Studies on the Mechanism of Hydrogen Transfer in the Coenzyme B_{12} Dependent Dioldehydrase Reaction II[1]

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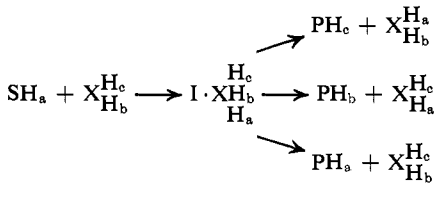
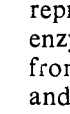
Abstract: Rates of the following processes which occur during the conversion of DL-1,2-propanediol to propionaldehyde by dioldehydrase and coenzyme B_{12} were measured: tritium transfer from the C-1 position of the substrate to the C-5′ position of coenzyme B_{12}; tritium transfer from the C-5′ position of coenzyme B_{12} to the C-2 position of the product; and the overall tritium transfer from substrate to product. The kinetics of these transfer reactions were found to be consistent with a reaction sequence in which coenzyme B_{12} functions as an obligatory intermediate hydrogen carrier. When [5′-^3H]coenzyme B_{12} is used, tritium transfer to product occurs in one out of 250 turnovers. When deuterium is present on C-1 of the substrate, and the C-5′ positions of coenzyme B_{12} contain deuterium and tritium, tritium is transferred in one out of 14 turnovers. If a substrate contains deuterium on C-1, the probability of tritium transfer to the product derived from that molecule is enhanced 4–8 times over that with a substrate which does not contain deuterium. These results support a previous suggestion that an intermediate must exist containing three hydrogens, one contributed by the substrate and the two C-5′ hydrogens of coenzyme B_{12}, and that in any one turnover any of the three hydrogens can be transferred to the reaction product. 5′-Deoxyadenosine could fulfill the role of this intermediate, but its participation in the reaction has not yet been convincingly established. When sufficient [^3H]coenzyme B_{12} is added to enzyme, the amount of tritium released to the reaction product is independent of the amount of [^3H]coenzyme B_{12} added, indicating that enzyme and coenzyme combine to form an undissociable complex.

Dioldehydrase (DL-1,2-propanediol hydrolyase, E.C. 4.2.1.28), an enzyme which requires coenzyme B_{12},[3] catalyzes the conversion of L- or D-1,2-propanediol to propionaldehyde and of ethylene glycol to acetaldehyde. When a substrate containing tritium at C-1 is used, both C-5′ hydrogens of coenzyme B_{12} become labeled, and when coenzyme B_{12} containing tritium at C-5′ is used, the tritium is transferred to the α position of the product aldehyde. In any single turnover, the α hydrogen of the product is either derived from coenzyme B_{12} or is the same hydrogen which was originally present in the substrate, so that both intermolecular and intramolecular hydrogen transfers occur.[1,4] Similar conclusions have been reached with methylmalonyl coenzyme A isomerase.[5] These results have led to the suggestion that in these reactions coenzyme B_{12} functions as an intermediate hydrogen carrier, and reaction sequence I (Scheme I) has been proposed.[1,5] SH

Scheme I. Reaction Sequence I



and PH represent the substrate and product, X^H_H represents the enzyme-bound coenzyme B_{12}, I is an enzyme-bound intermediate derived from the substrate, and



represents an enzyme-bound intermediate form of coenzyme B_{12} containing three hydrogens, two derived from the C-5′ position of coenzyme B_{12} (H_c, H_b) and one from the substrate (H_a). In any one turnover any of the three hydrogens can appear in the product, although not necessarily with the same probability. Since transfer of tritium from coenzyme B_{12} to product has been observed with other enzymic reactions in which coenzyme B_{12} participates,[6-9] Scheme I may be applicable to these reactions also.

No kinetic confirmation of this mechanism has been reported, *i.e.*, it has not been demonstrated that the rate of tritium introduction into coenzyme B_{12} from the substrate is sufficiently fast to account for the rate of tritium appearance in the product. Experiments reported here indicate that the mechanism represented by Scheme I is kinetically feasible. Other experiments, also consistent with this mechanism, are reported.

Experimental Section

Enzymes, Coenzyme, and Substrates. Dioldehydrase was isolated from *Aerobacter aerogenes* (ATCC 8724) as previously described.[10] When it was necessary to remove DL-1,2-propanediol, which is present in the purified enzyme as a stabilizing agent, dioldehydrase was dialyzed for 2 hr against 0.01 *M* K_2HPO_4 at 0–5°, treated with 1 μg of coenzyme B_{12}/200 units of enzyme at 37° for 10 min, and then dialyzed an additional 2 hr against 0.01 *M* K_2HPO_4 at 0–5°. Horse liver alcohol dehydrogenase was purchased from Worthington Biochemical Corp. Crystalline coenzyme was pre-

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(2) Charles Revson Fellow, 1967–1969.
(3) The following abbreviations are used: coenzyme B_{12}, α-(5,6-dimethylbenzimidazolyl)-Co-5′-deoxyadenosylcobamide; [^3H]coenzyme B_{12}, [5′-^3H]-α-5,6-dimethylbenzimidazolyl)-Co-5′-deoxyadenosylcobamide; enzyme–coenzyme B_{12}, the dioldehydrase–coenzyme complex.
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pared by the procedure of Hogenkamp and Pailes.[11] Commercial DL-1,2-propanediol, ethylene glycol, propionaldehyde, and acetaldehyde were redistilled before use.

Isotopically Labeled Substrates. [^{14}C]Ethylene glycol and [1,1,2,2-$^{2}H_4$]ethylene glycol were purchased from New England Nuclear Corporation. [1-^{14}C]-1,2-Propanediol was prepared by reduction of DL-[1-^{14}C]sodium lactate with $LiAlH_4$ according to a published procedure.[12] It was purified by chromatography on Dowex 1-X8 ion exchange resin in the hydroxide form and was eluted with water. It was subjected to descending paper chromatography using the upper phase of 1-butanol–acetic acid–water (4:1:5). Unlabeled propanediol was spotted beside the radioactive material. The single radioactive peak migrated the same distance as the unlabeled propanediol, which was identified by periodate–benzidine stain. The radioactive propanediol was eluted with water. DL-[1,1-$^{2}H_2$]-1,2-Propanediol was prepared by reduction of ethyl lactate with $LiAlD_4$ (Metal Hydrides Inc., Beverly, Mass.) according to a published procedure.[13] It was purified by gas–liquid chromatography with an F & M Model 720 gas chromatograph. Samples (0.25 ml) were injected into a 220 cm × 0.9 cm column filled with Porapak Q, 100–120 mesh (Waters Assoc., Inc., Framingham, Mass.). The helium carrier gas flow rate was 120 cc/min, and the column temperature was raised from 170 to 250° at 4°/min. DL-1,2-Propanediol was eluted after 18 min and was collected in a liquid nitrogen cooled trap.

D-[1-^{3}H]-1,2-Propanediol, stereospecifically labeled at C-1 so that ^{3}H is transferred to C-2 of the product aldehyde, was prepared by reduction of D-lactaldehyde by ^{3}H-NADH and horse liver alcohol dehydrogenase according to the procedure described for D-[1-^{2}H]-1,2-propanediol.[14] Since any racemization of the lactaldehyde would have led to preparation of L-[1-^{3}H]-1,2-propanediol labeled in the C-1 hydrogen which is not transferred by dioldehydrase, the stereospecificity of labeling of the [1-^{3}H]-1,2-propanediol obtained was tested. [1-^{3}H]-1,2-Propanediol (14 μmol) was incubated with 19 units of enzyme–coenzyme B_{12} for 50 sec at 10°. The reaction was stopped with 0.1 ml of 20% trichloroacetic acid (TCA). The reaction mixture, containing 4.9 μmol of aldehyde, was neutralized, added to carrier propionaldehyde, and steam distilled into an ice-cooled receiver. One aliquot of the propionaldehyde was converted to the propionaldomethone derivative and another to propionic acid by oxidation with $KMnO_4$. The specific activity of the acid was lower than that of the propionaldomethone, and this difference was a measure of tritium on C-1 of propionaldehyde. Sixteen per cent of the tritium in the substrate was in the nontransferable position on C-1. It was assumed that during the kinetic experiment in which this substrate was used, 16% of the radioactive substrate was converted to product without kinetic isotope effect. Radioactivity due to this incorrectly labeled compound was subtracted from experimental values of tritium in propionaldehyde to give the values presented in Figure 3 and Tables II and III.

