Studies on the Mechanism of Hydrogen Transfer in the
Cobamide Coenzyme-dependent Dioldehydrase Reaction*

(Received for publication, June 19, 1967)

PERRY A. FREY,‡ MARGARET KOTTKE ESSENBERG,§ and ROBERT H. ABELES¶
From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154

SUMMARY

When dl-1,2-propanediol-1-3H is converted to propionaldehyde in the presence of dioldehydrase and cobamide coenzyme, tritium is transferred to the coenzyme. The tritiated coenzyme so obtained transfers tritium to the reaction product when reacted with dl-1,2-propanediol and apoenzyme. The coenzyme is tritiated exclusively at the C-5' position of the adenosyl moiety. The location of tritium was established by chemical degradation and further confirmed by showing that chemically synthesized cobamide coenzyme, containing tritium at the C-5' position, transferred tritium to the product when added to enzyme and unlabeled substrate. The conversion of dl-1,2-propanediol-1-3H to propionaldehyde proceeds with inter- and intramolecular tritium transfer. Approximately 1% of the tritiated substrate reacts by intramolecular transfer, i.e. the hydrogen abstracted from C-1 of a substrate molecule is found in the α position of the aldehyde derived from that molecule. A partial reaction occurs, as evidenced by tritium exchange between tritiated coenzyme and propionaldehyde or acetaldheyde under conditions in which no net reaction occurs. The results obtained have led to the following tentative reaction sequence. Hydrogen is abstracted from C-1 of dl-1,2-propanediol and transferred to the coenzyme, where it becomes equivalent with at least one, but probably both, hydrogens of the C-5' position. This results in the formation of a reduced form of the coenzyme and a molecule derived through the oxidation of the substrate. In a subsequent step the hydrated form of propionaldehyde is formed by a transfer of hydrogen from the reduced coenzyme to the intermediate derived from the substrate.

Dioldehydrase (dl-1,2-propanediol hydro-lyase, EC 4.2.1.28) requires a cobamide coenzyme and catalyzes the intramolecular dismutations of D- and L-1,2-propanediol to propionaldehyde and of ethylene glycol to acetaldehyde (1). Other rearrangements which require a cobamide coenzyme are: glutamate mutase, methylmalonyl-coenzyme A mutase, glycerol dehydrase, and ethanolamine deaminase (2, 3). These reactions have a common feature, in that they involve the exchange of a hydrogen atom and some other group —X between adjacent carbon atoms of the substrates. This is shown in the following scheme.

\[
\begin{align*}
\text{C-C} \quad \text{C-C} \\
\text{H} \quad \text{X} \\
\text{H} \quad \text{X}
\end{align*}
\]

\[X = -\text{CH-COOH}, -\text{COSCoA}, -\text{OH, -NH}_2^+\text{NH}_2^+\]

The hydrogen migrations in these reactions occur without exchange with solvent hydrogen (4-6). The cobamide coenzyme-dependent ribonucleotide reductase catalyzes the reduction of ribonucleoside triphosphates to the corresponding deoxyribonucleotides (7, 8). This reaction is similar to the dioldehydrase reaction, in that both involve the displacement of a hydroxyl group by hydrogen, but it differs in that a second substrate, usually reduced lipoyl acid, serves as the hydrogen donor.

We have previously reported that the hydrogen transfer in the dioldehydrase reaction does not occur exclusively by an intramolecular 1,2-hydride shift. Thus, when mixtures of dl-1,2-propanediol-1-3H and unlabeled ethylene glycol are converted to products, acetaldehyde-2-3H is isolated (9). On the basis of these data we advanced the hypothesis that the enzyme-coenzyme complex functions as a hydrogen transfer agent in these reactions. Hydrogen, as H⁺, H⁻ or H₂, is transferred from the substrate to the enzyme-coenzyme complex and an equivalent hydrogen from the complex is transferred to the product. According to this hypothesis, the use of substrate containing tritium at C-1 should lead to tritium introduction into the coenzyme. The experiments reported here confirm this expectation. Additional properties of the hydrogen transfer reaction are described which confirm the hypothesis that the coenzyme functions as a hydrogen transfer agent.

* Publication No. 000 from the Graduate Department of Biochemistry. This work was supported in part by Public Health Service Grant 12633 (to R. H. Abeles) from the National Institute of General Medical Sciences. A preliminary report of portions of this work has appeared (Frey, P. A., and Abeles, R. H., J. Biol. Chem., 241, 2732 (1966)).

‡ National Institute of General Medical Sciences Predoctoral Fellow, 5-Fl-GM-20, 226-03, 1966-67. Taken in part from a dissertation submitted by P. A. Frey to the Graduate Department of Biochemistry, Brandeis University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.


¶ To whom inquiries regarding this paper should be addressed.
Enzyme, Coenzyme, and Substrates—Diolehydrase was isolated from Aerobacter aerogenes as previously described (1). In certain experiments, in which substrate-free enzyme was required, it was necessary to remove dl-1,2-propanediol which is present in the purified enzyme as a stabilizing agent. In these experiments diolehydrase was dialyzed for 2 hours against 0.01 M K₂HPO₄ at 0-5°C, treated with 1 μg of DBC coenzyme per 200 units of enzyme at 37°C for 10 min, and then dialyzed an additional 2 hours against 0.01 M K₂HPO₄ in the cold.

Crystalline DBC coenzyme was prepared by the procedure of Pailes and Hogenkamp (10). Commercial dl-1,2-propanediol, ethylene glycol, propionaldehyde, and acetaldehyde were redistilled before use.

Chromatographic Systems—Whatman No. 1 or No. 3MM filter paper and the following solvent systems were used for paper chromatography of DBC coenzyme and adenine nucleosides: A, H₂O-saturated 2-butanol; B, 1-butanol-acetic acid-water-2-propanol (100:1:99:70); C, 2-butanol-ammonium hydroxide-water (100:36:14); D, 1-butanol-water (172:28); E, 1-butanol-acetic acid-water (4:1:5, organic layer); and F, 1-butanol-ethanol-water (10:3:7). Paper electrophoresis was carried out on Whatman No. 3MM filter paper at 10 volts per cm with 0.5 M NH₃ as the electrolyte.

