid reacts with aqueous vitamin B_{12s} at pH = 2.2 – 6.8 to yield succinic acid and vitamin B_{12s} at about 5 % of the rate of maleic acid. Similarly, diethyl fumarate reacted with hydridocobalamin in 1 : 1 (v/v) ethanol/acetic acid to yield 50 % diethyl succinate, with half of the diethyl fumarate remaining [13]. The present study shows that if hydridocobalamin is reacted in glacial acetic acid with excess maleic or bromosuccinic acid, **10** becomes detectable spectroscopically *in situ*. On attempted isolation of **10**, however, only B_{12r} was obtained.

Optical absorption spectra of carboxyalkylcobalamins

While most of the carboxyalkylcobalamins were found to have absorption spectra identical with those of the corresponding alkyl analogs, *i. e.* 2-carboxyethylcobalamin spectroscopically resembles *n*-propylcobalamin, the spectra of carboxymethyl, 1-carboxyethyl, 1-carboxypropyl, 1-carboxybutyl, and succinylcobalamin differed from those of their alkyl analogs (Table 2). Carboxymethylcobalamin and 1-carboxyethylcobalamin had identical *base-off* spectra that resembled the base-off spectra of cyclopropylcobalamin, those of the transient cobalamins **3**, **4**, and **10** resembled that of isopropylcobalamin (Figs. 1 and 2).

The *base-on* spectrum of carboxymethylcobalamin resembled that of isobutylcobalamin, and 1-carboxyethylcobalamin was similar to cyclobutylcobalamin. The existence of 1-carboxyethylcobalamin in the base-on form is attributed mainly to the smaller steric demand of the -COOH group compared to *e. g.* an ethyl group. For similar reasons, cycloalkylcobalamins with a ring size of five or less carbons can exist in the base-on form in solution [8].

Axial base coordination

The carboxyl group influences the primary alkylcobalamin pK_a values of the axial base coordination when the group is up to two carbons removed from the cobalt-bonded carbon and affects the pK_a value of the secondary alkylcobalamins when the carboxyl group is adjacent to the cobalt-bonded carbon (Table 3). The pK_a values of the primary carboxyalkylcobalamins increase as the carbon chain of the carboxyalkyl group

increases in a manner similar to that of the alkylcobalamins [18]. In the series of carboxymethyl to 3-carboxypropyl, this difference in pK_a values between the carboxyalkylcobalamin and the alkyl analogs decreases as the distance of the carboxyl group from the cobalt-bonded carbon increases. Although secondary alkylcobalamins do not exist in the base-on form, the base-on form of **2** is stable and has a pK_a of 3.7 – even lower than that of ethylcobalamin. The pK_a values indicate stronger bonding of the benzimidazole nitrogen to the cobalt atom, which may be a result of electron density withdrawal from cobalt by the carboxyalkyl ligand.

Dealkylation reactions

The anaerobic thermodealkylation of the primary carboxyalkylcobalamins was studied in 1 M NaOH aqueous solution. Carboxymethylcobalamin, **1**, was found to be surprisingly stable under these conditions: In 1 M NaOH it required several days of heating at 70 °C for decomposition. In contrast, 2-carboxyethylcobalamin, **5**, and 2-carboxypropylcobalamin, **6**, dealkylated rapidly in aqueous 1 M NaOH to yield vitamin B_{12s}. This rapid dealkylation is attributable to the presence of the hydrogen atoms in β-position relative to cobalt and adjacent to the carboxy group, providing a facile dealkylation pathway through intermediate carbanions formed by hydrogen abstraction. One additional methylene group as in 3-carboxypropylcobalamin, **7**, stabilizes the cobalamin against alkaline degradation. Heating solutions of **7** in 1 M NaOH first produced a typical base-off cobalamin sprectrum, suggesting that the hydrolysis of the phosphate side-chain occurs prior to Co-C bond cleavage. Among the secondary carboxyalkylcobalamins studied, 1-carboxyethylcobalamin, **2**, exhibited a stability similar to the primary 3-carboxypropylcobalamin, **7**. The increased stability of **2** relative to isopropylcobalamin may be due partly to the smaller spacial demand of the carboxyl group relative to the methyl group, as well as to an electron-withdrawing effect of the carboxyl group. Except for **2**, the secondary carboxyalkylcobalamins studied dealkylated rapidly in pH = 7.0 buffered aqueous solutions to B_{12s} in a fashion similar to their alkyl analogs. Interestingly, the stability of **2** was comparable to that of **7**, a primary carboxyalkylcobalamin, and not to a secondary alkylcobalamin.

pH-Rate profiles of the spontaneous dealkylation of carboxyalkylcobalamin

Because of the rapid rate the reaction of vitamin B_{12s} with oxygen, the rates of dealkylation of the carboxyalkylcobalamins could be measured under aerobic conditions. The observed pseudo first order rate constants and half-lives are summarized in Table 4.