[^{3}H]Coenzyme B_{12}. Coenzyme B_{12} containing tritium on the 5′ carbon atom of the adenosyl moiety was prepared synthetically as previously described.[1] [^{3}H]Coenzyme B_{12} of higher specific activity than had been obtained from the chemical synthesis was prepared enzymatically. Dioldehydrase (330 units) and 100 μg of coenzyme B_{12} were incubated at 10° with 11 μmol of DL-[1-^{3}H]-1,2-propanediol (2.9×10^{7} cpm/ μmol), which had been prepared as previously described,[1] in a volume of 2.5 ml. The reaction was initiated by addition of the substrate. After 33 sec, the reaction was stopped by addition of 0.1 ml of 20% TCA. The mixture was centrifuged and decanted, and the precipitate was washed with 1 ml of water. The combined supernatant fluids were extracted three times with equal volumes of diethyl ether, which removed excess TCA and brought the pH up to 2.8. The solution was then passed through a 1 × 5 cm column of Dowex 50-X8, 200–400 mesh ion exchange resin which had been equilibrated with 2.0 *M* sodium phosphate buffer, pH 3.0,[15] and the column was washed with 35 ml of water. [^{3}H]Coenzyme B_{12} was eluted with 30 ml of 0.1 *N* NH_3 and further purified by paper electrophoresis, using 0.5 *N* NH_3 as the electrolyte, and by descending paper chromatography, using water-saturated 2-butanol as the developing solvent. The material eluted from the first chromatogram gave a single radioactive peak when it was chromatographed a second time. Since [^{3}H]coenzyme B_{12} could not always be detected visually, it was necessary to spot markers of authentic coenzyme on either side of the radioactive material. After electrophoresis and chromatography, the area between the markers was eluted and assayed enzymatically and radiochemically for [^{3}H]coenzyme B_{12}. This procedure has been shown to produce radiochemically pure [^{3}H]-coenzyme B_{12}.[1] As explained in the Results, a reaction time which is just long enough to allow complete reaction of ^{3}H substrate is of great advantage for preparing [^{3}H]coenzyme B_{12} of high specific activity, since the radioactivity of coenzyme B_{12} becomes very much higher than that of the substrate near the end of the reaction. Prolonged incubation, on the other hand, reduces the radioactivity in coenzyme B_{12} by exchange with propionaldehyde, and also leads to excessive oxygen inactivation of the enzyme–[^{3}H]coenzyme B_{12} complex.

Assays. Dioldehydrase was assayed as previously described,[10] except that sodium pyruvate was used as a standard for the colorimetric assay. Coenzyme B_{12} was assayed enzymatically with dioldehydrase.[16] Aldehydes were assayed by the procedure used in the enzyme assay,[10] and ethanol and propanol were assayed with alcohol dehydrogenase.[17] Radiochemical assays were performed by liquid scintillation counting with a solvent system consisting of 7 g of 2,5-diphenyloxazole, 300 mg of *p*-bis[2-(5-phenyloxazolyl)]benzene, and 100 g of naphthalene made to 1 l. with dioxane. The Ansitron and the Nuclear-Chicago Mark I liquid scintillation spectrometers were used for radioactivity measurements. Radioactive areas on paper chromatograms were detected with a Tracerlab 4π scanner.

Isolation of Products. The product solutions obtained from the experiments with mixtures of ethylene glycol and DL-1,2-propanediol described in the legend to Table IV were immediately adjusted to pH 5–6 with potassium phosphate. Charcoal (10–20 mg) was added to remove coenzyme B_{12}, and the tubes were shaken and allowed to stand for 5 min at 0°. The tubes were centrifuged in a clinical centrifuge for 5 min, and the supernatant fluids were decanted and distilled into an ice-cooled receiver. Small aliquots were taken for aldehyde and radiochemical assays before and after the distillations. Recoveries of aldehyde were 30–75% and recoveries of radioactivity were 0–14% lower than aldehyde recoveries. The aldehydes were oxidized by treatment with 50–150 μmol of potassium permanganate for 30–40 min in an ice–water bath. During the oxidation the pH was maintained at 6.5 with additions of 0.1 *N* NaOH. Excess permanganate was titrated with sodium bisulfite, the mixture was centrifuged, and the solution was decanted from precipitated manganese dioxide. The pH was adjusted to greater than 10 with NaOH. Acetic and propionic acids were separated by chromatography on columns of silicic acid, titrated, and assayed for radioactivity as described in an earlier publication.[1]

The propionaldomethone derivative was prepared by adding to reaction mixtures containing propionaldehyde 1 ml of a solution of 1% 5,5-dimethylcyclohexane-1,3-dione and 0.5% Na_2CO_3 for each 10 μmol of aldehyde. The resulting solution was adjusted to pH 4.0 with HCl and refrigerated overnight. The precipitate was filtered, dried, and recrystallized from ethanol–water. The melting point (155–156°, uncorrected) was in agreement with the published value[18] and remained unchanged after recrystallization. The specific radioactivity changed no more than 10% after first recrystallization and no more than 3% between second and third recrystallizations.

Kinetic Measurements. The rates at which tritium is released from enzyme–[^{3}H]coenzyme B_{12} to acetaldehyde or propionaldehyde at 0–20° were measured for comparison with the rates of the corresponding overall reactions. All reactions were carried out in the dark to prevent photolysis of coenzyme B_{12}. The rates were measured by two procedures. In one set of experiments KBH_4 was present throughout the reaction to reduce product aldehyde to ethanol. It was shown that KBH_4 does not affect the overall rate or maximum amount of tritium released to aldehyde. Reaction components were equilibrated at the reaction temperature in the dark. For each time point 0.020 ml containing 3.4×10^{-3} or 6.8×10^{-3} μmol of [^{3}H]coenzyme B_{12} was added to 6–9 units of

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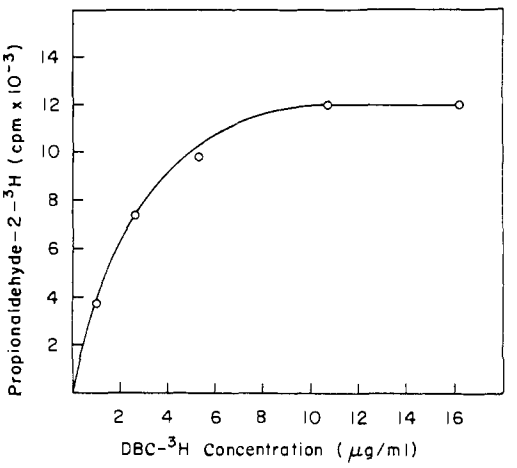


Figure 1. Saturation of dioldehydrase with [^3H]coenzyme B_{12}. Each reaction mixture consisted of 10 units of dioldehydrase, 4.5 μmol of potassium phosphate buffer, pH 8.0, 5 μmol of DL-1,2-propanediol, and 6.9×10^{-4}–1.03×10^{-2} μmol/of [^3H]coenzyme B_{12} (1.93×10^7 cpm/μmol) in a total volume of 0.50 ml. Enzyme, buffer, and [^3H]coenzyme B_{12} were incubated for 2.5 min at 10° in the dark before starting the reactions by adding 0.2 ml of 0.025 *M* substrate. After 2 min at 10° the reactions were stopped by adding 0.5 ml of 0.02 *N* HCl containing 104 μmol of carrier propionaldehyde. The solutions were treated with 1–2 mg of charcoal to remove excess [^3H]coenzyme B_{12} and then centrifuged in a clinical centrifuge. The supernatant fluids were assayed for propionaldehyde recovery and radioactivity content.

