The Mechanism of Action of Ethanolamine
Ammonia-Lyase, a B₁₂-dependent Enzyme

VIII. FURTHER STUDIES WITH COMPOUNDS LABELED WITH ISOTOPES OF HYDROGEN:
IDENTIFICATION AND SOME PROPERTIES OF THE RATE-LIMITING STEP*

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SUMMARY

The previous observation (BABIOR, B. M., J. Biol. Chem., 244, 449 (1969)) that the tritium isotope effect for the coenzyme B₁₂-dependent deamination of ethanolamine was smaller than the deuterium isotope effect prompted a re-examination of the various isotope effects observed in this reaction. Measurements of the rates of deamination of [1-D]- and [1,1-D₂]ethanolamine showed that no secondary isotope effect was detectable within the limits of experimental error. Activation parameters for the deamination of unlabelled ethanolamine and [1,1-D₂]ethanolamine at 23° were as follows: for the unlabelled substrate, $E_a = 3.9$ kcal per mole and $S = -9.7$ e.u.; for the deuterated substrate, $E_a = 5.5$ kcal per mole and $S = -9.4$ e.u. The similarity of these values implies that the rate-limiting step does not change on passing from the unlabelled to the deuterium-labeled substrate. Measurement of the tritium isotope effect for each of the two individual hydrogen transfer steps of the reaction gave the following results: for the first step (transfer of hydrogen from substrate to coenzyme), $k_T/k_H = 4.7$; for the second step (transfer of hydrogen from coenzyme to product), $k_T/k_D = 160$ and $k_T/k_D = 22$. The over-all deuterium isotope effect was found to be 7.4, confirming previous results. From these and other observations the following conclusions were drawn: (a) the rate-limiting step is the transfer of hydrogen from coenzyme to product; (b) an irreversible step occurs between the first hydrogen transfer step and the second; and (c) exchange between free and enzyme-bound coenzyme B₁₂ during the course of the reaction is slow.

CARBON CARBON OF ETHANOLAMINE MIGRATES TO THE ADJACENT CARBON ATOM (1). THE OVER-ALL REACTION SHOWS A DEUTERIUM ISOTOPE EFFECT OF ABOUT 7, INDICATING THAT THE RATE-LIMITING STEP INVOLVES THE Rupture OF A CARBON-HYDROGEN BOND (1). EXPERIMENTS WITH TRITIATED ETHANOLAMINE SHOWED THAT THIS HYDROGEN TRANSFER IS STEREOSPECIFIC—that is, that the enzyme is capable of distinguishing between the R and S hydrogen atoms on the carbiniol carbon of ethanolamine, selecting one to migrate and leaving the other behind to become the aldehydic hydrogen of the product. However, contrary to expectations, the tritium isotope effect, which according to the Swain relationship should have been in the vicinity of 17 (2), was observed to be smaller than the deuterium isotope effect.

This anomaly, which was unexplained at the time, prompted a reinvestigation of the various isotope effects observed in the ethanolamine ammonia-lyase reaction. From the results of this study, it became possible to specify the rate-limiting step in the reaction.

MATERIALS AND METHODS

Ethanolamine ammonia-lyase (EC 4.1.3) was prepared and resolved of bound cobalamins by the method of Kaplan and Stadtman (3). Enzyme concentration was determined by the spectrophotometric assay of Kaplan and Stadtman (3) or the radioactive assay of Babior and Li (6). Coenzyme B₁₂ was purchased from Calbiochem. Yeast alcohol dehydrogenase (lyophilized, salt free) and DPNH were purchased from Sigma. 2-Aminoacetaldehyde diethyl acetal was obtained from Aldrich. [3H]Ethanolamine was obtained from New England Nuclear, and [1-T]ethanolamine was synthesized as previously described (1); before use, both compounds were treated with activated charcoal (Dareo G-60, acid washed) to remove charcoal-adsorbable radioactive impurities. [1,1-D₂]-

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Ethanolamine (1) and [14C]adenosyl-DBC (8) were prepared as previously described. Other materials were reagent grade, and were used without further purification.