The 2,4-dinitrophenylhydrazone derivatives of propionaldehyde, acetaldehyde, and formaldehyde were separated by chromatography on Whatman No. 1 filter paper which was impregnated with N,N-dimethylformamide.¹ The paper was treated by dipping in acetone-N,N-dimethylformamide (1:1) and then drying in the air for 10 min. Chloroform solutions of the hydrazones were quickly applied to the paper, and development of the chromatograms was begun immediately. The developing solvents were cyclohexane or decalin, both of which were isolated in the degradation experiments, were assayed by paper chromatography of DBC coenzyme and adenine nucleosides: A, H₂-saturated 2-butanol; B, 1-butanol-acetic acid-water-2-propanol (100:1:99:70); C, 2-butanol-ammonium hydroxide-water (100:36:14); D, 1-butanol-water (172:28); E, 1-butanol-acetic acid-water (4:1:5, organic layer); and F, 1-butanol-ethanol-water (10:3:7).

Assays—Diolehydrase was assayed as previously described (1) and the same activity units were used. DBC coenzyme was assayed enzymatically with diolehydrase (11). Carrier DBC coenzyme used in the degradation experiments was assayed enzymatically with diolehydrase (11). The activity units were used. DBC coenzyme was assayed spectrophotometrically, by converting a diluted aliquot to acetate for measurement of their absorbance at 345 μ. The extinction coefficient, 19 × 10³ M⁻¹, was determined with the use of standard solutions of 2,4-dinitrophenylhydrazones. 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 in 1 liter of dioxane solution. The Anisotron 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.

Enzymatic Preparation of DBC-3H—This reaction and all the operations connected with reisolation of DBC-3H were carried out in a dark room. Prepurified nitrogen was freed of oxygen by passing it through a column containing copper deposited on diatomaceous earth (19). The reaction vessel used had three chambers. Chamber A contained diolehydrase, chamber B contained DBC coenzyme, and chamber C contained dl-1,2-propanediol-1-H. The solutions in the three chambers were carefully deaerated by bubbling oxygen-free nitrogen through them individually for 40 min with the use of small bore polyethylene tubes inserted through the stopcock and into each chamber. During all subsequent operations, care was taken to keep nitrogen gas bubbling through all the solutions at all times. The E·DBC complex was formed by tipping the enzyme from chamber A into chamber B. After 2 min, chambers A and B were immersed in an ice-water bath for 5 min. The substrate in chamber C was then tipped into chamber A together with the E·DBC complex from chamber B. During the reaction 0.7 ml of 10% TCA was introduced into chamber C by injection through the polyethylene tube used for deaeration, and was then deaerated for the remainder of the incubation period. After 8 min the reaction was stopped and the coenzyme was dissociated from the protein by tipping the TCA from chamber C into the reaction mixture. The mixture was maintained anaerobically for 5 min and then opened to the atmosphere. The precipitated protein was removed by centrifugation and the clear yellow supernatant fluid was decanted.

Radioactive DBC coenzyme was isolated by adjusting the supernatant fluid to pH 3.1 with 1 N NaOH, concentrating it to a volume of about 2.5 ml with a rotary evaporator, and passing it through a column (3 × 50 mm) of Dowex 50-X2 resin previously equilibrated with 2 M NaH₂PO₄·H₂PO₄ buffer at pH 3.0. DBC coenzyme was adsorbed as a sharp orange band at the top of the resin bed. After washing the column with 5 ml of water, the DBC coenzyme was eluted with 5 ml of 0.1 M sodium acetate buffer at pH 6.4. The coenzyme was desalted by extracting the eluate once with 1 ml of liquified phenol and then four times with 0.5 ml of phenol. The combined phenol layers were washed three times with 2 ml of water, and the combined water layers were back-extracted with 0.5 ml of phenol. DBC coenzyme with displaced back into water by diluting the phenol extract with 3 volumes of diethyl ether and 1 volume of acetone and extracting five times with 1 ml of water. The combined aqueous extracts were washed three times with 2 ml of ether to remove residual phenol.

The combined aqueous extracts were washed three times with 2 ml of ether to remove residual phenol. DBC coenzyme was purified from this solution by paper electrophoresis and paper chromatography using Solvent Systems A and B.

Synthetic Preparation of DBC-3H—DBC coenzyme containing

¹ The abbreviations used are: DBC coenzyme, 5,6-dimethyl-benzimidazolyloleobamide 5'-deoxyadenosine; DBC-3H, 5,6-dimethylbenzimidazolyloleobamide 5'-deoxyadenosine-5'-3H; E·DBC, the diolehydrase-DBC coenzyme complex; TCA, trichloroacetic acid; B₁₂, vitamin B₁₂ containing cobalt in the +1 oxidation state.

² B. Zagalak, personal communication.
troduced by the following sequence of reactions where DCC is dicyclohexylcarbodiimide and DMSO is dimethylsulfoxide.

\[
\begin{align*}
2',3'-\text{isopropylidenedenosine} & \xrightarrow{\text{DCC, DMSO}} \text{COCl} \rightarrow \\
2',3'-\text{isopropylidenedenosine-5'-aldehyde} & \xrightarrow{\text{NaBH}_4-3\text{H}} \\
2',3'-\text{isopropylidenedenosine-5'-H} & \xrightarrow{\text{TeCl}} \\
2',3'-\text{isopropylidene DBC-3H} & \xrightarrow{\text{H}_2\text{O}^+} \text{DBC-3H}
\end{align*}
\]

Two milliliters of anhydrous pyridine were added to the flask containing the thoroughly dried crude 2',3'-isopropylidenedenosine-5'-3H. Only part of the solid material dissolved, therefore the suspension was filtered by suction through a pyrex wool plug overlaid with a layer of anhydrous NaSO. The filtrate was transferred to a small flask fitted with a CaCl₂ drying tube and cooled in an ice-water bath. After adding 50 mg of p-toluenesulfonfyl chloride, which has been reprecipitated from petroleum ether (bp 40–60°), the solution was swirled gently for 5 min and then allowed to react at room temperature for 10 hours. The resulting brown solution was concentrated to dryness at reduced pressure on a rotary evaporator. The residue, dissolved in 5 ml of ice-cold chloroform, was partially purified by extracting twice with 10 ml of ice-cold 3 N H₂SO₄ once with 10 ml of water, and twice with 10 ml of a saturated solution of NaHCO₃ at room temperature. The pale yellow chloroform layer was filtered and evaporated to dryness. The residue was dissolved in 5 ml of ethanol and used directly for the alkylation of vitamin B₁₂.