substrate-free dioldehydrase and 4–5 μmol of potassium phosphate buffer, pH 8.0, in a volume of 0.18 ml and allowed to react for 4–5 min in order to ensure complete formation of the active enzyme–[^3H]coenzyme B_{12} complex. Potassium borohydride (5–8 μmol) was added in 0.10-ml volumes 30 sec before starting the reaction, and the reaction was started by adding 0.5, 2.0, or 20 μmol of ^{14}C substrate in 0.2-ml volumes with rapid mixing. After the appropriate time, 2–120 sec, the reaction was stopped and the enzyme irreversibly inactivated by adding 0.5 ml of 0.1 *N* HCl containing a known amount of carrier ethanol or propanol greatly in excess of the amount produced by the reaction. A zero time point was obtained by adding the HCl–alcohol to one of the tubes before adding the substrate. In each experiment one of the reaction mixtures was incubated at 37° for 2 min before adding HCl–alcohol, in order to determine the maximum amount of tritium which could be released to aldehyde, that is, the amount of [^3H]coenzyme B_{12} complexed with the enzyme. After the reaction was stopped, 0.5 ml of 0.1 *N* NaOH and 3–5 mg of activated charcoal were added to each tube. The tubes were thoroughly shaken and centrifuged for 10 min in a clinical centrifuge. The supernatant fluids were decanted and diluted to 4 ml with water. Each of these solutions was distilled into an ice-cooled receiver until 2 ml of distillate had been collected, and the distillates were diluted to 5.0 ml. Aliquots of these solutions were assayed radiochemically to determine their ^{14}C and ^3H contents; separate aliquots were assayed for carrier alcohol recovery by the alcohol dehydrogenase method,[17] and the ^{14}C and ^3H contents of alcohol before losses were calculated. From these values the amount of product formed and the amount of tritium transferred to the product were calculated for each time point. The amount of catalytically active holoenzyme was calculated from the amount of tritium transferred to aldehyde in 2 min at 37° and the known specific activity of [^3H]coenzyme B_{12}. The results were plotted, and first-order rate constants for product formation (k_{cat}) and for tritium transfer ($k_{^3H}$) were calculated.

Rates of tritium release to aldehyde and rates of the overall reactions with DL-1,2-propanediol, ethylene glycol, and [1,1,2,2-^3H]-ethylene glycol as substrates were measured by a second procedure which was similar to that described above except that no KBH_4 was used and the rate of aldehyde production was measured directly.[10] The reactions were carried out in 1-ml volumetric tubes at 10°. For each point, 0.010 or 0.020 ml of [^3H]coenzyme B_{12} (1.5–6.3×10^{-4} μmol) was added to 0.04–0.09 ml of substrate-free dioldehydrase (4–12 units) and allowed to react for 4–5 min. A solution of 5 μmol of potassium phosphate, pH 8.0, in sufficient volume to bring the reaction mixture to a volume of 0.40 ml was added 30 sec before starting the reaction, and the reaction was started by adding 1–5 μmol of substrate in 0.1-ml volume with rapid mixing. The reaction was stopped by adding 0.1 ml of 0.1 *N* HCl from a syringe, and the solution was neutralized by adding 0.1 ml of 0.1 *N* NaOH. The early reaction mixtures (0–40 sec) were used to measure the rate of appearance of tritium in aldehyde. A known amount of carrier aldehyde (40–50 μmol) was added to these reaction mixtures, water was added to 1.0 ml, and the mixtures were treated with 3–5 mg of charcoal and centrifuged. The supernatant solutions were assayed colorimetrically for aldehyde recovery, and aliquots were subjected to radiochemical assay. From these values and the original amount of carrier, the total amount of tritium transferred to aldehyde was calculated. Three additional points were taken at later times, and these reaction mixtures were used for measuring the rate of acetaldehyde production, as well as the maximum amount of tritium released. No carrier aldehyde was added to these tubes. Water was added to 1.0 ml, and an aliquot was assayed colorimetrically for aldehyde. Another aliquot was added to a chilled solution of carrier aldehyde for radiochemical assay by the procedure used for the early reaction mixtures. The same amount of tritium was always detected in these three late reaction mixtures. This amount was used as the value at infinite time for the purpose of calculating k_T. The rate constant for the overall reaction, k_{cat}, was calculated from the rate of production of aldehyde and the amount of holoenzyme.

Results

Saturation of Dioldehydrase with [^3H]Coenzyme B_{12}. Previous studies suggest that the dioldehydrase–coenzyme B_{12} complex does not dissociate at an appreciable rate relative to the rate of the catalytic reaction.[1] The results of experiments designed to establish this point more conclusively are depicted in Figure 1. A constant amount of dioldehydrase was incubated with increasing concentrations of [^3H]coenzyme B_{12}, and the amount of tritium released to propionaldehyde was measured. As expected, at low [^3H]coenzyme B_{12} concentrations, the amount of tritium released to propionaldehyde was dependent upon the [^3H]coenzyme B_{12} concentration, whereas at high concentrations it was not. Experiments described below show that the half-time for tritium release is less than 10 sec, so that longer incubation times would not result in the release of more tritium. These data establish that enzyme-bound coenzyme B_{12} does not turn over at an appreciable rate and that 1 μg of coenzyme B_{12} (6.3×10^{-4} μmol) activates approximately 10 units of dioldehydrase. Determination of total tritium released is therefore a measure of the concentration of active enzyme–coenzyme B_{12}. The amount of enzyme activated by 1 μg of coenzyme B_{12} may be used with the specific activity of our most highly purified enzyme preparation (60 units/mg) to estimate that the equivalent weight of dioldehydrase is 2.6×10^5 g/mol.[19]

Rate of Tritium Transfer from Enzyme–[^3H]Coenzyme B_{12} to Reaction Products Compared with Rate of the Overall Reaction. In these experiments, substrate was added to enzyme–[^3H]coenzyme B_{12} and the rates of product formation and tritium appearance in the reaction product were measured. In all of the experiments the appearance of tritium in product followed a first-order rate equation in which the rate was proportional to the amount of radioactivity in enzyme–[^3H]coenzyme B_{12}. The rate constants, $k_{^3H}$, were measured graphically. A typical experiment is shown in Figure 2. A small amount of tritium released before addition of substrate at $t = 0$ was probably due to reaction of en-

(19) Unpublished determinations made in our laboratory with a calibrated Sephadex G-200 column indicated that the molecular weight and equivalent weight are 2.2–2.4×10^5.

zyme-[^3H]coenzyme B_{12} with traces of substrate in the enzyme solution and was subtracted from subsequent points. As expected, the appearance of reaction product was linear with respect to time. The rate constant for the overall reaction, k_{cat}, was calculated as the rate of product appearance divided by the amount of enzyme-coenzyme B_{12} present. The amount of enzyme-coenzyme B_{12} in the reaction mixtures was calculated from the total amount of tritium released to product at infinite time and the known specific activity of the [^3H]coenzyme B_{12}.

Results of a typical experiment with each substrate and the ranges of ratios $k_{^3H}/k_{cat}$ which were obtained are presented in Table I. The rate constant for the

Table I. Rates of Tritium Transfer from Coenzyme B_{12} to Product and Rates of Product Formation[a]

Substrate	Temp, °C	k_{cat}, sec^{-1}	$k_{^3H}$, sec^{-1}	$k_{cat}/k_{^3H} \times 10^{-2}$
DL-1,2-Propanediol[b]	10	48	0.20	2.4
Ethylene glycol[b]	10	23	0.091	2.5
Ethylene glycol[c]	0	1.1	0.0052	2.7
DL-1,2-Propanediol[c]	10	44	0.13	3.3
Ethylene glycol[c]	10	12	0.038	3.1
Ethylene glycol[c]	20	24	0.083	2.9

[a] The procedures used for carrying out the measurements and for calculating k_{cat} and $k_{^3H}$ are described in the Experimental Section under "Kinetic Measurements." [b] Three determinations of these rates were made with each substrate. Results of a typical experiment with each are given above. The highest and lowest values of k_{cat} for each substrate varied by about a factor or two. Since this variation is much greater than can be accounted for by random errors in measurement, we feel that separate preparations of the enzyme may have somewhat different catalytic efficiencies. The observed ratios of $k_{cat}/k_{^3H}$ were less variable: for DL-1,2-propanediol, 2.0–2.4 $\times 10^2$; for ethylene glycol, 2.0–2.7 $\times 10^2$. [c] KBH_4 was used in these reactions to trap acetaldehyde or propionaldehyde.

transfer of tritium from enzyme-[^3H]coenzyme B_{12} to propionaldehyde or acetaldehyde is smaller by a factor of approximately 250 than the corresponding k_{cat}. Therefore, tritium originally present in coenzyme B_{12} is discriminated against for transfer to the product by a factor of about 250. If it is assumed that an intermediate containing three equivalent hydrogens occurs, a statistical factor would enter into the discrimination against tritium. Even if this is taken into account, the tritium isotope effect would be approximately 125.