Primary carboxyalkylcobalamins, in general, are kinetically less stable than the comparable unsubstituted alkylcobalamins, and their decomposition rates are strongly pH-dependent. The half-lives for the primary carboxyalkylcobalamins in neutral solutions were 2 – 7 d, whereas the half-lives of the alkylcobalamins were 4 – 6 month. Specifically, the pH-rate profile for the aerobic dealkylation of **5** is shown in Table 5. The half-life of **5** reaches a maximum of 35 d at pH = 5.0 but is significantly shorter than that observed for higher primary alkylcobalamins. As primary alkylcobalamins also decomposed in alkaline solution, the decreased cobalt-carbon bond stability expected with increasing pH did not show in a decrease in reaction half-life for the primary alkylcobalamins in increasingly acidic solutions. Other primary carboxyalkylcobalamins, except carboxymethylcobalamin, exhibited a similar pH-rate profile, but the half-lives of these compounds in pH = 5.0 buffered aqueous solutions decreased as the alkyl chain length increased (Table 5). In neutral solutions of alkylcobalamins, a similar decrease of the half-lives with increasing alkyl chain length is not observed.

Secondary carboxyalkylcobalamins. The half-lives of the secondary carboxyalkylcobalamins differed from those of the corresponding alkylcobalamins (Tables 6 and 7). The secondary 1-carboxyalkylcobalamins were less stable in neutral and acidic solution than the corresponding 2-butylcobalamin. The 2-carboxy-1-methylethyl- and 3-carboxy-1-methylpropylcobalamins, **8**, **9**, proved to be more stable than the 2-butyl- and 2-pentylcobalamins in neutral and acidic solutions. It is noteworthy that 1-carboxyethylcobalamin, **2**, exhibited a pH-rate profile similar to that of a primary carboxyalkylcobalamin, but in neutral solution it was more stable than 2-carboxyethylcobalamin, **5**, and less stable than ethylcobalamin. The stability maxima at pH = 5.0 for primary carboxyalkylcobalamins and the secondary 1-carboxy-ethylcobalamin suggests that Co–C bond cleavage is impeded due to hydrogen bonding between the carboxyl group and the cor-

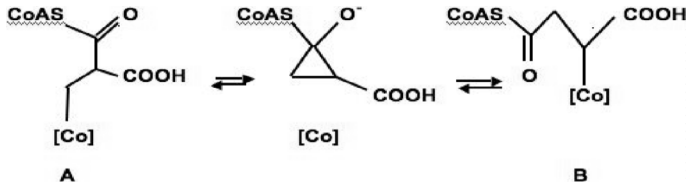
rin amide side chains; at increasingly higher pH, the number of carboxyl groups that are deprotonated increases, diminishing this stabilizing effect. No such stability maxima are seen in the pH-rate profiles of alkylcobalamins where similar hydrogen-bonding interactions could not occur.

Co–C bond strengths and activation parameters of Co–C bond cleavage. Plots of log *k*/T *versus* 1/T produced straight lines from which the numerical values given in Table 6 and 7 of the activation enthalpy $\Delta H^{\neq}$, the activation entropy, $\Delta S^{\circ\neq}$, and the Co–C bond dissociation energy, $\Delta G^{\circ\neq}$, were obtained. Compared to secondary alkylcobalamins, 1-carboxyalkylcobalamins are, in general, more resistant to spontaneous Co–C bond cleavage, which applies even to their base-on forms. The increased strength of the coordinative Co–N (DMBZ) bond is attributable to the inductive effect of the COOH group. The latter is also responsible, in part, for the greater strength of the Co–C bonds, as reflected by the increased $\Delta G^{\circ\neq}$ values, but is also influenced by structural and steric effects of the carboxyalkyl substituent. For example, the $\Delta G^{\circ\neq}$ of 1-carboxymethylcobalamin, **2**, of 25.9 ± 0.7 kcal mol^{-1} is nearly 5 kcal mol^{-1} larger than that of isopropylcobalamin, **14**, of 20.8 ± 1.0 kcal mol^{-1} at pH = 7. The difference of the corresponding $\Delta H^{\circ\neq}$ values is even larger and close to 8 kcal mol^{-1} and is mainly caused by the large negative $\Delta S^{\circ\neq}$ associated with the Co–C bond cleavage of 1-carboxyethylcobalamin (Table 7). Whereas the presence of the methyl group in α-position effects no major weakening of the Co–C bond, its replacement by an ethyl group in α position as in 1-carboxypropylcobalamin, **3**, reduces the $\Delta G^{\circ\neq}$ by nearly 3 kcal mol^{-1} to 22.2 ± 0.7 kcal mol^{-1} and changes the $\Delta S^{\circ\neq}$ from strongly negative to positive. This suggests that the Co–C bond in **3** is cleaved without significantly involving DMBZ-induced 'upward' distorted corrin configurations. A large negative $\Delta S^{\circ\neq}$ is indicative of the opposite, *i. e*., that the Co–C bond cleavage involves a highly ordered, upward-distorted corrin structure generated by strong axial interactions of the cobalt atom with DMBZ. This interpretation also applies to the thermal cleavage of primary carboxyalkylcobalamins, whose $\Delta S^{\circ\neq}$ values of Co–C bond cleavage are almost invariably negative (see Table 6). The observed larger negative values also suggest that there may be hydrogen bonding between the carboxyl group and substituents of the corrin, although the exact position of this site still remains to be identified. The site on the corrin where this hydrogen bonding occurs must be different from the site suspected to be responsible for the $t_{1/2}$ maxima observed around pH = 5.0 in several of the carboxyalkylcobalamins investigated (see Tables 4, 5).