DBC-3H was prepared from 2',3'-isopropylidene-5'-p-toluenesulfonfyladenosine-5'-3H and vitamin B₁₂, by general procedures previously described (10) with a few exceptions. Of greatest importance is the requirement that a considerable excess of B₁₂ should be used. Ordinarily, in this step the alkylation agent is present in excess. However, crude 2',3'-isopropylidene-5'-p-toluenesulfonfyladenosine-5'-3H was prepared as described above, is contaminated with impurities which consume B₁₂. Therefore, 350 mg of hydroxocobalamin reduced with NaBH₄ were used in the alkylation by the crude 2',3'-isopropylidene-5'-p-toluenesulfonfyladenosine-5'-3H. Isopropylidene-DBC-3H was isolated by phenol extraction and passage through a column (22 × 260 mm) of carboxymethyl cellulose in the acid form. The product was eluted with water as a prominent orange band, while unreacted vitamin B₁₂ remained on the column. Hydrolysis of isopropylidene-DBC-3H was effected by concentrating the eluate to 80 ml and combining it with 10 ml of 2 N HCl for 24 hours at room temperature (10). After neutralization of the hydrolysate, DBC-3H was purified by phenol extraction, paper electrophoresis, descending paper chromatography with Solvent System A and ascending paper chromatography with Solvent System B. The yield was 3.8 mg of pure material, as determined by both enzymatic and spectrophotometric assays, and the specific activity was 1.7 × 10⁷ cpm per μmole.

\[
\text{dl-1,2-Propanediol-1-H and Ethylene Glycol-H—These substrates were prepared by reduction of dl lactaldehyde and glycolaldehyde with NaBH}_4\text{-H. dl-Lactaldehyde was obtained by reduction of pyruvaldehyde according to a published procedure (16). dl-Lactaldehyde (36 mg) was dissolved in 2 ml of 95\% ethanol and reduced with 9.2 mg of NaBH}_4\text{-H (New England Nuclear, 200 mC per mmole) dissolved in 0.5 ml of ice-cold water. The sodium borohydride was added in 5-drop increments over a 15-min period with gentle stirring. After an additional 15 min, the reaction mixture was diluted with 5 ml of water and reduced to a volume of about 10 ml on a rotary evaporator. The resulting heterogeneous mixture was extracted three times with 5-ml volumes of chloroform. The combined chloroform extracts were washed once with 10 ml of water. The organic layer was transferred to a 50-ml round bottom flask and evaporated to dryness at reduced pressure by a rotary evaporator. The product was deposited on the sides of the flask as a glassy pale yellow residue. This crude product was not purified further; instead it was dried under reduced pressure over P₂O₅ for 24 hours and then used directly for the next reaction.}
\]

2 We wish to thank Dr. Moffatt for advice on the preparation of this compound.
The radiochemical purity of \(\text{dl-1,2-propanediol-1-}^3\text{H}\) was established by two criteria. When samples of this material were subjected to paper chromatography with the Solvent Systems E, G, and H, all of the detectable radioactivity was associated with the position of \(\text{dl-1,2-propanediol}\). In addition, when \(\text{dl-1,2-propanediol-1-}^3\text{H}\) containing a known amount of radioactivity was converted to 2-(\(p\)-nitrophenyl)-4-methyl-1,3-dioxolane by reaction with \(p\)-nitrobenzaldehyde (17), the crystalline derivative contained all of the radioactivity.

**Isolation of Products—**Propionaldehyde, acetaldehyde, and ethylene glycol were isolated from enzymatic reaction mixtures in several labeling experiments. Propionaldehyde was isolated as the 2,4-dinitrophenylhydrazone, propionaldomethene, and propionic acid. Acetaldehyde was isolated as acetic acid. The following procedures were used for the isolation of these materials.

**Propionaldehyde 2,4-Dinitrophenylhydrazone—**Two milliliters of 0.2\% 2,4-dinitrophenylhydrazine in 3 \(\times\) \(H_2SO_4\) were added directly to the 1-ml reaction mixture. After 15 min the precipitate was centrifuged and washed with water. The derivative was dissolved in benzene and passed through a column (0.5 \(\times\) 2 cm) of Fisher neutral adsorption alumina to remove excess 2,4-dinitrophenylhydrazine. The yellow effluent was evaporated to dryness, and propionaldehyde 2,4-dinitrophenylhydrazone was purified from the residue by paper chromatography.

**Propionaldomethene—**Propionaldomethene was prepared by lyophilizing the reaction mixtures and adding to the distillate 20 ml of a solution consisting of 1\% 5,5-dimethylcyclohexane-1,3-dione and 0.5\% \(Na_2CO_3\). The solution was adjusted to \(pH 3.9\) and refrigerated overnight. The precipitate was filtered, dried, and crystallized from ethanol-water. The melting point (155-156\°) and specific radioactivity remained unchanged after recrystallization.

**Propionic and Acetic Acids—**The reaction mixtures were adjusted to \(pH 5.5\) with 0.25 \(\times\) \(H_2SO_4\) and lyophilized. The aldehydes in the distillates were oxidized to the corresponding acids with \(KMnO_4\) (17), while maintaining at \(pH 5.5\) to 6.5 with 0.1 \(\times\) \(NaOH\). Propionic and acetic acids were separated and purified by chromatography on columns of silicic acid (18). The acids were quantitatively resolved by elution with 2\% 1-butanol in chloroform. The individual fractions from the columns were titrated with standard sodium hydroxide to determine their acid content. The acids were assayed radiochemically as their sodium salts, by counting aliquots of the aqueous layers resulting from the titrations.

