Hydrogen Transfer in Catalysis by Adenosylcobalamin-Dependent Diol Dehydratase†

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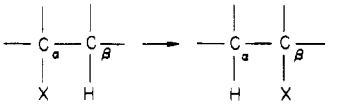
ABSTRACT: Studies [Bachovchin, W. W., et al. (1978) *Biochemistry 17*, 2218] of the mechanism of inactivation of adenosylcobalamin-dependent diol dehydratase have led to the development of a general method to describe the kinetics of a reaction pathway containing a reservoir of mobile hydrogen. Analysis by this method of catalytic rate measurements for mixtures of 1,2-propanediol and 1,1-dideuterio-1,2-propanediol supports a mechanism involving an intermediate with three equivalent hydrogens, in which hydrogen transfer from this intermediate to product is the major rate-contributing step. Other results using tritium as a trace label [Essenberg, M. K., et al. (1971) *J. Am. Chem. Soc. 93*, 1242] are considered in light of these deuterium isotope studies.

Diol dehydratase is an adenosylcobalamin-dependent enzyme which catalyzes the conversion of 1,2-propanediol to propionaldehyde, of ethylene glycol to acetaldehyde (Lee & Abeles, 1963), and of glycerol to β-hydroxypropionaldehyde (Toraya et al., 1976; Bachovchin et al., 1977). The reactions catalyzed by diol dehydratase share several features with eight other adenosylcobalamin-dependent rearrangements (Babior, 1975) which are of the type shown in Scheme I, where X can be an amino, hydroxyl, acyl or alkyl group. The migration of X is known to occur intramolecularly in the reactions catalyzed by diol dehydratase (Retey et al., 1966b), glutamate mutase (Barker et al., 1964a,b), methylmalonyl coenzyme A mutase (Kellermayer & Wood, 1962; Wood et al., 1964; Phares et al., 1964), and ethanolamine deaminase (Babior, 1969). The hydrogen which is transferred does not exchange with water and, during the course of the rearrangement, apparently becomes one of three equivalent hydrogens attached to C-5′ of the cofactor (Abeles & Zagalak, 1966; Retey & Arigoni, 1966; Frey et al., 1967a,b; Miller & Richards, 1969; Switzer et al., 1969; Babior, 1970; Eagar et al., 1972; Babior et al., 1973).

The stereochemistry of these rearrangements has also been extensively investigated. The carbon to which hydrogen migrates undergoes inversion in the diol dehydratase (Retey et al., 1966a; Zagalak et al., 1966) and glutamate mutase reactions (Sprecher & Sprinson, 1964). In contrast, one observes retention of configuration at the carbon to which hydrogen migrates for methylmalonyl coenzyme A mutase (Sprecher et al., 1964; Retey & Zagalak, 1973). Hydrogen is stereospecifically abstracted from C-1 of substrate (Zagalak et al., 1966; Babior, 1969; K. W. Moore and J. H. Richards, 1978, unpublished experiments). In the case of glycerol, which functions both as a substrate and an inactivator of diol dehydratase, catalysis occurs when the substrate binds in the "R" conformation, while binding in the "S" conformation leads to irreversible inactivation of the enzyme (Bachovchin et al., 1977). Use of ^{18}O-labeled (*R*)- and (*S*)-1,2-propanediol allowed the identification of 1,1-propanediol as an intermediate which is then stereospecifically dehydrated by the enzyme to propionaldehyde (Retey et al., 1966b).

The purpose of the work described in this paper is to investigate the nature of the intermediate(s) on the catalytic pathway between 1,2-propanediol and 1,1-propanediol. Experimental values of k_{cat} were determined for several mixtures of 1,2-propanediol and 1,1-dideuterio-1,2-propanediol. These were then analyzed in terms of an equation derived to describe the kinetics which would result for several different mechanisms. This method facilitates discrimination among the suggested mechanisms and, also, allows calculation of isotope effects on k_{cat} caused by substitution of deuterium for hydrogen in a single step of the reaction pathway.

Scheme I



The experiments and analysis of this work are somewhat similar to those previously applied to methylmalonyl coenzyme A mutase (Miller & Richards, 1969) and to glutamate mutase (Eagar et al., 1972). Moreover, these methods should prove applicable to the study of intermediates in other enzymatic processes.

Experimental Section

Propanediol dehydratase was obtained from *Klebsiella pneumoniae* (ATCC 8724) by a procedure adapted from Lee & Abeles (1963). Fraction E-8 with a specific activity of 35–58 was used for all determinations. Enzyme free of 1,2-propanediol was prepared by dialysis against several changes of 0.01 M K_2HPO_4 at 4 °C.

Adenosylcobalamin (AdoCbl) was purchased from Sigma Chemical Co.

Assays were performed as previously described (Bachovchin et al., 1977, 1978). Solutions generally contained: yeast alcohol dehydrogenase (ADH, Sigma), 10 units; β-nicotinamide adenine dinucleotide (reduced form, NADH, Sigma), 0.2 mM; apodiol dehydratase, 0.15 unit; bovine serum albumin, 0.1 mg; potassium phosphate buffer, pH 8.0, 30 mM; AdoCbl, 0.02 mM; and the desired mixture of deuterated and undeuterated substrate, 20 mM. Total volume was 2.3 mL, and all reactions were carried out at 37 °C. The reaction was initiated by addition of AdoCbl in a 30-μL aliquot. The rate for each substrate mixture was determined from at least three kinetic experiments.

Substrate. (*R,S*)-1,2-Propanediol was purchased as the reagent grade chemical (MCB) and distilled before use.

(*R,S*)-*1,1-Dideuterio-1,2-propanediol* (*diol-d*$_2$) was prepared as described (Bachovchin et al., 1977).