Optical spectroscopy was performed on a Cary model 15 recording spectrophotometer, with 1-ml quartz cuvettes with path lengths of 1 cm. Sample temperature was controlled by means of a thermostated cell holder. Nuclear magnetic resonance spectroscopy was performed on a Varian T-60 instrument. Radioactivity was measured with a Nuclear Chicago Mark II liquid scintillation counter, with Bray’s solution (9) as scintillant. Counting efficiency was determined by internal standardization with radioactive solutions of known specific activity.

**dL-[1-D]Ethanolamine—**Racemic [1-D]ethanolamine was prepared by the reduction of 2-aminoacetaldehyde with NaBD₄. To prepare 2-aminoacetaldehyde, 0.8 ml of 2-aminoacetaldehyde diethyl acetal was added to 9.2 ml of 3.3 n HCl. After 12 hours at room temperature, the acetal was completely hydrolyzed. Without further treatment, the acidic solution of aminoacetaldehyde was added dropwise with constant stirring to a solution containing 150 mg of NaBD₄ in 5 ml of 7 n NaOH. The entire reaction mixture was then distilled to separate the product from nonvolatile substances present in the mixture. The distillate was brought to pH 7.0 with HCl and taken to dryness on a rotary evaporator. The crude [1-D]ethanolamine-HCl was purified by recrystallization from 1-propanol. (Yield after recrystallization 30%; m.p. 77–80°C (10) for unlabeled ethanolamine-HCl.) When chromatographed on Whatman No. 1 paper with 1-butanol-acetic acid-water (4:1:5) (organic phase) as the developing solvent, the product had the same mobility as authentic ethanolamine. The nuclear magnetic resonance spectrum of the product is shown in Fig. 1.

**Tritiated DBC—**This compound was prepared by incubating unlabeled DBC with [1-T]ethanolamine in the presence of ethanolamine ammonia-lyase. During the course of the reaction, tritium from the substrate was transferred to the 5’-carbon of the adenosyl group of the coenzyme (11, 12).

The reaction mixture contained 0.12 μmole of DBC, 0.17 μmole of ethanolamine ammonia-lyase, 6.2 μmole (0.5 μCi) of [1-T]ethanolamine, and 10 μmole of potassium phosphate buffer (pH 7.4) in a total volume of 0.616 ml. The incubation, which was begun with enzyme, was carried out in dim light at room temperature. After 5 min, the incubation was terminated by heating for 30 s in boiling water. The coenzyme was separated from salts and unreacted substrate according to the method of Barker (13). After removal of these substances, the coenzyme solution was taken to dryness on a rotary evaporator, and the residue was dissolved in 1 ml of water and used without further purification.

The specific activity of the tritiated coenzyme was 1.8 nCi per μmole. Activated charcoal (Dancoel G-60, acid washed) took up 100% of the radioactivity from a solution of the tritiated coenzyme. When a reaction mixture containing 0.79 μmole of labeled coenzyme, 0.62 μmole of ethanolamine ammonia-lyase, 0.108 μmole of ethanolamine-HCl (pH 7.4), and 0.2 μmole of potassium phosphate buffer (pH 7.4) in a total volume of 0.085 ml was incubated for 2 min at room temperature in the dark, 80% of the tritium originally in the coenzyme was transferred to product, as determined by the appearance in the reaction mixture of tritium which was not taken up by charcoal (see below).

**Secondary Isotope Effect—**In the previous paper (1), a large secondary isotope effect was proposed as a possible explanation for the anomalous relationship between the deuterium and tritium isotope effects. Such an isotope effect would lead to a decrease in the rate of deamination of deuterated substrate (in which both carbinol hydrogen atoms are replaced by the isotope) but not tritiated substrate (in which only one such hydrogen atom is replaced by the isotope). The existence of such an isotope effect was investigated by studying how the rate of conversion of ethanolamine to acetaldehyde is affected by the substitution of deuterium for the pro-aldehydic hydrogen of the substrate. For reasons which will become clear as the results are presented, the experiments were conducted with substrate in which the mobile hydrogen atom was replaced by deuterium. Thus, the determination of the secondary isotope effect involved comparison of the rate of deamination of [1-D]ethanolamine with that of [1,1-D]ethanolamine.

