Mechanism of Action of Ethanolamine Ammonia-lyase, an Adenosylcobalamin-dependent Enzyme

INTERACTION BETWEEN THE ENZYME AND A POSTULATED ORGANOCOBALAMIN INTERMEDIATE*

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Ethanolamine ammonia-lyase catalyzes the adenosylcobalamin (AdoCbl)-dependent conversion of ethanolamine to acetaldehyde and ammonia. It has been proposed that the mechanism of this and other AdoCbl-dependent rearrangements involves the formation and rearrangement of an organocobalamin in which a substrate fragment is coordinated to the corrin metal by a carbon-cobalt bond. In the case of ethanolamine ammonia-lyase, this mechanism states that one of the catalytic intermediates is a complex in which 5'-deoxyadenosine and β-amino-β-hydroxyethylcobalamin are bound to the active site of the enzyme. This complex, which arises when the substrate-containing organocobalamin initially formed at the active site undergoes rearrangement, then reacts further to release the products (acetaldehyde and NH₃) and regenerate the original cofactor, AdoCbl.

To obtain evidence regarding this proposal, experiments were conducted to determine whether this complex, if constructed from its constituents, would react to form AdoCbl. For these experiments, formylmethylcobalamin plus ammonia (β-amino-β-hydroxyethylcobalamin is the NH₃ adduct of formylmethylcobalamin) were incubated with 5'-deoxyadenosine and enzyme, and AdoCbl formation was assayed by determining whether added [14C]ethanolamine was converted to [14C]acetaldehyde. All three of the low molecular weight constituents of the complex, namely, formylmethylcobalamin, 5'-deoxyadenosine, and ammonia, have been shown in this and other studies to bind to the active site of ethanolamine ammonia-lyase. Nevertheless, the formation of AdoCbl was not detected by an assay sensitive enough to measure the conversion to cofactor of 0.0006% of the added formylmethylcobalamin. This finding supports other data which suggest that the alkylation of the corrin metal by the substrate does not occur during rearrangements catalyzed by AdoCbl-requiring enzymes.

Ethanolamine ammonia-lyase from Clostridium sp. is an adenosylcobalamin-dependent enzyme which catalyzes the conversion of ethanolamine and propanolamine to acetaldehyde and propionaldehyde, respectively, with the elimination NH₃. The reaction path involves an exchange of places between a hydrogen atom and the amino group to form a 1-amino alcohol, which then eliminates ammonia to generate the aldehyde (Reaction 1) (1, 2).

Extensive study of this and similar reactions has provided an understanding of the mechanism of hydrogen migration. This process has been shown to involve homolysis of the carbon-cobalt bond of the cofactor followed by the abstraction of the migrating hydrogen atom from the substrate by the resulting 5'-deoxyadenosine-5'-yl radical, to form a substrate radical and 5'-deoxyadenosine. Rearrangement to the product radical followed by the reversal of the above steps yields the final products and regenerates the original cofactor. The mechanism of hydrogen migration has recently been reviewed in detail (3-5).

In contrast to hydrogen migration, studies in biochemical systems have produced almost no evidence concerning the mechanism of amino group migration. However, chemical investigations have provided a number of model reactions on the basis of which several mechanisms for amino group migration have been proposed. One particularly attractive mechanism (6-9), first supported by the studies of Golding et al. (10) and later by work from other laboratories (11-18), involves substrate radical alkylation of the cob(II)alamin generated at the active site by the homolysis of the cofactor, followed by rearrangement of the resulting organocobalt compound by a σ → π interconversion (Reaction 2). This and related mechanisms (hereinafter called "transalkylation mechanisms") are currently accepted as the most likely paths for the interconversion of substrate and product radicals in adenosylcobalamin-requiring rearrangements.