Since it was previously shown that tritium can be transferred from enzyme-[^3H]coenzyme B_{12} to propionaldehyde or acetaldehyde, the possibility was considered that some of the transfer of tritium measured in these experiments occurred as a result of direct interaction between the enzyme-coenzyme B_{12} complex and the reaction product.[20] To evaluate the magnitude of this contribution, experiments were carried out in which the product was immediately removed by reduction with potassium borohydride. The results of such kinetic experiments, carried out in the presence of potassium borohydride, are also included in Table I. The rate of tritium transfer relative to the overall reaction was somewhat slower since $k_{cat}/k_{^3H}$ was found to

(20) This interaction could account for only a minor part of the transfer observed, since the half-time for transfer of tritium from [^3H]-coenzyme B_{12} to propionaldehyde at 10° is 13 sec (T. H. Finlay and R. H. Abeles, unpublished results) and the corresponding half-time for DL-1,2-propanediol is 2–4 sec.

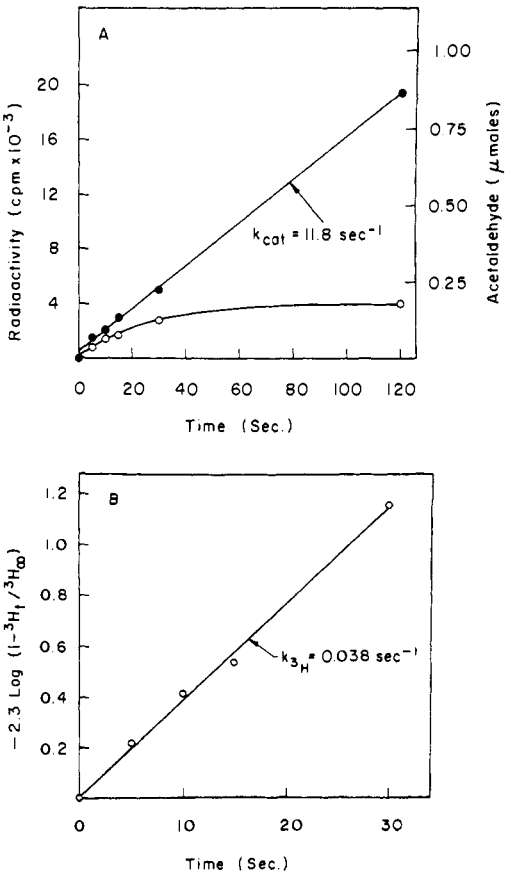


Figure 2. (A) The rate of appearance of tritium in acetaldehyde compared with the overall rate during reaction of [^{14}C]ethylene glycol with enzyme-[^3H]coenzyme. The following symbols are used: ●, micromoles of acetaldehyde produced; ○, tritium (counts per minute) in acetaldehyde. (B) First-order plot of tritium appearance in acetaldehyde. The reaction was carried out at 10°. For each time point there was a separate reaction mixture containing 8.6 units of dioldehydrase, 3.4×10^{-3} μmol of [^3H]coenzyme B_{12} containing 2.62×10^4 cpm, 4.8 μmol of potassium phosphate buffer, pH 8.0, 5.5 μmol of KBH_4, and 2.0 μmol of [^{14}C]ethylene glycol. The procedure of the experiment is described in the Experimental Section.

be approximately 300. Apparently, the aldehyde product may contribute slightly to the rate of removal of tritium from enzyme-[^3H]coenzyme B_{12}.

Tritium Content of Coenzyme and Rate of Tritium Appearance in Propionaldehyde during Reaction of D-[1-^3H]-1,2-Propanediol. This experiment was done to decide whether all of the tritium found in the product is derived directly from coenzyme B_{12} or whether another significant pathway exists for transfer of tritium from substrate to product. By procedures similar to those described above, the rate constant for transfer of tritium from coenzyme B_{12} to propionaldehyde, using D-1,2-propanediol as substrate and [^3H]coenzyme B_{12}, was determined. In addition, the incorporation of tritium into coenzyme B_{12} and into propionaldehyde from D-[1-^3H]-1,2-propanediol was determined as a function of time. Assuming that the only route for overall transfer of tritium from substrate to product involves intermediate attachment of the migrating tritium to coenzyme B_{12}, and knowing the rate of transfer of tritium from D-[1-^3H]-1,2-propanediol to coenzyme B_{12} and the rate of tritium transfer from coenzyme B_{12} to propionaldehyde, one can compute the rate of overall transfer of tritium from D-[1-^3H]-1,2-propanediol to

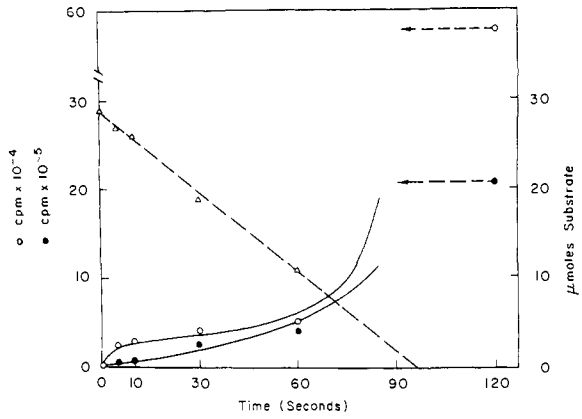


Figure 3. The appearance of tritium in coenzyme B_{12} and propionaldehyde during the reaction of stereospecifically labeled D-[1-^3H]-1,2-propanediol. The following symbols are used: ○, total radioactivity (counts per minute) in coenzyme B_{12}; ●, total radioactivity (cpm) in propionaldehyde; △, micromoles of residual substrate. The solid lines are computed values. The dotted arrows directed toward the left of the 120 second points are included to indicate that these points were taken after the end of the reaction and presumably are values which would correspond to somewhat earlier times. The experiment was carried out at 10° in the dark. A separate reaction mixture containing 98 units of dioldehydrase, 4 μmol of potassium phosphate, pH 8.0, 3.2×10^{-3} μmol of coenzyme B_{12}, and 29.8 μmol of substrate containing 1.17×10^7 cpm in a volume of 0.55 ml was used for each point. The reaction was started by adding the substrate in 0.10-ml volume, and, after the appropriate time, 0.1 ml of 20% TCA was added to stop the reaction. A 0.05-ml aliquot was immediately withdrawn, diluted with water, and assayed colorimetrically for its propionaldehyde content. A separate 0.10-ml aliquot was withdrawn, diluted into 1.0 ml of a solution containing 240 μmol of propionaldehyde carrier and 200 μmol of potassium phosphate, pH 8.0, treated with 1–2 mg of charcoal, and centrifuged. Propionaldehyde was isolated from the supernatant fluid as the dimethone derivative, which was assayed radiochemically to determine the amount of radioactivity in propionaldehyde. [^3H]Coenzyme B_{12} was isolated from the remainder of the reaction mixture according to the procedure described in the Experimental Section.

[2-^3H]propionaldehyde. Agreement between this computed rate and the experimentally observed rate for this transfer would strongly suggest that all tritium found in the product had come from coenzyme B_{12}, that is, the obligatory pathway for tritium transfer is from substrate to coenzyme to product.