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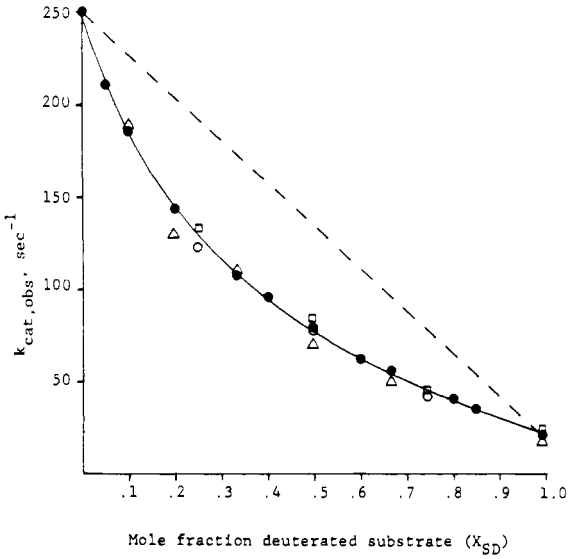


FIGURE 1: $k_{cat,obsd}$ as a function of X_{SD}, the mole fraction of deuterated substrate, for mixtures of 1,2-propanediol with diol-d_2, diol-d_3, and diol-d_6 at different substrate concentrations: diol-d_2, 20 mM (●); diol-d_2, 8 mM (○); diol-d_3, 23.4 mM (△); diol-d_6, 10.3 mM (□).

(R,S)-1,1,2-Trideuterio-1,2-propanediol (diol-d_3) was prepared by reduction of ethyl pyruvate with lithium aluminum deuteride (Stohler). Deuterium content in the labeled positions was at least 98% as determined by ^{1}H NMR.

(R,S)-Perdeuterio-1,2-propanediol (diol-d_6) was prepared by lithium aluminum deuteride reduction of ethyl 3,3,3-trideuteriopyruvate which had been synthesized from $Cd(CD_3)_2$ and ethyloxalyl chloride (Aldrich) by the general procedure outlined by Kollonitsch (1966a,b). CD_3I (Stohler) was used in the synthesis of the organocadmium reagent.

Computing. Computer programs based on the algorithms described by Marquardt (1963) were used to fit the experimental rate data to an equation derived to describe the kinetics of AdoCbl-dependent reactions.

Results

Figure 1 illustrates the variation in the observed catalytic rate constant as a function of mole fraction of deuterated substrate in mixtures of 1,2-propanediol with diol-d_2, diol-d_3, or diol-d_6. The rate data obtained for these three substrates are virtually identical which indicates that substitution of deuterium for hydrogen at C-2 or C-3 of 1,2-propanediol does not significantly affect the reaction. Several substrate concentrations were used; since all were at least 250-fold greater than K_m (Bachovchin et al., 1977, 1978), saturation kinetics were observed in each case. Moreover, as a maximum of 1.7% of the original substrate was converted to product during any particular experiment, we eliminated complications resulting from preferential depletion of undeuterated substrate, with a concomitant change in mole fraction of deuterated substrate.

Derivation of Rate Expressions. A quantitative interpretation of the data of Figure 1 requires an equation which relates the observed catalytic rate to the isotopic composition of substrate. Such an equation may be derived by consideration of the generally accepted mechanism of catalysis, which involves a minimum of two steps: (i) transfer of hydrogen (or deuterium) from C-1 of substrate to an intermediate reservoir of hydrogen and (ii) transfer of hydrogen (or deuterium) from this reservoir to C-2 of product. Figure 2 outlines a simple hypothetical mechanism in which hydrogen abstracted from substrate becomes one of two equivalent hydrogens in an intermediate reservoir. Substrate may contain either deuterium (mole fraction X_{SD}) or hydrogen (mole fraction $X_{SH} = 1 - X_{SD}$), and the reservoir may likewise have either deuterium (mole fraction X_D) or hydrogen (mole fraction X_H). As illustrated in Figure 2, conversion of one molecule of substrate to product may occur by any one of six microscopic pathways (a–f) each of which is characterized by a particular overall rate constant (k_{HH}, k_{HD}, k_{DH}, or k_{DD}). The contribution of each pathway to the overall observed rate constant ($k_{cat,obsd}$) is the rate constant appropriate for that particular path multiplied by the probability that such a path will be followed. Accordingly, the catalytic rate observed for a given isotopic composition of substrate will be the sum of the terms at the bottom of Figure 2.

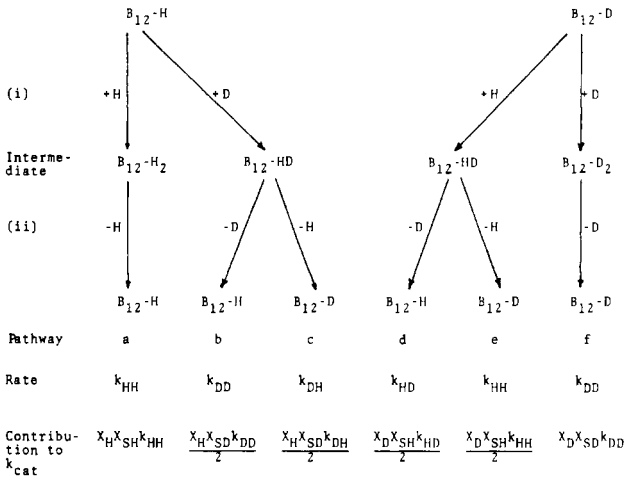


FIGURE 2: Reaction scheme for a hypothetical mechanism in which the intermediate carries two equivalent hydrogens.

$$k_{cat,obsd} = X_H\left[X_{SH}k_{HH} + X_{SD}\left(\frac{k_{DH} + k_{DD}}{2}\right)\right] + X_D\left[X_{SH}\left(\frac{k_{HH} + k_{HD}}{2}\right) + X_{SD}k_{DD}\right] \quad (1)$$

This expression can be simplified by deriving X_D (and X_H) in terms of X_{SD}, assuming that the isotopic composition of the coenzyme has reached a steady state. (This is a reasonable expectation, as each molecule of holoenzyme turns over thousands of molecules of substrate even during the very early stages of the reaction.) In the mechanism of Figure 2, only pathways c and d affect the isotopic composition of hydrogen attached to coenzyme. At steady state, their contributions to $k_{cat,obsd}$ become equal so that

$$(1/2)X_H X_{SD} k_{DH} = (1/2)X_D X_{SH} k_{HD}$$

This leads, on substitution of $X_H = 1 - X_D$ and rearrangement, to

$$X_D = \frac{X_{SD}k_{DH}}{X_{SD}k_{DH} + X_{SH}k_{HD}} \quad (2)$$

Equation 2 indicates that coenzyme will become enriched in deuterium if $k_{HD} < k_{DH}$; for $k_{HD} > k_{DH}$, the intermediate will be depleted in heavy isotope relative to substrate.