Referring specifically to the ethanolamine ammonia-lyase reaction, the transalkylation mechanism postulates the existence along the reaction path of an intermediate in which β-amino-β-hydroxyethylcobalamin (Compound I, Reaction 2) and 5'-deoxyadenosine are bound to the active site of the enzyme. This mechanism further predicts that, if it were possible to construct this intermediate from its constituents, the intermediate so constructed should react in such a way as...
to complete the sequence stipulated in the mechanism, with the production of acetaldehyde and ammonia and the formation of adenosylcobalamin. \( \beta \)-Amino-\( \beta \)-hydroxyethylcobalamin is the ammonia adduct of formylmethylcobalamin, a compound whose synthesis and properties have been described by Silverman et al. (19). In the following, we report the results of experiments designed to test the above prediction, using formylmethylcobalamin and \( \mathrm{NH}_4^+ \) as precursors of \( \beta \)-amino-\( \beta \)-hydroxyethylcobalamin, the corrinoid constituent of the hypothetical intermediate.

**MATERIALS AND METHODS**

Ethanolamine ammonia-lyase (EC 4.3.1.7) from Clostridium sp was prepared and resolved of bound cobamides by the method of Kaplan and Stadtman (20). Enzyme concentration was calculated on the basis of a molecular weight of 520,000. The enzyme has been shown to possess two active sites/molecule (21). Commercial AdoCbl, cyanocobalamin, yeast alcohol dehydrogenase, and NADH were purchased from Sigma. AdoCbl and cyanocobalamin were purified by passage over a column (1 x 6 cm) of carboxymethyl-cellulose (H\(^+\) form), eluting with water. 3-Chloro-1,2-propanediol was obtained from Aldrich. [U \(^{14}\)C]Ethanolamine HCl was purchased from New England Nuclear. Other reagents were the best grade commercially available and were used without further purification.

Formylmethylcobalamin (\(\text{OHCMe}\text{Cbl}\)) - This compound was prepared from 2,3-dihydroxypropylcobalamin by the method of Silverman et al. (19). 2,3-Dihydroxypropylcobalamin was synthesized from hydroxocobalamin (21) and 3-chloro-1,2-propanediol by the reaction (19, 22):

\[
\text{Hydroxocobalamin} + 3\text{-chloro}-1,2\text{-propanediol} \rightarrow \text{2,3-dihydroxypropylcobalamin.}
\]

The spectrum of the purified material is shown in Fig. 1. This material was divided into aliquots and stored at -70°. Each day's experiments were performed with a single aliquot of (OHCMelCbl) which was thawed just before it was required, used only when spectrophotometry showed no evidence of a shoulder between 350 and 360 nm (indicating no decomposition to hydroxocobalamin (cf. Fig. 2), and discarded at the end of the day.

The spectrum of (OHCMelCbl) was observed to vary depending on the buffer in which the cobalamin was dissolved. Fig. 2, left, shows the spectrum of (OHCMelCbl) in 0.5 M \(\text{NH}_3\text{OH}\) (pH 11.4), taken immediately after periodate oxidation of the precursor but before gel filtration. Also shown is the spectrum of (OHCMelCbl) prepared in exactly the same way except in 0.5 M \(\text{CH}_3\text{NH}_2\text{HCl}\) buffer (pH 11.4) (Fig. 2, right). Differences between the two spectra are particularly noticeable in the region between 400 and 450 nm. The significance of these spectral differences are discussed below.

**Fig. 1.** Spectrum of purified \(\text{OHCMelCbl}\) in 0.5 M \(\text{NH}_3\text{OH}\).

**Fig. 2.** The dependence of the spectrum of (OHCMelCbl) on buffer composition. Left, the spectrum of (OHCMelCbl) in ammonia. The reaction mixture initially contained 0.01 ml of 2.0 M 2,3-dihydroxypropylcobalamin, 0.01 ml of 0.75 M \(\text{NaIO}_3\), and 0.49 ml of 0.5 M \(\text{NH}_3\text{OH}\). Right, the spectrum of (OHCMelCbl) in methylamine. The reaction mixture initially contained 0.01 ml of 2.0 M 2,3-dihydroxypropylcobalamin, 0.01 ml of 0.75 M \(\text{NaIO}_3\), and 0.55 ml of 0.5 M \(\text{CH}_3\text{NH}_2\text{HCl}\) (pH 11.4). In both cases, the oxidation of 2,3-dihydroxypropylcobalamin to (OHCMelCbl) was virtually instantaneous. The spectra were taken within 2 min of preparing the reaction mixtures. Gel filtration was not performed before spectroscopy in either case because (OHCMelCbl) in methylamine was found not to survive the purification procedure.