Measurement of the reaction rate with [1,1-D]ethanolamine was straightforward, since the doubly deuterated substrate was available. Measurement of the rate of deamination of the monodeuterated substrate was more difficult, however, since the sub-

![Fig. 1. Nuclear magnetic resonance spectrum of [1-D]ethanolamine in D₂O.](https://example.com/fig1.png)
The Mechanism of Action of Ethanolamine Ammonia-Lyase. VIII

Vol. 246, No. 19

FIG. 2. Time course of the deamination of singly and doubly deuterated ethanolamine. Reaction mixtures contained 74 μg of ethanolamine ammonia-lyase, 10 μmoles of DBC, 0.4 μmole of DPNH, 0.2 mg of yeast alcohol dehydrogenase, 50 μmoles of potassium phosphate buffer (pH 7.4), and substrate as noted (0.2 μmole of \([1-D]\) ethanolamine or 0.16 μmole of \([1,1-D]\) ethanolamine) in a total volume of 1.0 ml. The reaction was begun with enzyme, and was carried out at 24°. The disappearance of DPNH was followed spectrophotometrically at 340 nm as described under "Methods."
Examples of such plots are shown in Fig. 3. It is seen that these points fall close to a straight line. This result indicates that the decrease in rate as the reaction approaches completion can be entirely attributed to the fall in substrate concentration, and implies that there is no other superimposed process (e.g., inactivation of enzyme or product inhibition) which would tend to slow the reaction down with time.

By plotting the results of several experiments according to the integrated Michaelis-Menten equation, values were obtained for $V_m$ and $K_m$ with both the deuterated and slowly reacting monodeuterated substrates. These are shown in Table I. It is apparent that within the limits of experimental error the substitution of deuterium for the nonmobile hydrogen atom has a negligible effect on the rate of deamination of ethanolamine by coenzyme $D_2$-dependent ethanolamine ammonia-lyase.$^4$

**Activation Energies**—Another explanation for the discrepancy between the deuterium and tritium isotope effects is the possibility that the rate-limiting step changes on passing from unlabeled to deuterium-labeled substrate. Discrepancies of this sort have been reported in reactions in which tunnelling is important in hydrogen transfer (16). This possibility was investigated by comparing the activation energy for the deamination of deuterated ethanolamine with that for the deamination of [1,1-$D_2$]ethanolamine, on the assumption that a change in the rate-limiting step would be reflected by a difference in the activation energies for these two reactions.

The Arrhenius plots for these two reactions are shown in Fig. 4. The plots for each of the substrates is linear over the temperature range investigated. According to these curves, the deuterium isotope effect at 23$^\circ$ was 7.4, confirming the results of a previous study (1).

The activation parameters (17) are as follows: for the unlabeled substrate, $E_a = 3.9$ kcal per mol and $S = -9.7$ e.u.; for the deuterium-labeled substrate, $E_a = 5.5$ kcal per mol and $S = -9.4$ e.u. Thus, there is relatively little difference between the activation parameters for the two substrates, suggesting that the rate-limiting step in the deamination of each of these compounds is the same.

**Effect of Isotopic Substitution on Rates of Individual Hydrogen Transfer Steps**—The foregoing results showed that the anomalous relation between the deuterium and tritium isotope effects could not be attributed to a secondary isotope effect or to a change in the rate-limiting step upon isotopic substitution. Anomalies of this sort have however been reported in reactions involving two sequential hydrogen transfer steps when the second transfer is rate-limiting (18). The ethanolamine ammonia-lyase-catalyzed deamination of ethanolamine, a reaction in which hydrogen is transferred first from substrate to coenzyme and then, in a subsequent step, from coenzyme to product, was shown to exemplify such a type of reaction by a series of experiments in which the flow of tritium from labeled substrate to coenzyme, the isotope effect is between free and enzyme-bound coenzyme, the isotope effect is between free and enzyme-bound coenzyme during the course of the reaction; if the exchange is rapid, so there is complete randomization between free and enzyme-bound coenzyme, the isotope effect is between 12 and 21, while if there is no exchange the isotope effect is between 750 and 1350.