Equation 1 may be generalized to describe a mechanism in which the intermediate hydrogen reservoir contains n hydrogens and in which that hydrogen transferred to the reservoir from substrate has a statistical probability f/n of being returned to product in step ii [$f = 1$ for a mechanism with n equivalent hydrogens and $f = 0$ for a "merry-go-round" mechanism in which hydrogen can never be returned to the

Table I[a]

n	f	k_{HH}	k_{HD}	k_{DH}	k_{DD}	σ
Equivalent Hydrogen Models						
2	1	250 ± 1	−11.6 ± 2.8	−56 ± 11	20.7 ± 1	1.22
3	1	250 ± 1	23.1 ± 2.1	112 ± 16	20.7 ± 1	1.22
4	1	250 ± 1	34.7 ± 1.8	168 ± 18	20.7 ± 1	1.22
5	1	250 ± 1	40.4 ± 1.7	196 ± 19	20.7 ± 1	1.22
Merry-go-round Models						
2-5	0	250 ± 1	57.8 ± 1.4	280 ± 23	20.7 ± 1	1.22

[a] Optimized rate constants [s^{-1}, based on a molecular weight of 250 000 and specific activity 60 units/mg (Essenberg et al., 1971)] for a least-squares fit to eq 3 of rate data obtained with 1,2-propanediol/diol-d_2 mixtures. Identical results were obtained for 2, 3, 4, and 5 hydrogen merry-go-round schemes.

same substrate molecule from which it was abstracted (Miller & Richards, 1969; Eagar et al., 1972)].

$$k_{cat,obsd} = \sum_{i=1}^{m} \binom{n-1}{i-1} X_H^{[n-i]} X_D^{[i-1]} \{X_{SH}[(f/n + B)k_{HH} + Ck_{HD}] + X_{SD}[Bk_{DH} + (f/n + C)k_{DD}]\} \quad (3)$$

where

$$B = \frac{(n-i)(n-f)}{n(n-1)}$$

$$C = \frac{(i-1)(n-f)}{n(n-1)}$$

and $\binom{n-1}{i-1}$ is the usual combinatorial notation. In a manner analogous to that used earlier in the case for $f = 1$, $n = 2$, one can show that eq 2 also applies to the general case.

Equation 3 includes several assumptions. Hydrogen in the intermediate reservoir may not exchange with product. In our experiments both the high substrate concentrations employed (which would saturate holoenzyme with propanediol) and the immediate reduction by ADH/NADH of propionaldehyde released from the enzyme preclude significant recombination between enzyme and product. In addition, hydrogen in the reservoir may not exchange with unreacted substrate to such an extent that the isotopic compositions of either are appreciably affected. The absence of appreciable exchange of hydrogen between cofactor and unreacted substrate has previously been demonstrated (Frey et al., 1967a; Carty et al., 1971). Furthermore, the finding that AdoCbl becomes considerably enriched in tritium relative to substrate during catalysis (Essenberg et al., 1971) indicates that the hydrogen of substrate does not equilibrate with that of AdoCbl. Equation 3 further requires all $(n - 1)$ hydrogens initially in the reservoir to become chemically equivalent in the intermediate. At least two results support this requirement. Both hydrogens attached to C-5′ AdoCbl can participate in the reaction (Frey et al., 1967b) and ^1H NMR studies of ethanolamine deaminase (Hull et al., 1975) indicate that the methyl group of enzyme-bound 5′-deoxyadenosine, the putative form of the coenzyme during the intermediate stages of the enzymatic reaction, rotates at a rate exceeding 10^7 s^{-1}, which is many orders of magnitude larger than the rate of hydrogen transfer from the intermediate to product. Lastly, eq 3 neglects any secondary isotope effect; for example, abstraction of hydrogen should occur with equal facility from intermediates CH_3Ad, CH_2DAd, and CHD_2Ad. Although no definitive support exists for this assumption, such secondary isotope effects should be small relative to the primary isotope effects included in eq 3 (Richards, 1970). Moreover, the kinetics of inactivation of diol dehydratase by glycerol of varying isotopic composition can be explained quantitatively without postulating such isotope effects (Bachovchin et al., 1978).

Table II[a]

n	k_{HH}	k_{HD}	k_{DH}	k_{DD}	f	n/f	σ
2	250 ± 2	20.6 ± 8	99.5 ± 36	20.7 ± 1	0.70 ± 0.11	2.9	1.30
3	250 ± 1	22.9 ± 3.4	110 ± 18	20.7 ± 2	1.0 ± 0.075	3.0	1.30
4	250 ± 1	24.9 ± 3.1	121 ± 15	20.7 ± 1	1.29 ± 0.11	3.1	1.30
5	250 ± 1	24.1 ± 3.1	116 ± 10	20.7 ± 1	1.63 ± 0.14	3.1	1.30

[a] Optimized rate constants (s^{-1}) and f for mechanisms with an intermediate containing n hydrogens.

Table III[a]

n	f	$\Sigma(k_{cat,obsd} - k_{cat,calcd})^2$
2	0	5814
2	1	1462
3	1	11.8
4	1	375
5	1	941

[a] Least-squares criterion as a function of n and f for fixed values of the rate constants $k_{HH} = 250$ s^{-1}, $k_{HD} = 23.1$ s^{-1}, $k_{DH} = 112$ s^{-1}, and $k_{DD} = 20.7$ s^{-1}.