**ASSAY METHODS** - Ethanolamine ammonia-lyase activity was measured by a spectrophotometric (20) or radioassay (21). Procedures were performed under Wratten 1A safelights. The activity was determined by the Lowry method (25) with the appropriate correction (1). Spectra were obtained on a Cary 118 C recording spectrophotometer. A microcell adapter was used where necessary. Radioactivity was determined by liquid scintillation counting, using Bray's solution (26) as scintillant and carrying out determinations on a Packard model 3320 instrument.

**Use of Ribose** - All reaction mixtures contained a small quantity of ribose. The use of this sugar was begun after it was found that solutions of (OHCMelCbl), even after purification over Bio-Gel P2, contained a compound (unreacted \(\text{IO}_4^{-}\)) capable of oxidizing ethanolamine to products not taken up on Dowex 50-H\(^+\). This oxidizing...
agent was consumed by ribose. Control experiments showed that the activity of ethanolamine ammonia-lyase was not affected by ribose either in the absence or presence of (OHCMe)Cbl. Reaction mixtures were therefore prepared by mixing ribose with (OHCMe)Cbl before adding other constituents, in order to destroy the contaminating oxidizing agent.

RESULTS

Binding of Formylmethylcobalamin to Ethanolamine Ammonia-Lyase in Presence of NH₃. — Previous studies with cofactor analogs such as methylcobalamin and cyanocobalamin have revealed that these bind to the active site of ethanolamine ammonia-lyase, competing with AdoCbl, and when so bound induce the conversion of the enzyme to a form with a lower intrinsic activity and a higher affinity for the analog (27).

\[ \text{Enzyme} \rightarrow R\text{-Cbl} \rightarrow \text{Enzyme} \rightarrow R\text{-Cbl} \rightarrow \text{Enzyme} \rightarrow R\text{-Cbl} \]  

(3)

This conversion process has been termed "deactivation." Experiments with (OHCMe)Cbl have shown that this analog too deactivates ethanolamine ammonia-lyase. Thus, the spectrophotometric determination of the course of the deamination of ethanolamine in a reaction mixture containing (OHCMe)Cbl in addition to the usual constituents showed that the rate of substrate utilization declined in an exponential manner (Fig. 3), a result in accord with the mechanism described in Equation 3. From a series of such determinations, performed at varying concentrations of AdoCbl and (OHCMe)Cbl, initial rates of substrate consumption (i.e. rates obtained before the enzyme had undergone any deactivation) were calculated according to the method described under "Appendix." Dixon plots (28) of these initial rates (Fig. 4) showed (OHCMe)Cbl to be an inhibitor of the fully active enzyme, competing with AdoCbl (Kᵦ = 2.6 μM). This interpretation is supported by the finding that the Kᵦ for AdoCbl calculated from the y-intercepts of the Dixon plots agreed with the Kᵦ determined directly under the same incubation conditions (1.0 and 1.4 μM, respectively). These results imply that (OHCMe)Cbl binds to the active site of ethanolamine ammonia-lyase.

The binding of (OHCMe)Cbl to the enzyme leads to a significant change in the spectrum of the analog. In Fig. 5, the spectra of free and enzyme-bound (OHCMe)Cbl are compared. In the enzyme-bound (OHCMe)Cbl spectrum, the peak between 400 and 450 nm is much less prominent than in the spectrum of the free analog, and both that peak and the αβ (long wavelength) components of the spectrum moved a little to the right.