The first-order rate constant for the appearance of tritium in propionaldehyde was measured for the reaction of enzyme-[^3H]coenzyme B_{12} with D-1,2-propanediol at 10°. A value of 0.25 sec^{-1} was obtained. Another experiment was then carried out in which C-1 stereospecifically tritiated D-1,2-propanediol was added to unlabeled enzyme–coenzyme B_{12}. The tritium contents of coenzyme B_{12} and propionaldehyde as a function of time and the rate of product appearance were determined. The results of these experiments are summarized in Table II and Figure 3. Table II gives the amount of tritium in coenzyme B_{12} at various times during the course of the reaction. After 60 sec, approximately 62% of the substrate had been consumed, whereas only 3.4% of the radioactivity was detected in propionaldehyde. The very large amount of radioactivity in coenzyme B_{12} at 120 sec was measured after essentially all of the substrate had reacted. Under these conditions the substrate concentration was too low to be determined, but it was undoubtedly of exceedingly high specific activity. From the total radio-

Table II. Time Course of Tritium Content of Coenzyme B_{12} and Rate of Tritium Appearance on Propionaldehyde during the Reaction of D-[1-^3H]-1,2-Propanediol[a]

Time, sec	Total [^3H]coenzyme-B_{12}, cpm × 10^{-4}	Rates of tritium appearance in propionaldehyde, cpm/sec × 10^{-3} Calcd instantaneous	Calcd av	Exptl av
0	0.057			
5	2.3	5.8		
10	2.8	7.0	9.0	7.7
30	4.1	10		
60	5.1	13		

[a] The reaction mixtures are described in the legend to Figure 3. The calculated instantaneous rates in the third column were obtained by multiplying the total amount of radioactivity in [^3H]-coenzyme B_{12} at each time by 0.25 sec^{-1}, the observed rate constant for the removal of tritium from [^3H]coenzyme B_{12} during the reaction of D-1,2-propanediol at 10°. These products represent the expected rates of appearance of tritium in propionaldehyde due to the presence of [^3H]coenzyme B_{12}. The calculated average rate is the arithmetic average of these four calculated instantaneous rates. The experimental average rate of appearance of tritium was obtained from a straight line drawn through the 5–60-sec points shown in Figure 3.

activity in [^3H]coenzyme at various times and the known rate constant ($k_{^3H} = 0.25$ sec^{-1}) for transfer of tritium from [^3H]coenzyme to product, "instantaneous" rates of tritium transfer were calculated. These are listed in Table II. The average of these values is in close agreement with the observed average rate of tritium appearance in propionaldehyde.

Figure 3 represents an alternative way of examining the data without the use of average values. The total radioactivity in enzyme–coenzyme B_{12} and in propionaldehyde is plotted against time. Two curves were generated with a computer program based on the following two equations which define the rates of appearance of tritium in coenzyme and in propionaldehyde (eq 1 and 2). In these equations X_t represents the

$$\frac{d(X_t)}{dt} = k_1(S_T) - k_{^3H}(X_t) = k_1(E)(S^*) - k_{^3H}(X_t) \quad (1)$$

$$\frac{d(P_t)}{dt} = k_{^3H}(X_t) \quad (2)$$

total radioactivity in coenzyme B_{12} at time "t," k_1 is the rate constant for transfer of tritium from the enzyme-bound substrate to enzyme–coenzyme B_{12}, S_T represents the amount of tritium in enzyme-bound substrate, $k_{^3H}$ is the rate constant for transfer of tritium from coenzyme B_{12} to product, E represents the concentration of enzyme–coenzyme B_{12}, S^* is the specific radioactivity of the residual substrate, and P_t represents the total radioactivity in propionaldehyde at time "t." S^* was calculated from the experimental data as follows

$$S^*(t) = \frac{(\text{tritium in substrate at } t)}{(\mu\text{mol of substrate at } t)} = \frac{(\text{tritium in substrate at } t = 0) - (P_t) - (X_t)}{(\mu\text{mol of substrate at } t = 0) - (\mu\text{mol of substrate at } t)}$$

With the experimentally determined value of 0.25 sec^{-1} for $k_{^3H}$ and the experimentally measured radioactivity contents of coenzyme B_{12} at 5, 10, 30, and 60 sec, a value for k_1 was calculated which produced the close fit between predicted and observed radioactivity in coen-

zyme B_{12} shown in Figure 3. That value of k_1 was 4.8 sec^{-1}. The catalytic constant, k_{cat}, for D-1,2-propanediol at 10°, computed from the rate of conversion of substrate to product and the concentration of enzyme–coenzyme B_{12}, is 97 sec^{-1}. Previously reported results indicate that the transfer of hydrogen from enzyme-bound substrate to coenzyme B_{12} is the rate-determining step,[1] so that k_{cat} is the rate constant for that transfer, and the tritium isotope effect is $k_{cat}/k_1 = 20$. Using the value of 4.8 sec^{-1} for k_1, a curve, shown in Figure 3, which represents the integrated appearance of tritium in propionaldehyde was generated. The computed curves were only plotted to 90 sec, because soon thereafter the substrate fell below saturating concentration; under these conditions, the equations on which the computer program was based were no longer valid. The results of this kinetic experiment show that a major fraction, possibly all, of the tritium in the product is derived from coenzyme B_{12}, and therefore provide support for the sequential hydrogen transfer of reaction sequence I. A mechanism involving an intramolecular 1,2 shift cannot occur or can occur only to a minor extent.

Table III compares the specific activity of coenzyme B_{12} isolated from the reaction at various times with the specific activity of the product and the specific activity of the residual substrate. Although specific activities of coenzyme B_{12} and substrate increased as the reaction progressed, their ratio remained essentially constant at a value of about 19. The specific activity of product was lower than that of coenzyme B_{12} by factors of 280–2700, reflecting the isotope discrimination against transfer of tritium from [^3H]coenzyme B_{12} to product.

Table III. Specific Activities of Coenzyme B_{12} and Residual Substrate during the Conversion of D-[1-^3H]-1,2-Propanediol to Propionaldehyde [a]

Time, sec	Specific activity, cpm/μmol × 10^{-6}: Residual substrate	Coenzyme B_{12}	Product
0	0.40	0.18	
5	0.43	7.5	0.027
10	0.44	8.8	0.022
30	0.59	13	0.026
60	1.03	16	0.022
120		180	0.067

[a] The reaction mixtures are described in the legend to Figure 3. The specific activities of residual D-[1-^3H]-1,2-propanediol were calculated from the known initial specific activity and data on the amounts of propionaldehyde and [2-^3H]propionaldehyde produced at each time. The specific activities of [^3H]coenzyme B_{12} and [2-^3H]propionaldehyde at the various times were measured directly.

The very high radioactivity content of coenzyme B_{12} at the end of the reaction suggested a procedure for preparing [^3H]coenzyme B_{12} of very high specific activity, which is described in the Experimental Section.

Effect of Deuterium on the Transfer of Tritium to Reaction Product. Two types of experiments were done. In the first experiment [1,1,2,2-$^2H_4$]ethylene glycol was added to enzyme-[^3H]coenzyme B_{12}, and kinetic constants were determined at 10°, as in Table I. The following results were obtained: $k_{cat} = 3.6$ sec^{-1}, $k_{^3H} = 0.26$ sec^{-1}, $k_{cat}/k_{^3H} = 14$. Despite the small k_{cat} due to the deuterium isotope effect upon the overall reaction, the value of $k_{^3H}$ is increased, and the ratio $k_{cat}/k_{^3H}$ is decreased about 18–20 fold from the ratio obtained with undeuterated substrates. Under these conditions, the 5′-hydrogens of coenzyme B_{12} became rapidly replaced by deuterium, so that the intermediate complex (Scheme I) would contain one tritium and two deuterium atoms. The increased probability of tritium transfer to the product in comparison with that observed with an unlabeled substrate is therefore the effect of replacing two hydrogens of the intermediate by deuterium.

Another set of experiments was carried out specifically to study the effect of deuterium introduced from the substrate into the postulated intermediate upon the rate of tritium transfer to product. This was done by adding mixtures of ethylene glycol and DL-1,2-propanediol, in one of which the hydrogens subject to tranfer were replaced by deuterium, to enzyme-[^3H]-coenzyme. The deuterated substrate would contribute a deuterium atom to the postulated intermediate, and the undeuterated substrate would contribute a hydrogen atom. Therefore, the intermediate complex formed from the deuterated substrate differed from that formed from the undeuterated substrate only in that it contained one more deuterium atom.[21] The ratio of specific activities of the reaction products was compared to that obtained when neither substrate contained deuterium.[22] A difference in the two ratios would reflect exclusively the effect of the deuterium introduced by the substrate into the intermediate complex on the probability of tritium transfer from the intermediate complex to the reaction product.