Because of the uncertainty in the extent of exchange between

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$</th>
<th>$V_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1-$D$]Ethanolamine</td>
<td>9.0 $\pm$ 1.9</td>
<td>1.68 $\pm$ 0.16</td>
</tr>
<tr>
<td>[1,1-$D_2$]Ethanolamine</td>
<td>8.6 $\pm$ 1.5</td>
<td>1.68 $\pm$ 0.07</td>
</tr>
</tbody>
</table>

*Mean $\pm$ 1 S.E.*
The experiment was done in duplicate. The reaction mixtures contained 1.0 μmole of ethanolamine-HCl (pH 7.4) (including 4.0 nCi of [14C]ethanolamine and 100 nCi of [1-14C]ethanolamine), 0.077 nmoles of ethanolamine ammonia-lyase, 10.9 n mole of DBC (including 0.5 nCi of [14C]DBC) and 50 μmoles of potassium phosphate buffer (pH 7.4) in a total volume of 0.2 ml. The incubation was begun by adding enzyme, and was conducted in dim light at 23°C. After 5 min, the reaction was terminated with 0.2 ml of 6% (w/v) trichloroacetic acid. One-tenth milliliter of the reaction mixture plus 10 μl of 1.2 nCi of [14C]ethanolamine and 100 nCi of [14]D-tritiated ethanolamine (including 0.3 nCi of [14C]DBC) and 10 pmoles of potassium phosphate buffer (pH 7.4) in a total volume of 1.0 ml. The incubation was begun with enzyme, and was conducted in dim light at 23°C. At the times noted, 0.2-ml portions of the reaction mixture were added to 0.2-ml aliquots of 6% trichloroacetic acid (w/v). Two-tenths milliliter of the resulting mixture was chromatographed and tritium in the coenzyme was determined as described in Table II. To the remainder of the trichloroacetic acid-treated reaction mixture were added 1.8 ml of water. DBC was removed as described in Table II. The extent of deamination of ethanolamine in the coenzyme-free solution was then determined by the radioactive assay.

Table II. The extent of deamination in which concentration of active sites exceeds concentration of coenzyme

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mobile tritium</th>
<th>% of total</th>
<th>Mobile tritium</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In substrate</td>
<td>In DBC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>86.1</td>
<td>1.0</td>
<td>55.7</td>
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</tr>
<tr>
<td>2</td>
<td>92.7</td>
<td>3.2</td>
<td>60.7</td>
<td></td>
</tr>
</tbody>
</table>

*Mobile tritium in substrate = 100 × S/ (see “Appendix”).

Fig. 5. Transfer of tritium from labeled coenzyme to product at high coenzyme to enzyme ratios. The reaction mixtures contained 0.67 n mole of ethanolamine ammonia-lyase, 10.9 n mole of DBC (including 1.5 nCi of [14C]DBC and 17.5 nCi of [14C]DBC), 5 μmoles of ethanolamine-HCl (pH 7.4) (including 20 nCi of [14C]ethanolamine), and 50 μmoles of potassium phosphate buffer (pH 7.4) in a total volume of 1.0 ml. The incubation was begun with enzyme, and was conducted in dim light at 23°C. After 1 min, the reactions were terminated with 0.1 ml of 6% (w/v) trichloroacetic acid. Water (1.9 ml) was then added, and the tritium content of 0.5 ml of the diluted reaction mixture was measured. DBC was removed from the rest of the incubation mixture by charcoal treatment followed by Millipore filtration as described in Fig. 4, and the quantity of tritium which was not taken up by charcoal was determined on 1 ml of filtrate. The amount of tritium transferred from coenzyme to product was calculated from these figures, on the assumption that the tritium which was not absorbed by charcoal represented tritium in product.
zyme, so that essentially all the coenzyme was bound to enzyme. In these experiments, the amount of coenzyme present was a defined fraction of the quantity of substrate in the reaction mixture. Enzyme was added, the reaction was permitted to reach completion, and the amount of tritium transferred to product was determined by radioactive assay. In the experiments reported here, the duration of incubation was 1 min, although it can be calculated from turnover numbers (6) that in each case the reaction was over within 10 s. (In unpublished controls, it was shown that for each concentration of unlabeled substrate, the amount of tritium lost from the coenzyme at the end of 2 min was the same as the amount lost at the end of 1 min. This indicates that the loss of tritium from coenzyme in these experiments is not the result of exchange of tritium between coenzyme and product, although this process has been shown to take place under other conditions in the presence of ammonium ion.)*

By using [1,1-D]ethanolamine in addition to unlabeled ethanolamine, it was possible to determine the tritium-deuterium as well as the tritium-hydrogen isotope effect for the second hydrogen transfer step. The results, given in Table III, indicate that for transfer from coenzyme to product, hydrogen is selected over tritium by a factor of 160, while deuterium is selected by a factor of 22.