The parameters k_{HH}, k_{HD}, k_{DH}, and k_{DD} in eq 3 were varied by the method of least-squares estimation of nonlinear parameters to obtain the best fit to this equation of the experimental rate data obtained with various mixtures of 1,2-propanediol/diol-d_2. A number of different mechanistic schemes were considered, including those with 2, 3, 4, or 5 "equivalent" hydrogens ($f = 1$, and $n = 2$, 3, 4, 5) and "merry-go-round" schemes ($f = 0$ and $n = 2$, 3, 4, or 5). The results of these comparisons are summarized in Table I. The uncertainties listed for each rate constant are standard deviations obtained from the least-squares fitting program. The value of σ (column 7) was calculated from eq 4, where $k_{cat,calcd}$

$$\sigma = \left[\frac{\sum_{i=1}^{d} (k_{cat,calcd} - k_{cat,obsd})^2}{d - p} \right]^{1/2} \quad (4)$$

is the value of k_{cat} for a given value of X_{SD} calculated by optimization of eq 3; $k_{cat,obsd}$ is the experimentally observed rate for substrate with this particular isotopic composition; d is the number of data points used in the analysis ($d = 12$ for 1,2-propanediol/diol-d_2 mixtures); and p is the number of parameters which were varied during the optimization ($p = 4$ for k_{HH}, k_{HD}, k_{DH}, and k_{DD}).

In a second analysis, the parameter f, which is proportional to the likelihood of the hydrogen derived from substrate being returned to product, was also allowed to vary systematically. Table II summarizes these calculations. Although the value of σ is larger ($\sigma = 1.30$) than for the data in Table I ($\sigma = 1.22$), the agreement between experimental and calculated rates is actually slightly improved. Moreover, in the comparisons of Table II, $p = 5$, whereas in Table I, $p = 4$, and this decreases the denominator of eq 4 which will, accordingly, increase the calculated values of σ in Table II.

Table III summarizes the variation of $\sum(k_{cat,calcd} - k_{cat,obsd})^2$ with changing values of n and f for a given set of rate constants (data from Table I, second row). These comparisons emphasize that, for a given set of rate constants, the accuracy with which eq 3 describes the experimental data depends sensitively on the assumed values of n and f and, thereby, on the assumed mechanistic scheme.

Discussion

A number of explanations can be considered to account for the nonlinear variation of $k_{cat,obsd}$ (Figure 1) with the isotopic composition of substrate. Two such explanations can be eliminated. A difference in enzyme–substrate association constants as a function of the isotopic composition of substrate could account for our observations but seems unlikely in view of previous results showing that isotopic composition does not affect binding of 1,2-propanediol (Bachovchin et al., 1978) or of 3-fluoro-1,2-propanediol (Eagar et al., 1975).

A second possibility involves a reaction pathway with simultaneous transfer of two or more hydrogens. A relation between the isotopic composition of substrate and reaction velocity has been derived (Kresge, 1964; Gold, 1969) for such reactions. In the present case, in which no exchange of substrate hydrogens with water occurs (Abeles & Lee, 1962), such an explanation would require that $(k_{cat})^{1/n}$, where n is the number of hydrogens simultaneously in flight, should be a linear function of X_{SD}. (Such behavior has, in fact, been reported in studies of the mechanism of action of serine proteases (Hunkapiller et al., 1976; Schowen, 1977) and amidohydrolases (Schowen, 1977).) Our results do not show such a dependence. Moreover, a concerted transfer of two or more hydrogens appears unlikely in view of the finding that hydrogen abstracted from C-1 of substrate has a significant probability of being returned to its parent substrate molecule (Frey et al., 1967a).

The third explanation, which we favor, attributes the shape of the curve of Figure 1 to the existence of an intermediate, which is enriched in deuterium relative to substrate, hydrogen transfer from which is a major rate-determining step of the reaction. Substantial evidence for the existence of such an intermediate, in which the cofactor is present as 5′-deoxyadenosine, has been reported (Babior, 1975; Abeles & Dolphin, 1976). Furthermore, that this intermediate should be enriched in heavy isotope relative to substrate is supported by studies with tritiated substrates and AdoCbl (Essenberg et al., 1971; Weisblat & Babior, 1971).

Models for Hydrogen Transfer. Equation 3 allows evaluation of pathways with intermediates containing n hydrogens in which hydrogen derived from substrate has a probability f/n of participating in the second transfer step (Figure 2, reaction ii). The best fit of the experimental rate data to this equation was determined by systematic variation of the rate constants k_{HH}, k_{DH}, k_{HD}, and k_{DD} by the method of least-squares estimation of nonlinear parameters for 2–5 equivalent and 2–5-hydrogen merry-go-round mechanisms (Table I). In Table II, these rate constants as well as the parameter f were allowed to vary. The rate constants k_{HH} and k_{DD} are turnover rates when both transfer steps involve only hydrogen or only deuterium, respectively; k_{HD} and k_{DH} are turnover rates in which deuterium is transferred only in the second or only in the first step, respectively.

As shown in Table I, row 1, a close fit for a mechanism involving 2 equivalent hydrogens requires the physically meaningless condition that k_{HD} and k_{DH} assume negative values. On this basis, we may eliminate this proposed mechanism. Similar evidence also argues against the merry-go-round mechanisms in which $k_{DH} \sim 280\ s^{-1}$ compared with $k_{HH} \sim 250\ s^{-1}$ ($k_{HH}/k_{DH} \sim 0.89$). Such an inverse primary isotope effect has no precedent in studies of AdoCbl-dependent enzymes and little precedence in enzymology in general (Jencks, 1969; Richards, 1970). Thus, the mechanisms which most adequately describe the experimental data are those involving an intermediate with three or more equivalent hydrogens (Table I, rows 2-4). The analysis of Table II, in which f is allowed to vary as well as the rate constants, indicates that mechanisms in which the ratio $n/f \sim 3$ provide the best fit to the experimental data. The improvement in this second analysis between calculated and experimental data is, however, slight. Nevertheless, despite uncertainties inherent in the rate measurements, these data and their analysis provide evidence for a mechanism involving an intermediate containing three equivalent hydrogens, one of which was abstracted from substrate. (The possibility of more than three equivalent hydrogens cannot, however, be rigorously eliminated on the basis of our data alone.)