Mixtures of [1,1,2,2-$^2H_4$]ethylene glycol and DL-1,2-propanediol as well as of ethylene glycol and DL-[1,1-$^2H_2$]-1,2-propanediol were incubated with enzyme and [^3H]coenzyme B_{12}. Experiments were done with several substrate ratios for each combination of substrates. This was done to assure that the effects seen were not some fortuitous effect of a specific ratio of substrates but actually reflected the effect of deuterium.

The results of these experiments are summarized in Table IV. The numbers in the last column show that the presence of deuterium in the substrate increases the probability of tritium transfer to the product four–sevenfold.

(21) Since deuterated substrate was reacting, some of the [^3H]coenzyme B_{12} molecules became labeled with deuterium in the other C-5′ position. However, since both substrates were always present, they were exposed to coenzyme B_{12} molecules of the same isotopic composition.

(22) When neither substrate contained deuterium, the specific activity of propionate obtained was 2.4 ± 0.2 times greater than the specific activity of acetate. This ratio was independent of the relative concentrations of substrates. It was unexpected that this ratio differ from 1.0, since with each substrate the ratio of the observed catalytic constant to the rate constant for transfer of tritium from enzyme-[^3H]-coenzyme B_{12} to product at 10° was about 250. The procedures used in the mixed substrate experiments and in the kinetic experiments differed in a number of respects, but we have examined each one separately without discovering the cause of the apparent discrepancy. It is not due to the difference in temperature or in order of addition of enzyme, [^3H]coenzyme B_{12}, and substrates. Kinetic measurements at the high substrate concentrations of the mixed substrate experiments or of the reaction of mixtures of two substrates give $k_{cat}/k_{^3H}$ between 200 and 300. The low relative specific activity of acetate is not due to loss of tritium during oxidation of acetaldehyde and chromatography of acetate, since more than 80% of the tritium released during the conversion of ethylene glycol to acetaldehyde can be accounted for by the specific activity of the eluted acetate. The apparent discrepancy between the two experiments may be due to effects of one or both substrates upon the enzyme's catalytic properties with respect to the other substrate.

Table IV. Reactions of Mixtures of DL-1,2-Propanediol or DL-[1,1-$^2H_2$]-1,2-Propanediol and Ethylene Glycol or [1,1,2,2-$^2H_4$]Ethylene Glycol with Dioldehydrase and [$^3H_2$]Coenzyme B_{12}[a]

Substrates	Substrate, μmol	Total aldehyde produced, μmol	Propionate: acetate, μmol	Sp act of product, cpm/μmol	Ratio of sp act propionate: acetate	Enhancement[b] of tritium transfer
DL-1,2-Propanediol [1,1,2,2-$^2H_4$]Ethylene glycol	150 920	113	17	2.2×10^3 3.6×10^3	0.61	3.9
DL-1,2-Propanediol [1,1,2,2-$^2H_4$]Ethylene glycol	140 1,700	54	15	5.0×10^3 9.5×10^3	0.53	4.5
DL-[1,1-$^2H_2$]-1,2-Propanediol Ethylene glycol	210 3,150	92	0.4	7.2×10^3 0.76×10^3	9.5	4.0
DL-[1,1-$^2H_2$]-1,2-Propanediol Ethylene glycol	250 1,750	68	0.46	12×10^3 1.0×10^3	12	5.0
DL-[1,1-$^2H_2$]-1,2-Propanediol Ethylene glycol	1,600 800	170	0.98	1.31×10^3 0.077×10^3	17	7.1
DL-1,2-Propanediol Ethylene glycol	380 2,400	65	5.2	3.8×10^3 1.5×10^3	2.5	
DL-1,2-Propanediol Ethylene glycol	500 15,000	33	1.4	2.8×10^3 1.3×10^3	2.1	
DL-1,2-Propanediol Ethylene glycol	300 15,000	80	0.65	1.9×10^3 0.77×10^3	2.5	

[a] The reaction mixtures contained the substrates indicated above, 40–200 units of enzyme, 2×10^{-3} to 1.2×10^{-2} μmol of [^3H]coenzyme B_{12} containing 3.4–9.1 $\times 10^5$ cpm, and 0.04 *M* potassium phosphate, pH 8.0, in 3–10-ml total volume. The reactions were initiated by addition of [^3H]coenzyme B_{12} and were allowed to proceed in the dark for 8–17 min at 37°. They were stopped by addition of 0.04 ml of 20% TCA/ml of reaction mixture. The product aldehydes were purified, oxidized, and separated as described under "Isolation of Products" in the Experimental Section. [b] The enhancement of tritium transfer was computed as (specific activity of [^2H]acetate)(2.4)/(specific activity of propionate) or (specific activity of [^2H]propionate)/(specific activity of acetate)(2.4). The number 2.4 = (specific activity of propionate)/(specific activity of acetate) when neither substrate contains deuterium.[22]

Discussion

Experiments reported here show that the mechanism represented by Scheme I is kinetically permissible. In the course of these kinetic studies, an unusually large isotope discrimination was found in the transfer of tritium from coenzyme B_{12} to reaction product. When unlabeled substrate reacts with enzyme-[^3H]-coenzyme B_{12} complex, tritium is transferred from coenzyme B_{12} in approximately one out of every 250 turnovers ($k_{cat}/k_{^3H} = 250$). Since the postulated intermediate (Scheme I) contains three transferable hydrogens, this isotope effect is assumed to represent competition of tritium with two hydrogen atoms and therefore contains a statistical factor. Even when this is considered, the isotopic discrimination against tritium is very large. Quantum mechanical tunneling could be responsible for a large isotope effect. However, this appears to be an improbable explanation, since, as will be discussed below, the magnitude of the deuterium isotope effect relative to the tritium isotope effect is that which would be predicted from differences in zero-point energies alone, whereas a greater difference between deuterium and tritium isotope effects is characteristic of tunneling.[23] It is possible that the discrimination observed against tritium in coenzyme B_{12} is not entirely due to an isotope effect but is partially due to a discrimination against the hydrogens originally present in coenzyme B_{12}. Some evidence in favor of this hypothesis exists. It has been previously shown that when tritiated substrate is used, intramolecular hydrogen transfer occurs in at least one out of every hundred turnovers,[1] which means that when the tritium is contributed by the substrate the isotopic discrimination in the transfer of tritium from the intermediate to the reaction product is about 100, as opposed to 250 seen in the experiments described here. This suggests that tritium contributed by the substrate to the intermediate has a somewhat greater probability of being transferred to the reaction product than that which was originally present in coenzyme B_{12}. Additionally, the transfer of tritium from coenzyme B_{12} to the product may not be a one-step process and may therefore involve cumulative isotope effects, as for instance an equilibrium isotope effect and a kinetic isotope effect. A definitive explanation for the high isotope discrimination must await a more detailed investigation of the mechanism of this reaction.

The rate of tritium transfer from [^3H]coenzyme B_{12} to the reaction product relative to the rate of the overall reaction with unlabeled ethylene glycol and deuterated ethylene glycol indicated isotope discrimination against tritium of about 250 and 14, respectively. Thus, the probability of tritium being transferred to product was enhanced approximately 18-fold by the use of deuterated substrate. This enhancement is consistent with Scheme I. Since the substrate contains only deuterium, the C-5′ hydrogen atoms initially present in coenzyme B_{12} are probably replaced rapidly by deuterium to form [^2H,^3H]coenzyme B_{12}. The intermediate of reaction sequence I would contain two deuterium atoms and a tritium atom, any of which could be transferred to product. In Scheme I, the probability of transfer of any particular hydrogen atom, H_a, in any turnover is given by eq 3, in which k_a, k_b, and k_c are the rate constants for

(23) E. S. Lewis and J. K. Robinson, *J. Amer. Chem. Soc.*, 90, 4337 (1968).

$$\gamma_a = \frac{k_a}{k_b + k_a + k_c} \quad (3)$$

transfer of H_a, H_b, and H_c, respectively, from the intermediate to product; γ_b and γ_c are defined by similar expressions. One of the factors which determines the magnitude of each rate constant is the rate of dissociation of the hydrogen–coenzyme bond. Substitution of tritium or deuterium for hydrogen would affect the rate constants: $k_H > k_D > k_T$ (H, D, and T represent the isotopes of hydrogen). For reaction of deuterated substrate with [^2H,^3H]coenzyme B_{12}, probability of transfer of tritium would be

$$\gamma_T = \frac{k_T}{k_T + k_D + k'_D} \quad (4)$$

and the 18-fold enhancement of γ_T by deuterated substrate would be ascribed to the presence of two deuterium atoms in the intermediate. If only changes in zero point energies are involved in the observed isotope effects, then the isotope effect upon tritium transfer, when a deuterated coenzyme B_{12} is used, should be predictable from the relation $\log k_H/k_T = 1.44 \log k_H/k_D$.[24] Assuming that the three hydrogens are chemically equivalent, the tritium isotope effect for the transfer of tritium from coenzyme B_{12} to product is approximately 125, and one can calculate that k_H/k_D is about 28. The probability of tritium transfer to product during reaction of deuterated substrate, calculated from 4, would be $\gamma = {}^1/_{10}$, 25-fold greater than for nondeuterated substrate. This value is to be compared with the observed 18-fold increase in the probability of tritium transfer.