**Ethylene Glycol—**The reaction mixture was lyophilized, and ethylene glycol in the distillate was purified by chromatography on Whatman No. 3MM filter paper with Solvent System E. After elution from the paper it was rechromatographed in the same system. The two paper chromatography steps largely separated \(\text{dl-1,2-propanediol-1-}^3\text{H}\) which was present, but a small amount remained. This was removed by partition chromatography of 7 to 10 \(\mu\)moles of glycol on a column (7 mm \(\times\) 20 cm) of Celite 535 (19). The elution schedule consisted of 30 ml of water-saturated ethyl acetate followed by 40 ml of water saturated 1-butanol-benzene (3:1). Both glycols were present in the butanol-benzene eluate, and they were quantitatively resolved.

**Degradation of Radioactive Compounds—**Samples of DBC-\(^3\)H were combined with carrier DBC coenzyme to a final specific radioactivity of 33,000 cpm per \(\mu\)mole. In both the anaerobic and aerobic experiments, DBC-\(^3\)H was photolyzed by exposure to bright sunlight for 4 hours. In the anaerobic photolyses, DBC-\(^3\)H was photolyzed at a concentration of 0.25 \(\mu\)mole per ml in a sealed vessel after bubbling oxygen-free nitrogen through the solutions for 30 min. \(5',8\)-Cycloadenosine-\(^3\)H was isolated from the photolyzed solutions by paper electrophoresis. The nucleoside was eluted from the paper and further purified and identified by paper chromatography by a sequence of Solvent Systems D, E, and F. After each chromatographic step, the nucleoside was eluted and its specific activity was determined by spectrophotometric and radiochemical assays. The remaining material was then rechromatographed using the next solvent system, and so on. The specific activity remained constant after elution from each of the chromatograms.

In the aerobic photolyses, DBC-\(^3\)H was photolyzed at a concentration of 0.0675 \(\mu\)moles per ml after bubbling oxygen through the solutions for 15 min. Adenosine-5'-aldehyde-\(^3\)H was isolated by ion exchange chromatography on columns of Dowex 50 and then desalted as described by Hogenkamp et al. (20). A portion of this material was reduced to adenosine-\(^3\)H which was purified. In the reduction 1.7 \(\mu\)moles of adenosine-5'-aldehyde-\(^3\)H in 1.5 ml of water were treated first with 0.1 ml of 1 \(\times\) \(KH_2PO_4\) and then with 0.2 ml of a 10 mg per ml solution of \(NaBH_4\) in an ice-water bath. The buffer and \(NaBH_4\) additions were repeated twice more at 2-min intervals. In this way the \(pH\) of the solution never exceeded 6.8 during the reduction. After desalting (20), adenosine-\(^3\)H was purified by paper chromatography with Solvent Systems D, E, and F as for \(5',8\)-cycloadenosine-\(^3\)H. A second portion of adenosine-5'-aldehyde-\(^3\)H was oxidized to adenosine-5'-carboxylic acid by sodium hypochlorite oxidation as described by Hogenkamp et al. (20). This compound was also purified by paper chromatography with Solvents D, E, and F. Adenosine-5'-carboxylic acid was identified on paper chromatograms by comparison with a sample of synthetic material prepared according to a published procedure (21).

A sample of \(\text{dl-1,2-propanediol-1-}^3\text{H}\) was diluted with 50 \(\mu\)moles of unlabeled \(\text{dl-1,2-propanediol}\) and oxidized to acetaldehyde and formaldehyde by treatment with an excess of sodium metaperiodate at 0-5° for 15 min. The solution was acidified with 1 drop of 2 \(\times\) \(H_2SO_4\), and the excess periodate was destroyed by adding 1 drop of 10\% \(KI\) and titrating the \(I_2\) with a 10\% solution of sodium arsenite. The solution was adjusted to \(pH 6.0\) with 1 \(\times\) \(NaOH\), and the aldehydes were isolated by lyophilization. The 2,4-dinitrophenylhydrazones of acetaldehyde and formaldehyde were precipitated by adding to the distillate 50 ml of 0.1\% 2,4-dinitrophenylhydrazine in 2 \(\times\) \(H_2SO_4\). The hydrazones were purified as described above for propionaldehyde-2,4-dinitrophenylhydrazone. The results of radiochemical and spectrophotometric analyses of these derivatives showed that less than 0.2\% of the total radioactivity is present in carbons 2 and 3 of \(\text{dl-1,2-propanediol-1-}^3\text{H}\).

**RESULTS**

**Enzymatic Transfer of Tritium from \(\text{dl-1,2-propanediol-1-}^3\text{H}\) to DBC Coenzyme—**When \(\text{dl-1,2-propanediol-1-}^3\text{H}\) is converted to propionaldehyde in the presence of dioldehydrase and DBC coenzyme, tritium is incorporated into the coenzyme. The detailed procedure used for the transfer of tritium from substrate to the coenzyme, dissociation of the enzyme-coenzyme complex, and reisolation of radioactive coenzyme, is described under...
“Materials and Methods.” Thirty-five micromoles of dl-1,2-propanediol-1-3H, containing 9.1 × 10^6 cpm per μmole, were incubated with 820 units of dioldehydrase and 100 μg of DBC coenzyme. Upon completion of the reaction, 31 μg of DBC-3H were reisolated, and this coenzyme contained 2.0 × 10^7 cpm per μmole. The radiochemical purity of DBC-3H was established by the fact that the specific activity remained constant through a sequence of two different paper chromatographic purifications (Solvents A and B). Confirmation of radiochemical purity is also obtained from the degradation experiments and from experiments which establish that all of the tritium of the coenzyme can be transferred to the reaction product.