Rate-Determining Step. The value of k_{DD} (20.7 s^{-1}, $k_{HH}/k_{DD} = 12$) indicates that the transfer of hydrogen is an important rate-contributing step of the reaction. Which step, i or ii, is slower can be ascertained from the values of k_{DH} and k_{HD} in Table I. In the three equivalent hydrogen mechanism, substitution of deuterium for hydrogen in step i causes the turnover rate to decrease from 250 s^{-1} (k_{HH}) to 112 s^{-1} (k_{DH}) for an isotope effect of $k_{HH}/k_{DH} = 2.2$. The same substitution in reaction ii causes a much larger decrease, to $k_{HD} = 23\ s^{-1}$ for $k_{HH}/k_{HD} = 10.8$, an isotope effect similar to that observed when deuterium participates in both reactions ($k_{HH}/k_{DD} = 12$). (We emphasize that the rate constants k_{HD} and k_{DH} represent turnover rates when deuterium replaces hydrogen in only one of steps i or ii; they are not true "microscopic" rate constants for a particular step of the reaction.) These data do, nevertheless, clearly identify reaction ii, the transfer of hydrogen from cofactor–intermediate to product, as the slowest step in the conversion of 1,2-propanediol to propionaldehyde catalyzed by propanediol dehydratase. This differs from a previous conclusion (Essenberg et al., 1971), but accords with those drawn for ethanolamine deaminase (Weisblat & Babior, 1971) and for the inactivation of diol dehydratase by glycerol (Bachovchin et al., 1978).

Relation to Microscopic Rate Constants. Although the rate constants k_{HH}, k_{HD}, k_{DH}, and k_{DD} are overall turnover rates, their values as determined above may be used to deduce relationships between some of the microscopic rate constants. Based on previous studies, the minimum reaction scheme which may be drawn is

$$E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} ES \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES' \xrightarrow{k_2} E + P$$

k_1 is the rate constant for hydrogen transfer from substrate to cofactor, while k_2 is the net rate constant for the remainder of the pathway, which includes hydrogen transfer from cofactor to product. When holoenzyme is saturated with substrate, k_{cat} is given by (Cleland, 1975)

$$k_{cat} = V_{max}/E_0 = (k_1k_2)/(k_1 + k_{-1} + k_2) \qquad (5)$$

Each of the constants k_1, k_{-1}, and k_2 may manifest a primary deuterium isotope effect. This expression may be used to derive expressions for the observed isotope effects k_{HH}/k_{HD}, k_{HH}/k_{DH}, and k_{HH}/k_{DD} in terms of the microscopic constants. For the first of these

$$k_{HH}/k_{HD} = \frac{k_1^H k_2^H/[k_1^H + k_{-1}^H + k_2^H]}{k_1^H k_2^D/[k_{-1}^H + k_1^H + k_2^D]} = \frac{k_2^H/[k_1^H + k_{-1}^H + k_2^H]}{k_2^D/[k_1^H + k_{-1}^H + k_2^D]} \qquad (6)$$

Because the values of k_1, k_{-1}, and k_2 are unknown, k_{HH}/k_{HD} cannot be evaluated explicitly. However, limiting values of k_{HH}/k_{HD} may be obtained for the cases $k_2 >> (k_1 + k_{-1})$ for

Table IV: Limiting Values of Overall Isotope Effects for a Two-Step Reaction

	$k_2 << (k_{-1} + k_1)^a$	$k_2 >> (k_{-1} + k_1)$
k_{HH}/k_{HD}	k_2^H/k_2^{D*}	1
k_{HH}/k_{DH}	$1^\dagger$	k_1^H/k_1^D
k_{HH}/k_{DD}	k_2^H/k_2^D	k_1^H/k_1^D

[a] If the hydrogens in the intermediate are equivalent with respect to k_{-1}, the * and † labeled quantities will be somewhat reduced and increased, respectively.

which $k_{HH}/k_{HD} = 1$ and $k_2 << (k_1 + k_{-1})$ for which $k_{HH}/k_{HD} = k_2^H/k_2^D$. Similar consideration of k_{HH}/k_{DH} and k_{HH}/k_{DD} (neglecting equilibrium isotope effects) gives the results in Table IV.

In view of the values $k_{HH}/k_{HD} = 10.8$, $k_{HH}/k_{DD} = 12$, and $k_{HH}/k_{DH} = 2.2$ obtained experimentally, we conclude that the diol dehydratase reaction corresponds more closely to the left column of Table IV. In contrast, the glutamate mutase reaction (Eagar et al., 1972), with isotope effects of k_{HH}/k_{DD} ("k_H/k_D") = 7.5, $k_{HH}/k_{HD}((k_H/k_D)/\Delta_1) = 7.5/7.0 = 1.1$, and $k_{HH}/k_{DH}((k_H/k_D)/\Delta_2) = 7.5/1.5 = 5.0$, corresponds to the right side of Table IV, with k_1 the most likely rate-limiting step. Methylmalonyl CoA mutase (Miller & Richards, 1969) which exhibits values of 3.5, 1.1, and 1.2, respectively, constitutes an intermediate case in which both hydrogen abstraction from substrate and hydrogen return to form product influence significantly the overall reaction rate.

Relation to Previous Studies. The existence of an intermediate in which the cofactor exists as 5′-deoxyadenosine with three equivalent hydrogens attached to C-5′ has been inferred from studies of ethanolamine deaminase (Babior, 1969; Weisblat & Babior, 1971) and diol dehydratase (Frey et al., 1967a; Essenberg et al., 1971), using substrates and AdoCbl in which transferrable hydrogens are enriched with ^3H. The results and analysis described here accord with the qualitative conclusions reached earlier. More importantly, these deuterium isotope effects, observed during rearrangement of 1,2-propanediol catalyzed by diol dehydratase, unambiguously require the intervention of a "reservoir" containing at least three equivalent hydrogens. This same point has been previously established for two other AdoCbl dependent enzymes, methylmalonyl coenzyme A isomerase (Miller & Richards, 1969) and glutamate mutase (Eagar et al., 1972).