Fig. 3. The deactivation of ethanolamine ammonia-lyase by (OHCMe)Cbl. A reaction mixture containing 10 μmol of ethanolamine·HCl, 2.4 nmol of AdoCbl, 1.2 nmol of (OHCMe)Cbl, 0.1 μmol of NADH, 60 μg of yeast alcohol dehydrogenase, 0.8 μmol of ribose, and 27 μmol of NH₄Cl buffer (pH 9.2) in a total volume of 1.0 ml was placed in a quartz cuvette (1 cm path length). The reaction was begun by the addition (arrow) of 18 μg of ethanolamine ammonia-lyase (26 μl of a solution containing 0.75 mg of enzyme/ml of 0.01 M potassium phosphate buffer (pH 7.4)). The spectrophotometer pen was turned on 15 s later and consumption of NADH was followed spectrophotometrically at 340 nm. The entire experiment was conducted under safelights (Wratten 1A filters) at 24°C.

Fig. 4. Competitive inhibition of ethanolamine deamination by (OHCMe)Cbl. Reaction mixtures were prepared as described in Fig. 3, except that the concentrations of AdoCbl and (OHCMe)Cbl were as noted, and the quantity of enzyme was varied (range, 18 to 73 μg), larger amounts being employed for assay mixtures in which lower reaction rates were expected. The incubations were conducted under safelights (Wratten 1A filters) at 24°C, following the consumption of NADH spectrophotometrically at 340 nm. For accuracy in calculation, the recording of the spectrophotometer trace was begun exactly 20 s after adding enzyme, and the initial reaction rate was determined by extrapolation as described under "Appendix."

Fig. 5. The spectra of free and enzyme-bound formylmethylcobalamin. Top, enzyme-bound (OHCMe)Cbl. For the upper spectrum the reaction mixture contained 0.26 nmol of enzyme (0.52 nmol of active sites), 1.5 nmol of ethanolamine, 0.20 nmol of (OHCMe)Cbl (2 μl of a solution containing 0.1 mM (OHCMe)Cbl in 0.5 M NH₃OH), 0.1 μmol of ribose, and 4 μmol of NH₄Cl buffer, pH 9.2, in a total volume of 0.12 ml. For the lower spectrum the conditions were identical, except that (OHCMe)Cbl was replaced with water. The dotted curve, which represents the spectrum of enzyme-bound (OHCMe)Cbl is the difference between the upper and lower spectra. Bottom, free (OHCMe)Cbl. The reaction mixture contained 0.20 nmol of (OHCMe)Cbl (2 μl of a solution containing 0.1 mM (OHCMe)Cbl in 0.5 M NH₃OH), 0.1 μmol of ribose, and 4 μmol of NH₄Cl buffer, pH 9.2, in a total volume of 0.12 ml. All spectra were taken with a microcell. It is of interest that the spectrum of free (OHCMe)Cbl in this experiment is similar to that of (OHCMe)Cbl in 0.5 M NH₃OH (see Fig. 1), suggesting that the species predominating in solution is the same in both cases (see "Discussion."
peak are shifted to the blue. These spectral changes suggest a withdrawal of electron density from the cobalt when the analog binds to the enzyme (29). Along with the spectral change, binding to the enzyme leads to labilization of (OHCMe)Cbl. When the survival of (OHCMeCbl) was followed with time, it was found that the rate of cleavage of the carbon-cobalt bond increased 4-fold when the analog was incubated with active enzyme, while boiled enzyme had little effect on this process (Table I). Thus, the active enzyme causes a specific acceleration in the cleavage reaction, providing further evidence for a well defined association between the enzyme and (OHCMeCbl), i.e. binding of the cobamide to the active site. It is noteworthy, however, that the acceleration, while real, is rather modest, suggesting that it may not be a manifestation of the catalytic activity of the enzyme, but may rather reflect subtle alterations in the environment of the analog upon binding to the active site.

**Failure of Enzyme to Convert (OHCMe)Cbl into AdoCbl** – The ammonia adduct of (OHCMeCbl) has been postulated to participate in the transamination mechanism for amino group migration catalyzed by ethanolamine ammonia-lyase (see above). To obtain evidence for this mechanism, similar experiments were performed to determine whether the enzyme could catalyze the formation of AdoCbl from (OHCMeCbl) in the presence of 5′-deoxyadenosine and high concentrations of NH3 (the latter to force the formation of the ammonia adduct of the cobamide). Detection of the coenzyme was to be accomplished by carrying out the reaction in the presence of [14C]ethanolamine; the formation of AdoCbl would be established by the appearance of [14C]acetaldehyde in the reaction mixture during the course of the incubation. The sensitivity of the assay was ensured by using [14C]ethanolamine in concentrations comparable to the concentration of active sites. Because of the high turnover number of the enzyme–AdoCbl complex, it would be possible by this means to detect the production of a very small quantity of AdoCbl in the reaction mixture.