**DISCUSSION**

The tritium isotope effect of 160 for the second hydrogen transfer step indicates that this reaction must have some peculiar mechanistic features, the nature of which are obscure. A similar isotope effect has been reported by Eisenberg, Frey, and Abeles for the dehydration of propylene glycol, a coenzyme B₃₄-dependent reaction which is very similar to the reaction catalyzed by ethanolamine ammonia-lyase (19). In contrast, the tritium isotope effect of 4.7 observed for the transfer of hydrogen from substrate to coenzyme, a much more reasonable value, indicates that the isotope effect for this step can probably be explained by the conventional mechanism, in which differences in the rates of reaction of isotopically substituted compounds are attributed to differences in vibrational energy levels of the various species arising in the course of the reactions (16).

Since the tritium isotope effect for the first hydrogen transfer step is 4.7, the deuterium isotope effect for this step must be something less than this value. From the Swain relationship (2), it can be estimated that the latter isotope effect is of the order of 2.0. However, the deuterium isotope effect for the over-all reaction is about 7. As pointed out previously (1), this large over-all deuterium isotope effect indicates that the rate-limiting step in the over-all reaction involves the rupture of a carbon-hydrogen bond. If the first hydrogen transfer were the slow step in the reaction, it would be expected that the over-all deuterium isotope effect would be zero. Since the tritium isotope effect for this step is 4.7 predicted for the latter step on the basis of the isotope effect for the over-all reaction is confirmed by the results of Table III. Under the conditions of these experiments, the S' of the adenosyl residue of the coenzyme rapidly becomes saturated with substrate hydrogen atoms. Therefore, in the transfer step under study in these experiments (i.e., the transfer from coenzyme to product), tritium is competing against which-over hydrogen isotope was originally present in the substrate. The data indicate that tritium competes about 7 times as effectively against deuterium as it does against hydrogen. Therefore, tritium transferred to the coenzyme to product thus appears to take place at about one-seventh the rate at which hydrogen transfer takes place, confirming the prediction based upon the over-all deuterium isotope effect and the assumption that the second hydrogen transfer step is rate limiting.

In contrast to the over-all deuterium isotope effect, the tritium isotope effect for the over-all reaction depends only on the isotope effect for the first hydrogen transfer step. This is because tritium, unlike deuterium, is present in the substrate in trace amounts only. Therefore, tritium transferred to the coenzyme is always in equilibrium with 2 hydrogen atoms. Since hydrogen atoms as well as tritium atoms are available for transfer from coenzyme to product, the rate of the second transfer step will only be marginally decreased in a reaction involving a tritiated substrate molecule, because the overwhelming likelihood is that hydrogen will be transferred from the coenzyme to the product instead of the newly acquired tritium. The tritium is then washed out of the coenzyme by transfer to an unlabeled molecule. The number of these which are processed by the enzyme per min greatly exceeds the number of labeled molecules which react in the same interval. Consequently, despite the large tritium isotope effect seen in the second step, tritium appears in product about as fast as it is transferred from substrate, and there is little accumulation in the coenzyme when expressed in terms of the total amount of mobile tritium in the reaction mixture. The over-all tritium isotope effect therefore reflects the tritium isotope effect for the first transfer only (that is, the transfer from substrate to coenzyme). With the present enzyme, the relationships between the rates and isotope effects of the two hydrogen transfer steps are such that the over-all tritium isotope effect is smaller than the over-all deuterium isotope effect.