Indeed, eq 3 as developed to analyze the results with deuterium can also be informatively applied to the earlier studies employing ^3H as a trace label. These investigations involved two types of experiments: (a) transfer of ^3H from 5′-^{3}H-AdoCbl to product during catalysis of unlabeled substrate and (b) catalysis with [1-^{3}H]-1,2-propanediol or [1-^{3}H]ethanolamine as substrate.

In case a, the rate of tritium transfer from cofactor to product is given by the sum of those terms in eq 3 which involve k_{HT} (k_{HD} in eq 3). Thus

$$\frac{dT_P}{dt} = -\frac{dT_{AdoCbl}}{dt} = \sum_{i=1}^{n} \binom{n-1}{i-1} X_H^{[n-i]} X_T^{[i-1]} X_{SH} \frac{(i-1)(n-f)}{n(n-1)} k_{HT} \quad (7)$$

When $n = 3$, $f = 1$, and $X_{SH} = 1$ for unlabeled substrate this expression reduces to

$$\frac{-dT_{AdoCbl}}{dt} = \frac{2}{3} X_T k_{HT}$$

which upon integration becomes

$$T_{AdoCbl} = T_{0,AdoCbl} e^{-(2/3)k_{HT}(t)}$$

Accordingly, transfer of ^3H from AdoCbl to product should be first order in the ^3H content of AdoCbl; a semilogarithmic plot of the ^3H content of AdoCbl as a function of time should be linear with a slope of $-(2/3)k_{HT}$.

Analysis of an experiment of type b is somewhat more complex. One may monitor both the specific activity of unreacted substrate and the ratio of specific activity of substrate to that of AdoCbl. The rate of conversion of tritiated substrate to product is given by the sum of those terms of eq 3 which contain k_{TH} and k_{TT}

$$\frac{-dT_S}{dt} = \sum_{i=1}^{n} \binom{n-1}{i-1} X_H^{[n-i]} X_T^{[i-1]} X_{ST} [Bk_{TH} + (f/n + C)k_{TT}] \quad (8)$$

where B and C have been defined previously. On substitution of $n = 3$ and $f = 1$, and neglect of terms in which the mole fraction of tritium appears more than once, this expression simplifies to

$$\frac{-dT_S}{dt} = \frac{(2k_{TH} + k_{TT})X_{ST}}{3} \quad (9)$$

Equation 9 may be used to derive an expression for the specific activity of residual substrate as a function of the fraction of substrate converted to product x (Melander, 1960):

$$\frac{\text{sp act., } x}{\text{sp act., } x = 0} = (1 - x)^{[[(2k_{TH} + k_{TT})/3k_{HH}] - 1]} \quad (9a)$$

Thus, observation of the specific activity of residual substrate can give only a weighted average of k_{TH} and k_{TT}.

Despite the fact that X_{ST} increases continuously due to depletion of unlabeled substrate, eq 2 may be adapted directly to tritium kinetics for AdoCbl-dependent rearrangements if one assumes that a steady-state value of X_T is attained rapidly on the time scale of changes in X_{ST}. In fact, measurements of the specific activity of AdoCbl during catalysis with tritiated 1,2-propanediol as substrate support this assumption (Essenberg et al., 1971). Furthermore, with the approximation that $X_{ST}k_{TH} << X_{SH}k_{HT}$, eq 2 becomes

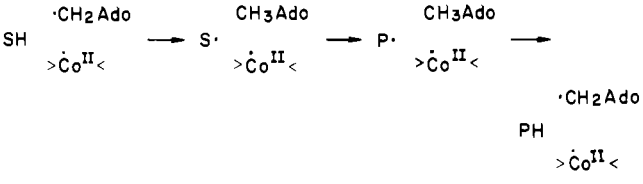
$$X_T = X_{ST}(k_{TH}/k_{HT}) \quad (10)$$

When X_T is given by eq 10, the amount of tritium entering the intermediate is equal to the amount transferred from intermediate to product. Under these steady-state conditions, the specific activity of product will be determined by the overall tritium isotope effect $k_{HH}/[(2k_{TH} + k_{TT})/3]$ (eq 9a) as follows:

$$\frac{\text{product sp. act., } x}{\text{substrate sp act., } x = 0} = \frac{1 - (1 - x)^{[(2k_{TH} + k_{TT})/3k_{HH}]}}{x} \quad (11)$$

In light of these equations, the information that can be derived from experiments using tritium kinetic isotope effects may be summarized. A value of k_{HT} can be determined by measuring the rate of tritium transfer from [5′-^{3}H]AdoCbl to product derived from unlabeled substrate. The ratio k_{TH}/k_{HT} may be determined by measuring the specific activities of substrate and AdoCbl during catalysis with tritiated substrate. Finally, measurement of the specific activity of unreacted substrate or of product as a function of extent of reaction can yield a value for $(2k_{TH} + k_{TT})/3$. However, in no case can one calculate the isotope effect for a single microscopic step of the reaction pathway from experiments of

Scheme II



this type in which either deuterium or tritium isotope effects are observed.