Among the primary carboxyalkylcobalamins, the carboxymethyl derivative, **1**, exhibited the highest Co–C bond strength, as evidenced by its $\Delta G^{\circ\neq}$ of 26.9 ± 0.3 kcal mol^{-1} (see Table 6). In this compound, steric strain and obstruction are expected to be largely absent, and indeed, in pH = 7 buffered solution the observed activation parameters of Co–C bond cleavage, including the positive $\Delta S^{\circ\neq}$ of 14.8 eu, are closely similar to those of an unsubstituted alkylcobalamin such as isobutylcobalamin, reported in [14]. However, in pH = 3 buffered solution, the $\Delta S^{\circ\neq}$ of **1** changes to -21.0 ± 1 eu and thus becomes comparable to the $\Delta S^{\circ\neq}$ values of the other carboxyalkylcobalamins studied; the cause(s) for this sign-switch remain to be elucidated. The $\Delta G^{\circ\neq}$ values of all other primary carboxyalkylcobalamins studied were lower by approximately 1 kcal mole^{-1}, and all $\Delta S^{\circ\neq}$ values were substantially negative, ranging from -29.2 ± 0.2 to -51 ± 5 eu. The observed values of $\Delta S^{\circ\neq}$ of thermal Co–C bond cleavage of these cobalamins were consistently more negative at pH = 7.0 than in pH = 2.0 buffered solutions and are indicative of a major role of the coordinated axial DMBZ in the Co–C bond cleavage process. In the secondary carboxyalkylcobalamins **8** and **9** of Scheme 1, the Co–C bond strength as determined in pH = 7 buffered solution is 21.2 ± 0.3 and 21.0 ± 0.1 kcal mol^{-1} (Table 7), indicating steric labilization of the Co–C bond with increasing length of the alkyl chain; the corresponding $\Delta S^{\circ\neq}$ values of -31.1 ± 1.0 and -17.2 ± 0.3 eu, do not affect the Co–C bond strength but are indicative of a less ordered transition state in the dealkylation process. By similar reasoning the observed activation parameters of the remaining primary and secondary carboxyalkyl cobalamins may be interpreted.

Biochemical implications and concluding remarks

Since coenzyme B_{12} is itself an organometallic compound it is plausible to assume that organocorrin intermediates with substrate-derived groups attached to cobalt are intermediates in the enzymatic reactions catalyzed by it. Although the view is also held by some that the corrin, after the initial cleavage of the Co–C bond in the coenzyme, behaves more like a specta-



(9)

tor than an active participant in the actual molecular transformation, the majority of mechanistic studies were performed with organocobalt derivatives of vitamin B_{12} or of simpler cobalt complexes as models of assumed intermediates of these enzymatic reactions. One such reaction is the conversion of methylmalonyl-CoA to succinyl-CoA by the coenzyme B_{12}-dependent methylmalonyl-CoA mutase, EC 5.4.99.2, for which several mechanisms have been proposed [19]. Work in our laboratory [11] led to the proposal that it could proceed *via* methylmalonyl-CoA- and succinyl-CoA-cobalamins as the intermediates in terms of Eq. 9.

Cobalamins structurally related to **A** of Eq. 9 have previously been prepared and were shown to yield rearranged products when reacted further, but so far no direct evidence was available for the existence of the terminal intermediate, succinyl-CoA-cobalamin, **B**. However, if the enzymatic rearrangement of methylmalonyl-CoA to succinyl-CoA should indeed proceed by way of intermediate **A**, then the principle of microscopic reversibility demands cobalamin **B** also to be an intermediate [11]. The present study indicates that cobalamin **B** could indeed be a catalytic intermediate in the enzymatic, coenzyme-B_{12}-dependent conversion of methylmalonyl-CoA to succinyl-CoA. In more generalizing term, based on the work described herein it will now be possible to further pursue mechanistic concepts of B_{12}-catalyzed reactions with a better understanding of the steric limitations imposed by the structure of this multifaceted biocatalyst.

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