**Enzymatic Transfer of Tritium from DBC-3H to Propionaldehyde**—The titrated DBC coenzyme obtained as described above and 5.6-dimethylbenzimidazolylcobamide 5'-deoxyadenosine-5'-3H, prepared chemically, were both tested for their ability to transfer radioactivity to propionaldehyde when incubated with dioldehydrase and unlabeled dl-1,2-propanediol. The data in Table I show that all of the radioactivity in both of these preparations is subject to this transfer. Suitable nonenzymatic control reactions were carried out, in which DBC-3H was incubated with buffer and propionaldehyde under the conditions of the enzymatic reactions. When propionaldehyde 2,4-dinitrophenylhydrazone was isolated, less than 5% of the radioactivity was present, and the derivative could not be purified to constant specific activity by the procedures used. Propionalidomethone was isolated from a similar nonenzymatic control and it contained no radioactivity. In an additional control experiment, propionaldehyde was generated from dl-1,2-propanediol with dioldehydrase and unlabeled DBC-coenzyme in the presence of photolyzed DBC-H. The resulting propionaldehyde was oxidized to propionic acid, which was purified by silicic acid chromatography and found to contain no radioactivity. Therefore, the transfer of radioactivity from DBC-3H to propionaldehyde is strictly dependent upon the presence of the active dioldehydrase-DBC-3H complex. The data in Table I show that only about 80% of the radioactivity present in the DBC-3H prepared either synthetically or enzymatically was isolated as propionaldehyde. Since essentially all of the radioactivity from synthetic DBC-3H was isolated in other propionaldehyde derivatives, we interpret this result to mean that some loss of radioactivity by exchange occurs when propionaldehyde is isolated as the dimethylone derivative under the conditions of Table I.

These results suggest that DBC coenzyme functions as a hydrogen transfer agent, and that the site involved in this transfer is C-5' of the 5'-deoxyadenosine moiety. The data in Table I also show that essentially all of the tritium of the synthetically prepared DBC-3H is subject to transfer. This is surprising since the C-5' hydrogens of the coenzyme are not sterically equivalent. The results therefore suggest that at some point during the reaction the carbon-cobalt bond is modified in such a way that the hydrogen atoms at the C-5' position become equivalent. The alternative possibility that the reduction with NaBH₄-3H showed considerable steric selectivity and that consequently the synthetically prepared DBC-3H is asymmetrically labeled must also be considered.

**Degradation of DBC-3H and Location of Tritium**—In order to locate the tritium in the enzymatic preparation of DBC-3H and to confirm the position of tritium at C-5' of the synthetically prepared coenzyme, we undertook the chemical degradation of these materials to known compounds. Fig. 1 shows the degradation scheme used and the radiochemical results obtained. The compounds isolated were 5',8-cycloadenosine, adenosine, and adenosine-5'-carboxylic acid. These compounds were identified by their rates of migration on paper chromatograms. Their specific radioactivities were determined after elution from the same chromatograms. The data in Table II show the behavior of these compounds in the chromatographic systems used.

DBC-3H was photolyzed under anaerobic conditions. The nucleoside product resulting from anaerobic photolysis has been tentatively identified as 5',8-cycloadenosine (22). This nucleoside was isolated after aerobic photolysis of both preparations of DBC-3H, and in both cases its specific activity was the same as that of DBC-3H. Therefore, all of the radioactivity in DBC-3H prepared either synthetically or enzymatically must be in the 5'-deoxyadenosine moiety.

DBC-3H was then photolyzed under aerobic conditions. The principal nucleoside product resulting from aerobic photolysis has been identified as adenosine-5'-aldehyde (20). After aerobic photolysis of both preparations of DBC-3H, this nucleoside was separated from hydroxycobalamin by ion exchange chromatography on columns of Dowex 50 as described by Hogenkamp et al. (20). Adenosine 5'-aldehyde obtained in this way was not radiochemically pure and could not be satisfactorily purified by chromatography on paper. Therefore, in order to determine its radioactivity content, part of it was reduced with sodium borohydride, and the adenosine formed was isolated by paper chromatography. The specific activity of the adenosine was 55% of that of DBC-3H in one case and 65% in the other, indicating that tritium had been lost during photolysis. Tritium would be liberated to water during aerobic photolysis if the hydrogen atoms at C-5' were labeled. The remaining adenosine-5'-aldehyde-3H was oxidized to adenosine-5'-carboxylic acid with sodium hypochlorite as described by Hogenkamp et al. (20). After purification by paper chromatography, adenosine-5'-car-

<table>
<thead>
<tr>
<th>Source of DBC-3H</th>
<th>Total radioactivity in DBC-3H (cpm)</th>
<th>Total radioactivity in propionaldehyde isolated as (cpm)</th>
<th>2,4-Dinitrophenylhydrazone</th>
<th>Pronic acid</th>
<th>Pronionaldehyde-methone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic</td>
<td>71,900</td>
<td>69,800</td>
<td>29,300</td>
<td>27,400</td>
<td>31,500</td>
</tr>
<tr>
<td>Synthetic</td>
<td>30,300</td>
<td>20,300</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthetic*</td>
<td>34,400</td>
<td>27,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzymatic</td>
<td>38,500</td>
<td>31,500</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* In this experiment the substrate was dl-1,2-propanediol.
Mechanism of Hydrogen Transfer by Cobamide

Aerobic

\[
\text{Adenosine-5'-aldehyde}
\]

Anaerobic

\[
\text{Adenosine-5'-carboxylic Acid}
\]

**Fig. 1.** The degradation scheme used for identifying the position of labeling in DBC-H. The numbers indicate the experimentally determined specific radioactivities (cpm per pmole) of DBC-H and of the degradation products. The numbers with asterisks represent data obtained in the degradation of DBC-3H prepared synthetically.

**Table II**

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>( \text{R}_{\text{adenosine}} ) movement of nucleoside/movement of adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>0.50 0.85 0.87</td>
</tr>
<tr>
<td>5',8-Cycloadenosine</td>
<td>0.50 0.81 0.81</td>
</tr>
<tr>
<td>Adenosine-5'-carboxylic</td>
<td>0.05 0.50 0.43</td>
</tr>
</tbody>
</table>

boxylic acid contained less than 1% of the radioactivity originally present in DBC-H. To show that the loss of radioactivity from adenosine-5'-aldehyde was not due to exchange from C-4 caused by the alkaline conditions of the sodium hypiodite oxidation, adenosine-5'-aldehyde was exposed to the same alkaline conditions used in the oxidation, but in the absence of KI-I₂. The solution was then lyophilized, and no radioactivity could be detected in the water which was collected. The results of the degradation experiments show that tritium is located exclusively on C-5' of both preparations of DBC.