During the course of the reaction, the specific activity of the tritiated coenzyme reaches values which greatly exceed that of the substrate. From the results in Table II, for example, the specific activity of the substrate and participating (i.e., enzyme-bound) coenzyme at the end of the incubation can be calculated to be 0.15 and 62 mCi per mmole, respectively, assuming no exchange between free and enzyme-bound coenzyme. This large difference indicates that the mobile tritium is not in equilibrium between the substrate and the coenzyme. Moreover, the departure from equilibrium cannot be attributed to a rapid irreversible loss of tritium from the coenzyme, since the fact that the coenzyme, rather than the substrate, has the greater specific activity indicates that tritium is being transferred to the coenzyme much more rapidly than it is being removed from the coenzyme. The failure of equilibration of tritium between substrate and coenzyme must therefore indicate the existence of an irreversible step occurring between the first hydrogen transfer and the second.

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and assistance for growing the bacteria from which ethanolamine ammonia-lyase is obtained. The superb technical assistance of Elizabeth A. Greene is gratefully acknowledged. We are indebted to Bernard Ransil for assistance in solving the equations presented in the "Appendix," and to Gustav Lienhard for many helpful discussions.

**APPENDIX**

**Calculation of Tritium Isotope Effects**

Isotope Effects Starting with Labeled Substrate (Table II)—The tritium isotope effect for the first hydrogen transfer step (the transfer of hydrogen from substrate to coenzyme) was determined as previously described (1), except that $S_t$, the fraction of mobile tritium atoms remaining in ethanolamine at the end of the reaction, was calculated from the following formula:

$$S_t = 1 - \frac{2(P_t - 0.5P_C - C_t)}{100}$$

where $P_t$ and $P_C$, respectively, represent the incorporation of tritium and $^4$C into acetylactdehyde and $C_t$ represents the incorporation of tritium into the coenzyme; all expressed as percentage of total isotope in the reaction mixture.

The tritium isotope effect for the second hydrogen transfer step was determined by comparing the rate constant for the loss of tritium from the coenzyme, all expressed as moles of substrate per mole of coenzyme per min. The model used to determine the rate constant for the loss of tritium from the coenzyme was the following:

$$S \xrightarrow{f} C \xrightarrow{k_2}$$

where $S$ and $C$ represent the quantities of tritium present at time $t$ in the substrate and coenzyme, respectively, and $f$ and $k_2$ are rate functions describing the flow of tritium to and from the coenzyme. The function $f$, which describes the transfer of tritium from substrate to coenzyme, is not a constant, but varies with time as a function of the specific activity of the tritiated substrate. This is true because the rate of conversion of tritiated substrate to product depends on the absolute quantity of tritiated substrate bound to the enzyme, and the higher the ratio of tritiated to tritium-free substrate, i.e. the higher the specific activity of the tritiated substrate, the greater this quantity will be. If it is assumed that the total quantity of tritiated substrate in the reaction mixture is constant with time (a good assumption in this case, since over the course of the reaction this quantity decreases by only 11%), and that unlabeled substrate disappears in a zero order reaction, then the rate function can be expressed by the following equation:

$$f = \frac{k_1}{1 - at}$$

where $k_1$ is the value of $f$ at the beginning of the reaction, and $a$ is the fraction of tritium-free substrate consumed per unit of time.

The set of equations describing the model is

$$-\frac{dS}{dt} = fS$$

$$\frac{dC}{dt} = fS - k_2C$$

(1)

The constant $k_2$ cannot be obtained in a straightforward way from these equations, since they cannot be solved in closed form because of the time dependence of $f$. However, from the data in Table II it is possible to establish upper and lower limits for $k_2$. This can be done by calculating $k_2$ under the assumption that $f$ is a constant. The limits of $k_2$ are obtained by using for this calculation the extreme values that $f$ assumes during the course of the reaction described in Table II.

With $f$ constant, the solution to the set of Equations 1 is as follows

$$C = \frac{fS_0}{k_2 - f}(e^{-t} - e^{-k_2 t})$$

where $f$, $k_2$, and $C$ are as defined above, and $S_0$ is the quantity of tritium in the substrate at $t = 0$. With the averages of the data of Table II, $S_0 = 1.0$ and $C = 0.021$, where $S_0$ and $C$ are expressed in terms of the fractions of total mobile tritium present in the substrate (at $t = 0$) and coenzyme (at $t = 5$ min), respectively. The extreme values for $f$ are 0.021 min$^{-1}$ (equal to $k_1$, the rate constant at the beginning of the incubation, assuming that tritium which is lost from the substrate disappears according to first order kinetics) and 0.037 min$^{-1}$ (equal to $k_2 (1 - at)$ at $t = 5$ min). From these numbers, $k_2$ for tritium can be calculated to lie between 0.91 and 1.6 min$^{-1}$.