In view of these conclusions, an earlier report (Essenberg et al., 1971) merits scrutiny. Based on an experiment of the type described by eq 8, a tritium isotope effect of 250(2/3) was calculated for hydrogen transfer from AdoCbl to product at 10 °C. This value is unprecedentedly large and is, further, not consistent with the deuterium isotope effects observed in this work. In a second experiment, Essenberg et al. measured the specific activities of AdoCbl, propionaldehyde, and residual substrate as a function of time with [1-^{3}H]-1,2-propanediol as substrate. Their first observation, that AdoCbl becomes enriched in tritium relative to unreacted substrate by a factor of 19, accords with the conclusions reached in the present work. From the present results, approximate values of k_{TH} and k_{HT} can be calculated (Swain et al., 1958):

$$k_{HH}/k_{HT} = (k_{HH}/k_{HD})^{1.44} \quad (12)$$

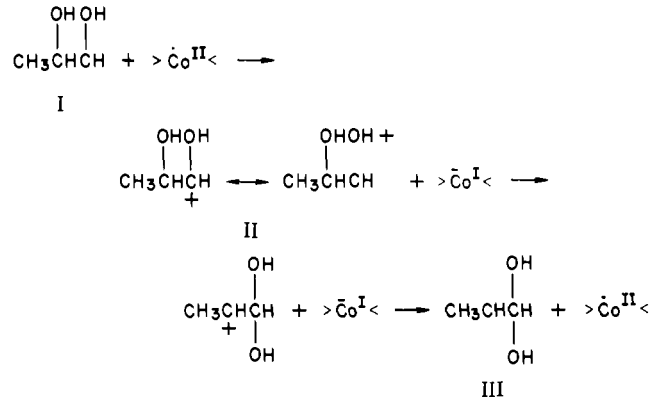
Thus, $k_{HH}/k_{TH} = 3.1$ and $k_{HH}/k_{HT} = 30.9$ which, though they do not represent true isotope effects for single steps in the reaction, do approximately reflect the tritium isotope effects for the first and second hydrogen transfer steps, respectively. These values, when substituted into eq 10, give a value of $X_T/X_{ST} \sim 10$ which must be multiplied by two to obtain a specific activity ratio of 20. This result agrees well with the experimental data and predicts a tritium isotope effect for the transfer of hydrogen from AdoCbl to product of 31, a magnitude frequently observed in enzymatic reactions (Jencks, 1969; Knowles & Albery, 1977).

In contrast, their second observation, that the specific activity of propionaldehyde product (given by eq 11) is lower by a factor of 20–47 than that of residual substrate (given by eq 9a), indicates that hydrogen transfer from cofactor to product exhibits an apparent tritium isotope effect approximately fivefold larger than that expected on the basis of our observed deuterium isotope effects.

Similar arguments also apply to the work on ethanolamine deaminase (Babior, 1969; Weisblat & Babior, 1971); they observed a tritium isotope effect of 5.8 for the conversion of [1-^{3}H]ethanolamine to acetaldehyde compared with a deuterium isotope effect of 6.8–7.4. This seemingly anomalous result (a deuterium isotope effect which is larger than the apparently analogous tritium isotope effect) in fact follows quantitatively from the treatment of this paper. The tritium isotope effect is dominated by k_{TH} and, therefore, depends principally on the isotope effect for the first hydrogen transfer step, a conclusion also reached qualitatively by Weisblat & Babior (1971). The overall deuterium isotope effect depends, in contrast, on k_{DD}. When the effect of isotopic substitution is $k_{DH} > k_{HD}$ (and $k_{TH} > k_{HT}$), for the overall reaction one observes that $k_H/k_D > k_H/k_T$. Indeed, when applied to the diol dehydratase reaction, our data on the deuterium isotope effects, together with this treatment, predict a tritium isotope effect of ~4.5 compared with a deuterium isotope effect of 12.

The deuterium isotope effects observed in this study are within the range of those reported for free radical reactions involving cleavage of a carbon–hydrogen bond. For example,

Scheme III



Wilen & Eliel (1958) reported isotope effects ranging from 3 to 10 for abstraction of hydrogen from α-deuterated toluene and *p*-xylene by various peroxides. Howard et al. (1968) observed isotope effects of 8 to 20 for the oxidation of deuteriocumenes and perdeuteriotetralin by cumene hydroperoxide and *tert*-butyl hydroperoxide. Thus, our data are compatible with a mechanism involving hydrogen transfer between free radicals (Babior, 1970; Finlay et al., 1972, 1973). See Scheme II.

However, with the exception of the work of Walling & Cioffari (1972) and Julia (1971), and of calculations carried out by Golding & Radom (1976), there is little precedent for a 1,2 rearrangement of a radical species. In contrast, 1,2 rearrangement of a carbonium ion, such as might be generated by oxidation of the 1,2-propanediol-1-yl radical by cob(II)-alamin, has ample precedent. In this event, the mechanism shown in Scheme III could obtain, as suggested earlier (Eagar et al., 1972; Halpern, 1974). Free radical I is more stable than its precursor, $\cdot CH_2Ad$. Similarly, the rearranged radical III is less stable than $\cdot CH_2Ad$, and formation of the latter, by transfer of a C-5′ hydrogen to C-2 of III, would be energetically favored. The relative stability of the oxocarbonium ion II could help to facilitate oxidation of radical I by Cbl^{II}, while the eventual elimination of H_2O could provide a driving force for rearrangement of II and the reduction of the resulting carbonium ion by Cbl^{I}.

Conclusions

By analysis of the variation of k_{cat} with mole fraction deuterated substrate in the AdoCbl-dependent conversion of 1,2-propanediol to propionaldehyde, we have inferred the obligatory intervention of an intermediate hydrogen reservoir in which the hydrogen derived from substrate becomes equivalent with at least two other hydrogen atoms; one of these three equivalent hydrogen atoms is returned to form product in the principal rate-determining step of the reaction. The magnitudes of the primary deuterium isotope effects observed in these two hydrogen transfer steps are consistent with these steps' involving cleavage of a carbon–hydrogen bond. These results should, therefore, be immune to the criticism (Schrauzer, 1971) of experiments which use tritium as a trace label. The role of the 5′-methyl group of 5′-deoxyadenosine as the reservoir of the three equivalent hydrogens has ample precedence (for example: Babior et al., 1974; Bachovchin et al., 1978).

References

Abeles, R. H., & Lee, H. A., Jr. (1962) *Brookhaven Symp. Biol. 15*, 310.

Abeles, R. H., & Zagalak, B. (1966) *J. Biol. Chem. 241*, 1245.