**Table I**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Hydrolysis of (OHCMe)Cbl in presence and absence of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>36 ± 1</td>
</tr>
<tr>
<td>Omit enzyme</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Boiled enzyme</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

*Enzyme replaced by an equal volume of water.*

*Enzyme heated for 1 min in boiling water.*

The results of this experiment are shown in Table II. Assuming that the production of 40 pmol of acetaldehyde (mean + 3 S.D. of the quantity of labeled nonbasic material measured in the absence of (OHCMeCbl) is significantly greater than control, employing a turnover number of 6400 min⁻¹ active site (the figure obtained from the AdoCbl control; last row, Table II, assuming that all the cofactor is taken up by the enzyme), and taking into consideration the Kₐ values for AdoCbl and ethanolamine (3), it can be calculated that the conversion of 0.0006% of the added (OHCMeCbl) to AdoCbl could be detected in this experiment. The data show that the fraction of analog converted to AdoCbl must be considerably less than this figure. This is true both in experiments in which the [14C]ethanolamine was present from the beginning of the reaction and in experiments in which the enzyme was incubated with the analog prior to the addition of the substrate. To allow for the possibility that the failure of substrate consumption was due to inactivation of the enzyme during the conversion of (OHCMeCbl) to AdoCbl, an experiment was performed in which, after incubation for 30 min, the reaction mixture was heated in boiling water (to release any AdoCbl from inactivated enzyme) and fresh enzyme was added; production of acetaldehyde still failed to take place. On the other hand, a reaction mixture initially containing AdoCbl instead of (OHCMeCbl) produced acetaldehyde at a rapid rate (see above), indicating that the enzyme is able to function under conditions similar to those of the control (without substrate), the substrates being added last. The experiments were conducted under safelights (Wratten 1A filters) at 24°C, incubating the reaction mixtures in a tungsten lamp until the spectrum remained constant. Per cent hydrolysis of (OHCMeCbl) was calculated by the formula: % = (A₅₅₀ - A₅₅₀t)/A₅₅₀, where A₅₅₀ is the absorbance at 550 nm measured at t minutes, and A₅₅₀ is the absorbance after exhaustive photolysis. The table shows the results of six separate experiments, expressed as mean ± 1 S.E.

**Table II**

**Failure of enzyme to form adenosylcobalamin from (OHCMeCbl)**

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>Acetaldehyde formation (% of total counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete reaction mixture</td>
<td>12.2 ± 0.5*(5)</td>
</tr>
<tr>
<td>Omit (OHCMeCbl)¹</td>
<td>0.9 ± 0.5*(6)</td>
</tr>
<tr>
<td>Complete reaction mixture, preincubated²</td>
<td>0.9 ± 0.2*(3)</td>
</tr>
<tr>
<td>Complete reaction mixture plus fresh enzyme²</td>
<td>0.9 (1)</td>
</tr>
<tr>
<td>Replace (OHCMeCbl) and 5′-deoxyadenosine with AdoCbl³</td>
<td>15.0 (1)</td>
</tr>
</tbody>
</table>

¹ Mean ± 1 S.E.
² (OHCMeCbl) was replaced with 2 μl of 0.5 M NH₃OH
³ All constituents except [14C]ethanolamine were preincubated together for 10 min. The reaction was then begun with substrate.

The incubation was conducted as described, except that the reaction was terminated by heating for 1 min in boiling water rather than by the addition of trichloroacetic acid. The reaction mixture was then cooled to 24°C, after which an additional 0.26 nmol of enzyme were added. Thirty minutes later, the reaction was terminated with trichloroacetic acid, and acetaldehyde formation was assayed as described.