**Reversibility of Reaction**—The conversions of glycols to aldehydes catalyzed by dioldehydrase are irreversible within the limits of chemical analysis (1). To further test reversibility of the overall reaction, we incubated 101 pmoles of ethylene glycol, 104 pmoles of dl-1,2-propanediol-1-H containing \( 4.8 \times 10^4 \) cpm per pmole of 0.08 M potassium phosphate buffer, pH 8, in a total volume of 2.0 ml at 37°C for 10 min, added 1 ml of 2 N HCl to stop the reaction, and reisolated the unreacted ethylene glycol. After extensive purification by paper chromatography and partition chromatography on columns of Celite 535, the ethylene glycol contained no significant radioactivity. In a second experiment we incubated 101 pmoles of dl-1,2-propanediol, 0.4 pmole of propionaldehyde-1-14C containing \( 4 \times 10^4 \) cpm, 7 units of dioldehydrase, 6 \( \times 10^{-3} \) pmoles of DBC coenzyme, and 20 pmoles of potassium phosphate buffer, pH 8, in a total volume of 2.5 ml for 7 min at 37°C, stopped the reaction, and reisolated the unreacted dl-1,2-propanediol as above. After purification by paper chromatography, the dl-1,2-propanediol again contained no significant radioactivity. Therefore, no reversibility could be shown.

In contrast, however, to the overall reaction, the reversibility of a partial reaction between \( E \cdot \text{DBC} \) complex and propionaldehyde could be shown. Dioldehydrase (107 units) was incubated with DBC-3H (5.1 \( \times 10^{-3} \) pmoles, 3.32 \( \times 10^4 \) cpm), propionaldehyde (810 pmoles), and K₂HPO₄ (8 pmoles) in a total volume of 4 ml for 10 min at 37°C. The propionaldehyde was then reisolated and converted to propionald Methone. This derivative was found to have a specific radioactivity of 99 cpm per pmole, which corresponded to 92% of the radioactivity originally present in DBC-H. In a similar experiment, in which acetaldehyde was substituted for propionaldehyde, the acetaldehyde was oxidized to acetic acid and purified by chromatography on a column of silicic acid. Radiochemical assays showed that the purified acetic acid contained 40% of the radioactivity originally present in DBC-H. In a control reaction mixture, in which dioldehydrase was omitted, the reisolated propionaldehyde contained no detectable radioactivity. Therefore, we conclude that dioldehydrase catalyzes the exchange of hydrogen between DBC coenzyme and acetaldehyde or propionaldehyde.

This result raised the question of whether exchange of hydrogen between propionaldehyde and \( E \cdot \text{DBC} \) occurs as a result of reversal of part of the normal pathway for conversion of glycols to aldehydes. All of the previous data reported in this paper could be explained on the basis of this partial reaction, which could conceivably be distinct from the normal catalytic pathway. Therefore, further experiments were carried out to establish that...
this reaction is related to the dehydration of dl-1,2-propanediol.
The data obtained are presented in Table III. dl-1,2-Propanediol and propionaldehyde were each incubated separately
with E-DBC-\(^3\)H, and, at the end of the incubation period,
propionaldehyde was reduced with sodium borohydride (Experiments 1 and 2). The propionaldehyde was isolated by lyophilizing
the reaction mixtures and collecting the distillates.
Radiochemical assays showed that in both reaction mixtures
equal amounts of tritium were in propanol. The amount of tritium transferred from DBC-\(^3\)H to propanol is therefore
the same in the presence of propionaldehyde and dl-1,2-propanediol.
In Experiments 3 and 4, propionaldehyde and dl-1,2-propanediol
were incubated with E-DBC-\(^3\)H in the presence of sodium borohydride,
which served as a chemical trap to remove propionaldehyde
as it was formed. Sodium borohydride effectively trapped
propionaldehyde. The radioactivity in propanol isolated in Experiment 4, in which propionaldehyde was the substrate was
markedly less than in Experiment 3, in which the substrate was
dl-1,2-propanediol. The presence of sodium borohydride did not
appreciably inhibit the transfer of tritium from E-DBC-\(^3\)H to
propionaldehyde when the substrate was dl-1,2-propanediol.

In view of the fact that the overall reaction is not detectably
reversible, the occurrence of a partial reaction between E-DBC-\(^3\)H
and propionaldehyde suggests a step-wise mechanism, in which
an early step is practically irreversible.

### Table III

**Relative effectiveness of dl-1,2-propanediol and propionaldehyde as substrates for enzymatic transfer of tritium from DBC-\(^3\)H to propionaldehyde in presence and absence of sodium borohydride**

The reaction mixtures contained 30 units of dioldehydrase, 3.2
\(\mu\)g of DBC-\(^3\)H (3.98 \(\times\) \(10^4\) cpm), 11 amoles of \(\text{K}_2\text{HPO}_4\), 1 mg of \(\text{NaBH}_4\), and 1 amole of either dl-1,2-propanediol or propionaldehyde
in a total volume of 1.0 ml. All of the components of the reaction mixtures except substrate and NaBH\(_4\) were preincubated
for 2 min at 25\(^\circ\). In Experiments 1 and 2 the reactions were
started by addition of substrate in the absence of NaBH\(_4\). After
2 min of incubation, NaBH\(_4\) was added, and 1 min later the excess
NaBH\(_4\) was destroyed with 0.1 ml of 2 \(\text{N} \text{HCl}\). In Experiments 3
and 4, NaBH\(_4\) was added following the preincubation, and 30 sec
later the reactions were started by addition of substrates. After
2 min of incubation, the excess NaBH\(_4\) was destroyed with 0.1 ml of 2 \(\text{N} \text{HCl}\). Each of the reaction mixtures was combined with 0.1
ml of carrier 1-propanol, diluted to 2.0 ml, and lyophilized. The
amount of radioactivity in propionaldehyde was determined by counting a 1.0-ml aliquot of each of the distillates. The data are corrected
for the presence of 0.28 \(\times\) \(10^6\) cpm of volatile radioactivity found
in a control experiment in which no substrate was added. This
amount of radioactivity could be attributed to a small amount of
propionaldehyde or dl-1,2-propanediol present in the enzyme.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Substrate</th>
<th>Radioactivity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>dl-1,2-Propanediol(^a)</td>
<td>2.3 (\times) (10^4)</td>
</tr>
<tr>
<td>2</td>
<td>Propionaldehyde(^a)</td>
<td>2.4 (\times) (10^4)</td>
</tr>
<tr>
<td>3</td>
<td>dl-1,2-Propanediol</td>
<td>1.9 (\times) (10^4)</td>
</tr>
<tr>
<td>4</td>
<td>Propionaldehyde</td>
<td>0.30 (\times) (10^4)</td>
</tr>
</tbody>
</table>