The data in Table IV show that the presence of deuterium in a substrate molecule enhances the probability of tritium transfer from [^3H]coenzyme B_{12} to the product derived from that substrate. This enhancement is predictable from Scheme I. According to that mechanism the intermediate derived from a deuterated substrate has the structure I and that from the undeuterated substrate has structure II. It is apparent that there is a higher probability of tritium transfer from I than from II. From eq 3, assuming the same isotope effects as before ($k_H:k_D:k_T = 125:4.5:1$), one would expect that the probability of tritium transfer to the product from [^3H]coenzyme B_{12} should be enhanced about twofold by introduction of a deuterium from the substrate. The observed enhancement was four–sevenfold. This higher probability of tritium transfer is attributed to the following. When a deuterated substrate is used, some deuterium will be introduced into coenzyme B_{12} so that some fraction of the intermediates formed from the deuterated substrate will be III and from the nondeuterated substrate will be IV. For these intermediates, it can be calculated from eq 3 that the probability of tritium transfer to the product derived from the deuterated substrate should be 13 times that from the nondeuterated substrate. One would therefore expect the experimental results to fall between 2 and 13, which is the case. Furthermore, the probability of tritium transfer from [^3H]coenzyme B_{12} to the product derived from the deuterated substrate should increase as the fraction of deuterated substrate in the reaction mixture increases, causing a concomitant increase in the concentration of coenzyme B_{12} with composition

$$X_T^D$$

The results in Table IV show that this is the case. These results are therefore in agreement with Scheme I.

Several alternative mechanisms consistent with previous isotope exchange experiments can be considered. A concerted mechanism in which a hydrogen is transferred to coenzyme B_{12} concomitant with a hydrogen transfer from the coenzyme to the product can be represented by Scheme II. The symbols used are as before.

Scheme II. Reaction Sequence II (Concerted)

$$SH_a + X^{H_b}_{H_c} \longrightarrow PH_b + X^{H_c}_{H_a} \quad \text{(first turnover)}$$

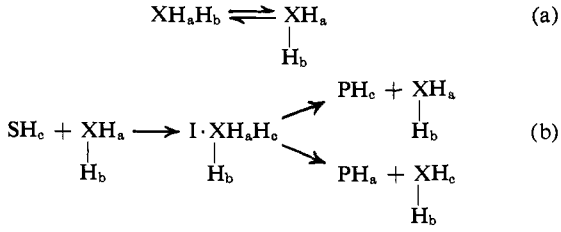
$$SH_d + X^{H_c}_{H_a} \longrightarrow PH_c + X^{H_a}_{H_d} \quad \text{(second turnover)}$$

Scheme II is a concerted process in which no intermediate containing three hydrogens exists. It is likely that such a process would be stereospecific for one of the two C-5′ hydrogens. Since the experimental data require that both C-5′ hydrogens participate, we have assumed that the displacement proceeds with inversion at the C-5′ carbon so that in two turnovers both C-5′ hydrogens of the coenzyme could be transferred to the reaction product. This mechanism is not consistent with the previous observations that in a single turnover intramolecular hydrogen transfer can occur and that tritium exchange occurs between enzyme–[^3H]coenzyme B_{12} and propionaldehyde under conditions where the overall reaction is not detectably reversible.[1] It is also difficult to see how the presence of deuterium in the substrate could enhance the probability of tritium transfer to the product. A mechanism similar to that above was considered for methylmalonyl CoA isomerase and rejected by experiments based on a different method of approach than those described here.[5] Thus, a considerable body of evidence now exists against the concerted mechanism.

Another possibility is a mechanism in which only one of the C-5′ hydrogens is subject to transfer to the product in any single turnover. This could occur if the enzyme-bound coenzyme B_{12} is in equilibrium with a form in which one of the C-5′ hydrogens is transferred to some other position in the complex (Scheme III, reaction a, below). This activated form of coenzyme B_{12} would then interact with the substrate to form an intermediate complex containing the remaining C-5′ hydrogen and a hydrogen contributed by the substrate (reaction b). When a deuterated substrate reacts with acti-

(24) C. G. Swain, E. C. Stivers, J. F. Reuwer, Jr., and L. J. Schaad, *J. Amer. Chem. Soc.*, 80, 5885 (1958).

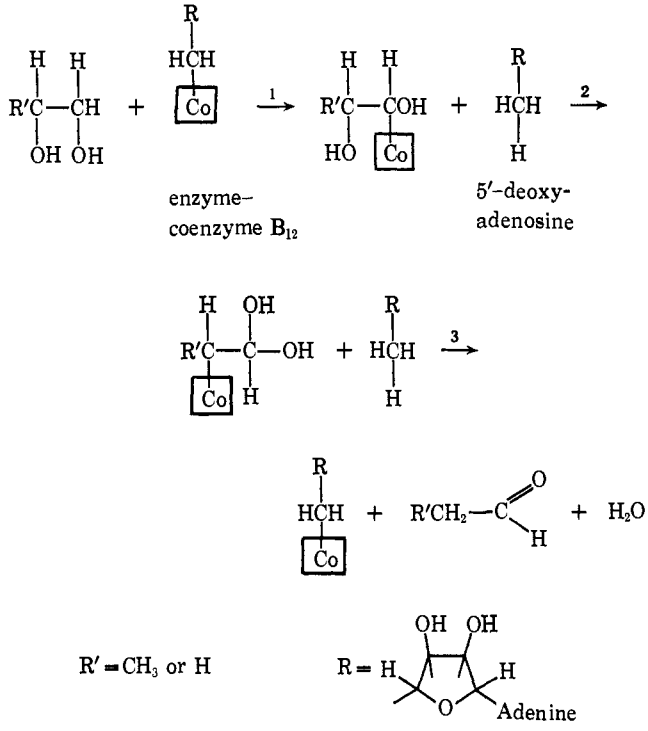
Scheme III. Reaction Sequence III



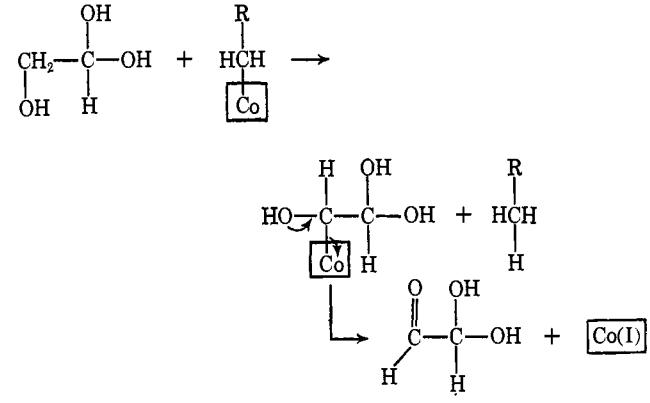
vated enzyme-[^3H]coenzyme B_{12} containing tritium on C-5′, the atoms available for transfer from the intermediate would be one deuterium and one tritium. There would be one transferrable deuterium in this intermediate regardless of the fraction of the substrate containing deuterium. Therefore, the fraction of deuterated substrate could not influence the probability of tritium transfer to product. The data in Table IV show that the probability of tritium transfer due to deuterium in the substrate increases as the relative amount of deuterated substrate in the mixture is increased. Therefore this mechanism appears to be inconsistent with the experimental results. A similar conclusion was reached by a different approach for methylmalonyl coenzyme A isomerase.[5]