For this reaction mixture, the quantity of substrate was 1.7 μmol (13,200 dpm), and the (OHCMeCbl) and 5′-deoxyadenosine were replaced with 0.08 nmol of AdoCbl. The incubation was conducted as usual, except that the reaction was terminated at 30 s. Downloaded from http://www.jbc.org/ by guest on September 22, 2017
the experimental conditions. It thus appears that the enzyme is unable to catalyze the formation of AdoCbl from (OHCMe)Cbl, NH₃, and 5'-deoxyadenosine.

**DISCUSSION**

Previous studies have shown that 5'-deoxyadenosine (30) and NH₃⁺ (2, 31) both bind to the active site of ethanolamine ammonia-lyase. Three lines of evidence indicate that (OHCMe)Cbl too binds to the active site of the enzyme. First is the fact that (OHCMe)Cbl is an inhibitor of the enzyme, competing kinetically with AdoCbl. Second is the shift in the spectrum of (OHCMe)Cbl on binding to ethanolamine ammonia-lyase, suggesting that the cobamide is in a special environment when associated with the enzyme. It is possible that this spectral shift represents protonation of the Coβ-ligand, as appears to occur when hydroxocobalamin binds to the enzyme and is converted to aquocobalamin (21). Finally, the observation that active, but not denatured, enzyme increases the rate of carbon-cobalt bond cleavage of (OHCMe)Cbl supports the notion that the cobamide is taken up at the active site, the specific configuration of which permits (weak) catalysis of the dealkylation of the analog.

In considering why (OHCMe)Cbl was not converted to AdoCbl, even though present at the active site, an issue arises having to do with the actual identity of the cobamide bound to the enzyme. (OHCMe)Cbl in NH₃OH probably exists as a mixture of rapidly interconverting species which are formed by the reversible addition of ammonia to the double bond of the ligand. Among these species is the ammonia adduct, and there are several pieces of evidence which suggest that this adduct is likely to constitute a significant fraction of the total cobamide in solution. That adducts are easily formed from (OHCMe)Cbl is indicated by the observations of Silverman and Dolphin (13), who showed that the major product of the reaction between hydroxocobalamin and 2-hydroxyethyl vinyl ether was the ethylene glycol acetal of (OHCMe)Cbl. Studies on the acetaldehyde-ammonia system (32) suggest that under the present experimental conditions, a substantial fraction of the cobamide is likely to be in the form of the ammonia adduct (assuming that (OHCMe)Cbl is too bulky to oligomerize in the presence of NH₃). Further evidence that NH₃ is a constituent of the cobamide is the difference seen in the spectrum of (OHCMe)Cbl depending upon whether it was dissolved in ammonia or methylamine (Fig. 2), as well as the difference in the stability of the cobamide at the same pH in different buffers, as indicated by our inability to recover (OHCMe)Cbl from the Bio-Gel column if the eluting solution was either KOH or methylamine (both at pH 11.4). The probable presence of the ammonia adduct in free solution implies that at least a portion of the (OHCMe)Cbl at the active site would be in the same form. This is particularly likely in view of the evidence that the active site of ethanolamine ammonia-lyase binds NH₃⁺, a circumstance which could favor the binding of (OHCMe)Cbl to the enzyme as its ammonia adduct. However, direct evidence for the presence of the (OHCMe)Cbl ammonia adduct at the active site of the enzyme is not available.

Another issue is concerned with the enzyme conformer to which (OHCMe)Cbl is bound. The kinetic data implies that (OHCMe)Cbl binds to the same conformer to which AdoCbl binds. Moreover, as mentioned above, the kinetics, together with the spectral data and the data showing cleavage of the analog by the enzyme, further suggest that the analog is attached to the enzyme at the active site. It could be argued, however, that catalysis involves (OHCMe)Cbl bound to an enzyme conformer which for kinetic reasons is not accessible when the enzyme in incubated directly with the analog in the presence of 5'-deoxyadenosine and NH₃, but can only be formed starting from enzyme, substrate, and cofactor.