\(^a\) In Experiments 1 and 2 the substrates were incubated with E-DBC-\(^3\)H complex for 2 min before adding sodium borohydride.

**Occurrence of Intramolecular Hydrogen Transfer**—It has been shown that the hydrogen which is abstracted from the C-1 posi-

The following mathematical treatment justifies this plot of the data. Concentration of dl-1,2-propanediol is \(C_1\), specific activity of dl-1,2-propanediol is \(S\); concentration of ethylene glycol is \(C_2\), fraction of total radioactivity transferred through intramolecular transfer is \(f_1\), and through intermolecular transfer is \(f_2\).

Total radioactivity in reaction product

\[
C_0Sf_1 + C_0S(1 - f_1)
\]

Total radioactivity in acetaldehyde

\[
C_0S(1 - f_2) \frac{kC_1}{kC_1 + C_0}
\]

\[
C_0S(1 - f_2) \frac{(C_2 + C_0)}{C_0}
\]

\(k\), a constant to convert actual concentration of \(C_1\) to effective concentration, which takes into account differences in \(K_m\). Specific radioactivity of acetaldehyde at \(C_2 \gg C_0\)
conversions occur in which the hydrogen which is abstracted from the dioldehydrase reaction.  $\Delta H$, substrate; $HA$, product.  a-H, hydroxyl group.  b-H, enzyme-bound coenzyme.  Hydrogen at positions a and b are the C-5' hydrogen atoms of the coenzyme.

The reaction sequence is consistent with all the known properties of diol dehydrogenase.  It accounts for tritium incorporation into the coenzyme from tritiated substrate as well as for tritium transfer from tritiated coenzyme to the reaction product.  As a result of the equivalence, or the potential equivalence, of the 3 hydrogens in Structure II (Fig. 3), it is possible in a single turnover of a tritiated substrate for either hydrogen or tritium to appear in the product, as is required by the experimental results.  Finally, tritium transfer from coenzyme to propionaldehyde or acetaldehyde without reversal of the overall reaction can be accounted for through steps 8, 6, 5, and 7.

The hydrogen transfer between substrate and coenzyme (I-11) occurs, an experiment was carried out in which a constant amount of $d\text{-}l$-1,2-propanediol-1-$\text{H}$ was converted to propionaldehyde in the presence of increasing concentrations of unlabeled ethylene glycol.  In such an experiment the following two possibilities will exist.  (a) If all hydrogen transfers are intramolecular, then the tritium originally present in the $d\text{-}l$-1,2-propanediol will be distributed only among propionaldehyde molecules and not among an infinite number of molecules.  Therefore, in this case, the specific activity of propionaldehyde will not approach zero as C-3-C-2 approaches zero, but that of acetaldehyde will.

Fig. 2 shows the results obtained in the experiment described above.  These results show that the specific activity of acetaldehyde approaches zero, but that of propionaldehyde does not.  It can be concluded that a certain fraction of the hydrogen transfers occur through an intramolecular process.  The specific activity of the propionaldehyde corresponds to the transfer of approximately 1% of the tritium atoms by an intramolecular process.  If it is assumed that all hydrogen transfer occurs via the coenzyme, then these results show that hydrogen from the C-5' position of the coenzyme is transferred to product approximately 100 times as fast as tritium which is introduced into the coenzyme by the substrate.

**DISCUSSION**

The results described in this paper have led us to postulate the tentative reaction sequence shown in Fig. 3.  This reaction sequence involves the formation of a Michaelis complex (I) between substrate (AH) and the enzyme-coenzyme complex, followed by a transfer of hydrogen from the substrate to the enzyme-bound coenzyme.

The hydrogen transfer results in the formation Complex II, which consists of an intermediate (A) derived from the substrate and a modified form of the coenzyme.

containing an additional hydrogen.  The 3 hydrogens may be equivalent or can become equivalent.  One of the 3 hydrogens of the modified coenzyme is then transferred to intermediate A to give the reaction product (HA).  When a tritiated substrate is employed, tritium will be introduced into the coenzyme (II).  In the subsequent step, either tritium or hydrogen can be transferred to A to form the reaction product.  The relative rates at which tritium and hydrogen are transferred will depend upon the following: (a) isotope effects; (b) the rate at which the 3 hydrogens of the intermediate form of the coenzyme become equivalent.  The relative importance of these two factors cannot be evaluated at this time.

The reaction sequence is consistent with all the known properties of diol dehydrogenase.  It accounts for tritium incorporation into the coenzyme from tritiated substrate as well as for tritium transfer from tritiated coenzyme to the reaction product.  As a result of the equivalence, or the potential equivalence, of the 3 hydrogens in Structure II (Fig. 3), it is possible in a single turnover of a tritiated substrate for either hydrogen or tritium to appear in the product, as is required by the experimental results.  Finally, tritium transfer from coenzyme to propionaldehyde or acetaldehyde without reversal of the overall reaction can be accounted for through steps 8, 6, 5, and 7.