Finally, it has been proposed that the conversion of the diols to aldehydes occurs by a 1,2-hydride shift, and that the resulting enzyme-bound aldehyde can undergo tritium exchange with an enzyme-activated coenzyme B_{12}.[25] The data in Table III show that the specific activity of the coenzyme during the course of the reaction is about 19 times that of the substrate and 280–2700 times that of the reaction product; therefore, tritium cannot be introduced through equilibration with substrate or product. This argument against the mechanism has been presented in greater detail elsewhere.[26]

Scheme IV



Evidence obtained with dioldehydrase[1,6] and by others with methylmalonyl coenzyme A isomerase[5] requires an intermediate which contains three equivalent hydrogens, the two C-5′ hydrogens and a hydrogen derived from the substrate. We tentatively propose the following reaction sequence which we believe to be consistent with all known experimental facts (Scheme IV). The first step involves an alkyl–metal exchange reaction; it is not necessarily a one-step reaction, and it is very likely that the enzyme plays an important part in activating the coenzyme and the substrate for this exchange. The activation may involve distortion of the corrin ring and reversible dissociation of one nitrogen ligand from cobalt, forming a coordinatively unsaturated complex to facilitate this exchange. The second step shown above is a rearrangement, in which the cobalt moves from C-1 of the substrate to C-2, and the 2-hydroxyl group moves to C-1. The final product is formed by an alkyl–cobalt exchange similar to the initial reaction. This mechanism meets the requirement for an intermediate in which any of the three hydrogens (two C-5′ hydrogens and a substrate hydrogen) can be transferred to product. A similar mechanism involving metal–ligand exchange reactions has been proposed for methylmalonyl coenzyme A isomerization.[5] The proposed reaction sequence also provides an explanation for the reaction of glycolaldehyde with enzyme–coenzyme B_{12}. This reaction leads to the formation of an inactive complex, which, upon dissociation, gives glyoxal and 5′-deoxyadenosine. 5′-Deoxyadenosine is a postulated intermediate in the reaction scheme. Furthermore, if glycolaldehyde were to react according to the proposed reaction sequence, an intermediate would be formed which could give rise to glyoxal



An additional advantage of the proposed mechanism is that it is isoenergetic, *i.e.*, a carbon–cobalt bond is preserved throughout the reaction sequence. The first and third steps involve very similar transformations, namely, the exchange of a C–H bond between substrate (or product) and the C-5′–Co bond of the coenzyme. Therefore, the very different tritium isotope effects for these steps (k_H/k_T is 20 for step 1 and approximately 125 for step 3) are noteworthy and may indicate that the actual mechanism involves more steps than are shown in Scheme IV.

At this time no obviously relevant chemical model systems are available for the proposed ligand exchange or the subsequent rearrangement, although the conversion of β-cyanoethylcobaloxime to α-cyanoethylcobaloxime[27] may provide a model for the rearrangement.

(25) G. N. Schrauzer and J. W. Sibert, *J. Amer. Chem. Soc.*, 92, 1022 (1970).

(26) P. A. Frey, M. K. Essenberg, S. S. Kerwar, and R. H. Abeles, *ibid.*, 92, 4488 (1970).

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We wish to emphasize that an enzymatic reaction is not meaningfully defined until it can be related to known nonenzymatic reactions and this cannot yet be done for reactions involving cobalamins. For these reactions it may well be that the relevant nonenzymatic chemistry has not as yet been discovered.

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Communications to the Editor

Electrophilic Reactions at Single Bonds. III.[1] Hydrogen–Deuterium Exchange and Protolysis (Deuterolysis) of Alkanes with Superacids. The Mechanism of Acid-Catalyzed Hydrocarbon Transformation Reactions Involving the σ Electron Pair Donor Ability of Single Bonds (Shared Electron Pairs) *via* Three-Center Bond Formation[2]

Sir:

The reactivity of olefins, acetylenes, and aromatic hydrocarbons toward electrophiles is based on the π-electron donor ability of the unsaturated C=C or C≡C bonds and π-aromatic systems. Unshared electron pair donor (n donor) heteroatom compounds represent the other major type of substrates in electrophilic reactions.[3] Contrary to frequent textbook references to electrophilic aliphatic substitution, authenticated examples are restricted to reactions involving organometallic compounds like organomercurials. No "pure" electrophilic substitutions of alkanes have been reported[4] with the exception of the recently observed hydrogen–deuterium exchange and protolytic cleavage reactions in FSO_3H–SbF_5, HF–SbF_5, and related[1,5,6] superacid media.

We now present evidence, detailed also in the accompanying two communications, for the *general electrophilic reactivity of covalent C–H and C–C single bonds of alkanes (cycloalkanes). This reactivity is due to what we consider the third major type of electron donor ability, i.e., the σ-donor ability (σ basicity) of shared electron pairs (single bonds) via two-electron, three-center bond formation.* It is our observation that C–C and C–H single bonds of all types (*i.e.*, tertiary, secondary, and primary) show substantial general reactivity in electrophilic reactions such as protolytic processes (isomerization, hydrogen–deuterium exchange, protolysis), alkylation, nitration, and halogenation. These observations promise to open up a new area of chemistry wherein alkanes and cycloalkanes are used as substrates in a wide variety of electrophilic reactions. Saturated single bonds, in general, can undergo electrophilic reactions.

The acid-catalyzed transformation reactions of saturated hydrocarbons (fragmentation, alkylation, isomerization) are generally considered to be carbocation reactions involving trivalent carbenium ions.[7] Whitmore[8] and subsequently Bartlett, Condon, and Schneider[9a] as well Schmerling[9b] in landmark publications developed the mechanistic concepts of acid-catalyzed alkane alkylations, isomerizations, and fragmentations. The major steps in the mechanism are the intermolecular transfer of hydride ion from a tertiary position in an isoparaffin to a carbenium ion and the ease of shift of a hydrogen atom or alkyl group from one carbon atom to another.

The key, we suggest, to the understanding of the hydrogen–deuterium exchange and protolytic reactions of alkanes is the realization (based on products obtained and theoretical calculations) that the *protolytic attack takes place on the C–H or C–C bonds where the major part of the electron density in covalent single bonds resides and not on the hydrogen or carbon atoms themselves.* The transition states of the reactions consequently are of three-center bound pentacoordinated carbonium ion nature.

It should be pointed out that the possibility of a triangular transition state was first suggested in a review article by Lewis and Symons[10] in 1958. It received relatively little interest until 1967–1968 when, based on chemical evidence and semiempirical self-consistent field calculations,[1,11,12] we concluded that protonation

(1) Parts I and II, respectively, are considered: G. A. Olah and R. H. Schlosberg, *J. Amer. Chem. Soc.*, 90, 2126 (1968), and G. A. Olah, G. Klopman, and R. H. Schlosberg, *ibid.*, 91, 3261 (1969).

(2) Presented in part at the Thirteenth Conference on Reaction Mechanisms, Division of Organic Chemistry of the American Chemical Society, University of California, Santa Cruz, Calif., June 1970, and at the Symposium on The Transition State of the French Physico Chemical Society, Paris, Sept 1970.

(3) G. N. Lewis, *J. Amer. Chem. Soc.*, **38**, 762 (1916); G. N. Lewis, "Valence and the Structure of Atoms and Molecules," Chemical Catalog Corp., New York, N. Y., 1923.

(4) F. R. Jensen and B. Rickborn, "Electrophilic Substitution of Organomercurials," McGraw-Hill, New York, N. Y., 1968.

(5) G. A. Olah and J. Lukas, *J. Amer. Chem. Soc.*, **89**, 2227, 4739 (1967); **90**, 933 (1968).

(6) H. Hogeveen and A. F. Bickel, *Chem. Commun.*, 635 (1967); H. Hogeveen, C. J. Gaasbeek, and A. F. Bickel, *Recl. Trav. Chim. Pays-Bas*, **88**, 703 (1969); H. Hogeveen and C. J. Gaasbeek, *ibid.*, **87**, 319 (1968).

(7) For a suggestion to name all cations of carbon compounds as carbocations and to differentiate trivalent carbenium ions as distinct from penta- or tetracoordinated carbonium ions, see G. A. Olah, *ibid.*, in press.

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