Abeles, R. H., & Dolphin, D. (1976) *Acc. Chem. Res. 9*, 114.
Babior, B. M. (1969) *J. Biol. Chem. 244*, 449.
Babior, B. M. (1970) *J. Biol. Chem. 245*, 6125.
Babior, B. M. (1975) *Acc. Chem. Res. 8*, 376.
Babior, B. M., Woodams, A. D., & Brodie, J. D. (1973) *J. Biol. Chem. 248*, 1445.
Babior, B. M., Carty, T. J., & Abeles, R. H. (1974) *J. Biol. Chem. 249*, 1689.
Bachovchin, W. W., Eagar, R. G., Jr., Moore, K. W., & Richards, J. H. (1977) *Biochemistry 16*, 1082.
Bachovchin, W. W., Moore, K. W., & Richards, J. H. (1978) *Biochemistry 17*, 2218.
Barker, H. A., Suzuki, F., Iodice, A. A., & Rooze, V. (1964a) *Ann. N.Y. Acad. Sci. 112*, 644.
Barker, H. A., Rooze, V., Suzuki, F., & Iodice, A. A. (1964b) *J. Biol. Chem. 239*, 3260.
Carty, T. J., Babior, B. M., & Abeles, R. H. (1971) *J. Biol. Chem. 246*, 6313.
Cleland, W. W. (1975) *Biochemistry 14*, 3220.
Eagar, R. G., Jr., Baltimore, B. G., Herbst, M. M., Barker, H. A., & Richards, J. H. (1972) *Biochemistry 11*, 253.
Eagar, R. G., Jr., Bachovchin, W. W., & Richards, J. H. (1975) *Biochemistry 14*, 5523.
Essenberg, M. K., Frey, P. A., & Abeles, R. H. (1971) *J. Am. Chem. Soc. 93*, 1242.
Finlay, T. H., Valinsky, J., Sato, K., & Abeles, R. H. (1972) *J. Biol. Chem. 247*, 4197.
Finlay, T. H., Valinsky, J., Mildvan, A. S., & Abeles, R. H. (1973) *J. Biol. Chem. 248*, 1285.
Frey, P. A., Essenberg, M. K., & Abeles, R. H. (1967a) *J. Biol. Chem. 242*, 5369.
Frey, P. A., Kerwar, S. S., & Abeles, R. H. (1967b) *Biochem. Biophys. Res. Commun. 29*, 873.
Gold, V. (1969) *Adv. Phys. Org. Chem. 7*, 259.
Golding, B. T., & Radom, L. T. (1976) *J. Am. Chem. Soc. 98*, 6331.
Halpern, J. (1974) *Ann. N.Y. Acad. Sci. 239*, 2.
Howard, J. A., Ingold, K. Y., & Symonds, M. (1968) *Can. J. Chem. 46*, 1017.
Hull, W. E., Mauck, L., & Babior, B. M. (1975) *J. Biol. Chem. 250*, 8023.
Hunkapiller, M. W., Forgac, M. D., & Richards, J. H. (1976) *Biochemistry 15*, 5581.
Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, Chapter 4, McGraw Hill, New York.
Julia, M. (1971) *Acc. Chem. Res. 4*, 386.
Kellermayer, R. W., & Wood, H. G (1962) *Biochemistry 1*, 1124.
Knowles, J. R., & Albery, W. J. (1977) *Acc. Chem. Res. 10*, 105.
Kollonitsch, J. (1966a) *J. Chem. Soc. A*, 453.
Kollonitsch, J. (1966b) *J. Chem. Soc. A*, 456.
Kresge, A. G. (1964) *Pure Appl. Chem. 8*, 243.
Lee, H. A., Jr., & Abeles, R. H. (1963) *J. Biol. Chem. 238*, 2367.
Marquardt, D. W. (1963) *J. Soc. Indust. Appl. Math. 11*, 431.
Melander, L. (1960) *Isotope Effects on Reaction Rates*, Chapter 3, Ronald Press, New York.
Miller, W. W., & Richards, J. H. (1969) *J. Am. Chem. Soc. 91*, 1498.
Phares, E. F., Long, M. V., & Carson, S. F. (1964) *Ann. N.Y. Acad. Sci. 112*, 680.
Retey, J., & Arigoni, D. (1966) *Experientia 22*, 783.
Retey, J., & Zagalak, B. (1973) *Angew. Chem. 85*, 721.
Retey, J., Umani-Ronchi, A., & Arigoni, D. (1966a) *Experientia 22*, 72.
Retey, J., Umani-Ronchi, A., Seible, J., & Arigoni, D. (1966b) *Experientia 22*, 502.
Richards, J. H. (1970) *Enzymes, 3rd Ed. 2*, 321–333.
Schowen, R. L. (1977) *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 79–89, University Park Press, Baltimore, MD.
Schrauzer, G. N. (1971) *Adv. Chem. Ser. No. 100*. 1.
Sprecher, M., & Sprinson, D. B. (1964) *Ann. N.Y. Acad. Sci. 112*, 655.
Sprecher, M., Clark, M. J., & Sprinson, D. B. (1964) *Biochem. Biophys. Res. Commun. 15*, 581.
Swain, C. G., Stivers, E. C., Reuwer, J. R., Jr., & Schaad, L. H. (1958) *J. Am. Chem. Soc. 80*, 5885.
Switzer, R. L., Baltimore, B. G., & Barker, H. A. (1969) *J. Biol. Chem. 244*, 5263.
Toraya, T., Shirakashi, T., Kosaga, T., & Fukui, S. (1976) *Biochem. Biophys. Res. Commun. 69*, 475.
Walling, C., & Cioffari, A. (1972) *J. Am. Chem. Soc. 94*, 6064.
Weisblat, D. A., & Babior, B. M. (1971) *J. Biol. Chem. 246*, 6064.
Wilen, S. H., & Eliel, E. L. (1958) *J. Am. Chem. Soc. 80*, 3309.
Wood, H. G., Kellermayer, R. W., Stjernholm, P., & Allen, S. H. G. (1964) *Ann. N.Y. Acad. Sci. 112*, 661.
Zagalak, B., Frey, P. A., Karabatsos, G. L., & Abeles, R. H. (1966) *J. Biol. Chem. 241*, 3028.