The value of $k_2$ for the transfer of hydrogen (rather than tritium) was equated with the turnover number, since 1 atom of hydrogen is transferred from the coenzyme for each molecule of substrate which is converted to product. The turnover number, however, depends upon the amount of coenzyme which actually participates in the reaction. Since the coenzyme is present in large excess over enzyme, it is conceivable that only a small fraction of the coenzyme is involved in the reaction. This would be the case if exchange between the free and bound coenzyme is slow with respect to the duration of the incubation. Under these circumstances, only those coenzyme molecules which are taken up by the enzyme at the start of the incubation would be able to participate in the reaction. Estimates of the turnover number can be made for the limiting cases of complete exchange or no exchange by dividing the zero order reaction rate for deamination of tritium-free substrate by the number of moles in the reaction mixture of coenzyme or active sites, respectively. The values are 28 min$^{-1}$ for complete exchange and 2500 min$^{-1}$ for no exchange.

To calculate the isotope effect, the turnover number was divided by $k_2$ for tritium, and this value in turn was divided by 2 to correct for the fact that a tritium atom on the methyl group of 5'-deoxyadenosine is more or less in equilibrium with 2 equivalent hydrogen atoms. A factor of 2 rather than 3 was used because of the fact that in the process under consideration, the transfer of hydrogen was much more rapid than the transfer of tritium, so that only the hydrogen atoms were taken into consideration in the determination of the statistical factor. To illustrate this point, consider the relative rates of loss of tritium and hydrogen from —CH$_2$T, where the rate constant for tritium loss is arbitrarily set at 1 and that for hydrogen loss as 100. From these figures, the rate of loss of hydrogen from —CH$_2$T would be 200 times that of tritium. Dividing this figure by 3, the number of equivalent atoms on the methyl group, yields a value for the tritium isotope effect of 67; the actual isotope effect as defined by the rate constants, however, is 100, which is the value obtained when only the 2 hydrogen atoms are considered.
in the calculation. The tritium isotope effects determined according to the above formulations are given under "Results."

Isotope Effects Starting with Labeled Coenzyme (Table III) —
In these experiments, incubations containing small fixed amounts of substrate were permitted to go to completion, and the amount of tritium transferred from coenzyme to product was measured. To avoid problems resulting from the low rate of exchange between free and bound coenzyme, ethanolamine ammonia-lyase (expressed as active sites) was present in molar excess over coenzyme. The ratio r, representing the rate of transfer of H (or D) out of the coenzyme with respect to the rate of transfer of T out of the coenzyme, could then be obtained directly from the following equation:

\[ \frac{dT}{dT} = \frac{r}{T} \]

where H, the amount of hydrogen transferred from coenzyme to product, is expressed in terms of turnover (moles of hydrogen transferred per mole of coenzyme), and T is equal to the amount of tritium in the coenzyme at the time the quantity H of hydrogen has been transferred from coenzyme to product. This equation is obtained from the equations describing the rates of transfer of the two isotopes from coenzyme to product. The latter equations are as follows

\[ \frac{dT}{dt} = k_H \]

for the zero order transfer of hydrogen from coenzyme to product, where k_H is expressed as a turnover number, and

\[ \frac{dT}{dt} = k_T \]

for the first order loss of tritium from coenzyme. Combining these equations yields Equation 2, with \( r = \frac{k_H}{k_T} \). Integrating Equation 2

\[ \ln \frac{T}{T_0} = \frac{H}{r} \]

where \( T_0 \) is equal to the amount of tritium in the coenzyme at the beginning of the reaction.

With the integrated equation, the parameter r was calculated from the results of Table III. The substrate to coenzyme mole ratio was equated with H, while the term on the left hand side of the equation was calculated according to the formula: \( T/T_0 = 1 - (0.01 \times \text{percentage of tritium in product}) \). The isotope effect is then equal to \( r/2 \), as described in the previous section. The values of the tritium isotope effects with respect to the transfer of hydrogen and deuterium from coenzyme to product are given under "Results."

REFERENCES