The hydrogen transfer between substrate and coenzyme (I-11) occurs, an experiment was carried out in which a constant amount of $d\text{-}l$-1,2-propanediol-1-$\text{H}$ was converted to propionaldehyde in the presence of increasing concentrations of unlabeled ethylene glycol.  In such an experiment the following two possibilities will exist.  (a) If all hydrogen transfers are intramolecular, then the tritium originally present in the $d\text{-}l$-1,2-propanediol will be distributed only among propionaldehyde molecules and not among an infinite number of molecules.  Therefore, in this case, the specific activity of propionaldehyde will not approach zero as C-3-C-2 approaches zero, but that of acetaldehyde will.

Fig. 2 shows the results obtained in the experiment described above.  These results show that the specific activity of acetaldehyde approaches zero, but that of propionaldehyde does not.  It can be concluded that a certain fraction of the hydrogen transfers occur through an intramolecular process.  The specific activity of the propionaldehyde corresponds to the transfer of approximately 1% of the tritium atoms by an intramolecular process.  If it is assumed that all hydrogen transfer occurs via the coenzyme, then these results show that hydrogen from the C-5' position of the coenzyme is transferred to product approximately 100 times as fast as tritium which is introduced into the coenzyme by the substrate.

**DISCUSSION**

The results described in this paper have led us to postulate the tentative reaction sequence shown in Fig. 3.  This reaction sequence involves the formation of a Michaelis complex (I) between substrate (AH) and the enzyme-coenzyme complex, followed by a transfer of hydrogen from the substrate to the enzyme-bound coenzyme.

containing an additional hydrogen.  The 3 hydrogens may be equivalent or can become equivalent.  One of the 3 hydrogens of the modified coenzyme is then transferred to intermediate A to give the reaction product (HA).  When a tritiated substrate is employed, tritium will be introduced into the coenzyme (II).  In the subsequent step, either tritium or hydrogen can be transferred to A to form the reaction product.  The relative rates at which tritium and hydrogen are transferred will depend upon the following: (a) isotope effects; (b) the rate at which the 3 hydrogens of the intermediate form of the coenzyme become equivalent.  The relative importance of these two factors cannot be evaluated at this time.

The reaction sequence is consistent with all the known properties of diol dehydrogenase.  It accounts for tritium incorporation into the coenzyme from tritiated substrate as well as for tritium transfer from tritiated coenzyme to the reaction product.  As a result of the equivalence, or the potential equivalence, of the 3 hydrogens in Structure II (Fig. 3), it is possible in a single turnover of a tritiated substrate for either hydrogen or tritium to appear in the product, as is required by the experimental results.  Finally, tritium transfer from coenzyme to propionaldehyde or acetaldehyde without reversal of the overall reaction can be accounted for through steps 8, 6, 5, and 7.
could be an oxidative process or a proton transfer. Currently available evidence suggests an oxidative process, i.e. a process in which the coenzyme functions as an oxidizing agent. A stoichiometric reaction between E-DBC and glycolaldehyde has been described in which glycolaldehyde is oxidized to glyoxal and E-DBC functions as an oxidizing agent (23). These results established that the enzyme-coenzyme complex can be reduced under the conditions of the enzymatic reaction. Further support for an oxidative process is provided by the elegant work of Retey et al. (24) which shows that oxygen migrates from C-2 of the substrate to C-1 of the product. The following reaction sequence was proposed:

\[
\begin{align*}
\text{CH}_2-\text{CH}_2-\text{CH}_2 & \rightarrow \text{CH}_2-\text{CH}_2-\text{CH}_2 \quad (1) \\
\text{OH} & \quad \text{OH} \\

\end{align*}
\]

\[
\begin{align*}
\text{CH}_2-\text{CH}_2-\text{C} & \rightarrow \text{CH}_2-\text{CH}_2-\text{C} \quad (2) \\
\text{OH} & \quad \text{OH} \\

\end{align*}
\]

The transfer of oxygen from C-2 to C-1 strongly suggests a hydride transfer and is difficult to reconcile with proton abstraction. Results obtained with a different enzyme, ribonucleotide reductase, are also most readily understood in terms of a reversible oxidation and reduction of the coenzyme. It was shown that ribonucleotide reductase catalyzed tritium exchange between solvent hydrogen and hydrogen at the C-5' position of DBC. This exchange was attributed to a reversible oxidation reduction of the enzyme-bound coenzyme.

\[
\begin{align*}
\text{SH} & \quad \text{SH} \quad + \quad \text{DBC coenzyme} \quad \Rightarrow \\
\text{reduced lipoic acid} & \\

\end{align*}
\]

\[
\begin{align*}
\text{S} - \text{S} & \quad + \quad \text{reduced DBC coenzyme} \quad + \quad \text{H}^+ \\
\text{oxidized lipoic acid} & \\

\end{align*}
\]

It appears probable that all rearrangement reactions in which a B-12-coenzyme participates have fundamentally similar mechanisms. This intuitive notion is supported by certain mechanistic similarities in these reactions. (a) All reactions involve the displacement of a group from a carbon atom by a hydrogen from an adjacent carbon atom. (b) In all rearrangement reactions, it has been shown that tritium from DBC-H is transferred to the product (26, 27). Currently available evidence therefore suggests the following generalization concerning the role of B-12-coenzyme in rearrangements. It is the function of the coenzyme to abstract a hydride ion from the substrate. The resulting carbonium ion, or potential carbonium ion, provides a driving force for the group migrations.

A question of major interest is the structure of the postulated reduced form of the coenzyme. No direct evidence bearing on the structure of this form of the coenzyme do not seem worthwhile.

Acknowledgments—We wish to thank Professor W. P. Jencks for many extensive and stimulating discussions. We also wish to acknowledge the technical assistance of Mrs. Patricia Auld.

REFERENCES

Studies on the Mechanism of Hydrogen Transfer in the Cobamide Coenzyme-dependent Dioldehydrase Reaction
Perry A. Frey, Margaret Kottke Essenber and Robert H. Abeles


Access the most updated version of this article at http://www.jbc.org/content/242/22/5369

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/242/22/5369.full.html#ref-list-1