The failure to form AdoCbl in the present system could thus be accounted for by either of the above two possibilities: the wrong cobamide at the active site or the right cobamide on the wrong conformer. However, a third possibility exists, namely, that the ethanolamine ammonia-lyase reaction does not involve transalkylation of the corrin metal by the substrate. The transalkylation mechanism, which has been proposed by several workers (6-9) and for which indirect support in the form of model reactions exists in abundance (10-18), has to date received no experimental confirmation in enzyme-containing systems. No cobin carrying a substrate fragment attached to the cobalt has yet been retrieved from an AdoCbl-requiring enzyme system. Carbon-cobalt bond cleavage in the presence of analogs of substrate has been shown to occur with several AdoCbl-dependent enzymes (3, 4), but in none of these reactions has transalkylation of the cobalt by analog been demonstrated. The present study has also failed to provide evidence for a mechanism involving transalkylation. Although negative results cannot be interpreted with certainty, the failure to find any evidence whatsoever in support of the transalkylation mechanism despite many attempts to do so by several groups of workers implies that the transalkylation mechanism may not be correct, and that mechanisms not involving transalkylation, such as the radical rearrangement mechanism proposed by Golding and Radom (33, 34), must be given serious consideration.

**APPENDIX**

The time course for the disappearance of substrate in the reaction mixture containing (OHCMe)Cbl in addition to enzyme and cofactor, shown in Fig. 3, is characterized by an exponential decline in the concentration of substrate, the rate of decline approaching an asymptote. It has previously been shown that this time course reflects the conversion of the enzyme from a fully active to a much less active form (deactivation) under the influence of the analog (27). For the purposes of this study, we wished to ascertain whether (OHCMe)Cbl was a competitive inhibitor of the fully active enzyme. It was therefore necessary to devise a method for calculating the initial reaction rate from the substrate disappearance curve.

The deactivation of the enzyme may be represented by the reaction

\[ E \xrightleftharpoons{c} E' \]

(\(E\), the fully active enzyme; \(E'\), the deactivated enzyme; and \(c\), the rate constant for the deactivation process), whose rate law is

\[ \frac{dE}{dt} = -cE \]

Solving,

\[ E = E_0 e^{-ct} \]

where \(E_0 = E + E'\), the amount of enzyme initially added.

Both \(E\) and \(E'\) catalyze the deamination of ethanolamine, but at different rates. The observed rate of appearance of product is the sum of these rates.
\[
d\frac{dP}{dt} = kE + k'E'
= (k - k') E_0 e^{-at} + k'E_0
\]

where \(k\) and \(k'\) are the rate constants for the deamination of ethanolamine by \(E\) and \(E'\), respectively, at saturating substrate concentrations. Solving,

\[
P(t) = E_0 \frac{k - k'}{c} (1 - e^{-kt}) + k't
\]

If \(S_0\) is the initial substrate concentration and \(S(t)\) the amount of substrate left at time \(t\), then by definition, \(S(t) = S_0 - P(t)\). Substituting into Equation 1 and rearranging,

\[
S(t) = S_0 - P(t) = S_0 - \left(\frac{k - k'}{c} E_0 e^{-at} + k't\right)
\]

The first term of this equation describes the exponential behavior of the substrate consumption curve. The equation also contains a linear term (the expression in brackets) which represents the asymptote. Using this equation, it was possible to calculate the initial rate constant corresponding to each experimental curve by the following procedure. The difference \(\Delta\) between the actual value for the substrate concentration and the value of the asymptote was plotted semilogarithmically against time. The value of \(\Delta\) at any time \(t\) is equal to the exponential term in Equation 2:

\[
\Delta(t) = \frac{E_0}{c} (k - k') e^{-at}
\]

This plot was extrapolated back to \(t = 0\), at which time, according to Equation 3, the value of \(\Delta\) is

\[
\Delta(0) = \frac{E_0}{c} (k - k')
\]

Dividing by \(E_0/c\), the value for \(c\) being obtained from the slope of the semilogarithmic plot, gave \(k - k'\), the difference between the two rate constants. Since \(k' < k\), this difference was taken as \(k\), the desired initial rate constant.

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