Mechanism of Action of Adenosylcobalamin: Glycerol and Other Substrate Analogues as Substrates and Inactivators for Propanediol Dehydratase—Kinetics, Stereospecificity, and Mechanism†

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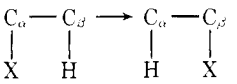
ABSTRACT: A number of vicinal diols were found to react with propanediol dehydratase, typically resulting in the conversion of enzyme-bound adenosylcobalamin to cob(II)alamin and formation of aldehyde or ketone derived from substrate. Moreover, all are capable of effecting the irreversible inactivation of the enzyme. The kinetics and mechanism of product formation and inactivation were investigated. Glycerol, found to be a very good substrate for diol dehydratase as well as a potent inactivator, atypically, did not induce cob(II)alamin formation to any detectable extent. With glycerol, the inactivation process was accompanied by conversion of enzyme-bound adenosylcobalamin to an alkyl or thiol cobalamin, probably by substitution of an amino acid side chain near the active site for the 5′-deoxy-5′-adenosyl ligand on the cobalamin. The inactivation reaction with glycerol as the inactivator exhibits a deuterium isotope effect of 14, strongly implicating hydrogen transfer as an important step in the mechanism of inactivation. The isotope effect on the rate of product formation was found to be 8.0. Experiments with isotopically substituted glycerols indicate that diol dehydrase distinguishes between "*R*" and "*S*" binding conformations, the enzyme-(*R*)-glycerol complex being predominately responsible for the product-forming reaction, while the enzyme-(*S*)-glycerol complex results primarily in the inactivation reaction. Mechanistic implications are discussed. A method for removing enzyme-bound hydroxycobalamin that is nondestructive to the enzyme and a technique for measuring the binding constants of (*R*)- and (*S*)-1,2-propanediols are presented.

Propanediol dehydratase ((*RS*)-1,2-propanediol hydro-lyase; EC 4.2.1.28) from *Klebsiella pneumoniae* (ATCC 8724) is one of nine known enzymes which utilize adenosylcobalamin as a cofactor to catalyze 1,2 rearrangements of the type



where X can be alkyl, NH_2, or OH (Hogenkamp, 1968; Babior, 1975a,b; Abeles and Dolphin, 1976). These enzymes exhibit a remarkable specificity for their respective substrates. For example, although diol dehydratase will catalyze the conversion of both (*R*)- and (*S*)-1,2-propanediols (albeit with different rates and different eventual fates for the hydroxyl oxygen at C-2 of the two isomeric substrates (Retey et al., 1966)), relatively minor structural modifications can abolish catalytic or inhibitory activity (Lee and Abeles, 1963). Toraya and Fukui (1972) found that 1,2-butanediol and styrene glycol, although catalytically inert, are weak competitive inhibitors. Eagar et al. (1975) showed that 3-fluoro-1,2-propanediol is a substrate for diol dehydratase with catalytic constants comparable to those of 1,2-propanediol itself. Recently Toraya et al. (1976) reported that glycerol functions both as a substrate and an inactivator for diol dehydratase.

This paper reports our results with a number of modified vicinal diols including glycerol, thioglycerol, 3-chloro-1,2-propanediol, 1,2-butanediol, 2,3-butanediol, isobutylene glycol, and 3,3,3-trifluoro-1,2-propanediol. All of these substances act as substrates (leading to product formation by the usual rearrangement) and also bring about irreversible inactivation of the enzyme. The inactivation reactions are all dependent on both apoenzyme and cofactor and independent of the presence of oxygen which, in the absence of substrate, is a potent inactivator of holoenzyme.

We have studied the mechanism of catalysis and inactivation for glycerol in some detail because of its close structural resemblance to the normal substrate, 1,2-propanediol, its presence in many biological environments (glycerol is, in fact, one of the ingredients of the growth medium used to induce propanediol dehydratase), and because of the existence of an adenosylcobalamin-dependent glycerol dehydratase that has been reported to be inactivated by its substrate, glycerol (Schneider and Pawelkiewicz, 1966). Moreover, even though glycerol is such a potent inactivator that significant product can be observed only when large amounts of enzyme are used, the catalytic rate constant for product formation actually exceeds that observed with the noninactivating substrate 1,2-propanediol.

Experimental Procedures

Enzyme Preparations. Propanediol dehydratase ((*RS*)-1,2-propanediol hydro-lyase; EC 4.2.1.28) was obtained from *Klebsiella pneumoniae* (ATCC 8724) by a procedure similar to that of Lee and Abeles (1963). Fraction E-8 with a specific activity of between 25 and 50 was used for all determinations. 1,2-Propanediol-free enzyme was prepared as previously reported (Frey et al., 1967).

Adenosylcobalamin. Adenosylcobalamin (AdoCbl)[1] was purchased from Sigma Chemical Co.

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[1] Abbreviations used are: AdoCbl, adenosylcobalamin; OH-Cbl, hydroxycobalamin; Cbl^{II}, Cob(II)alamin; DNP, dinitrophenylhydrazone; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NMR, nuclear magnetic resonance.

Hydroxycobalamin. Hydroxycobalamin (OH-Cbl) was purchased from Sigma Chemical Co.

Assays. All assays for product aldehyde were carried out in the dark or in dim red light at 37 °C. Two different methods were used and are described below. In several cases, results were duplicated by both methods.

DNP Assay. Aldehyde was assayed colorimetrically as the 2,4-dinitrophenylhydrazone using the method described by Eagar et al. (1975).

Alcohol Dehydrogenase–β-Nicotinamide Adenine Dinucleotide Assay. Yeast alcohol dehydrogenase (ADH, Sigma Chemical Co.) and β-nicotinamide adenine dinucleotide (reduced form, NADH, Sigma Chemical Co.) reduce propionaldehyde to 1-propanol. The maximum velocity of this reaction is 30% of that for acetaldehyde reduction (Bruemmer and Roe, 1971). The production of propionaldehyde by diol dehydratase can be measured by monitoring the decrease of absorbance at 340 nm, due to the oxidation of NADH to NAD in the presence of excess ADH.

Determination of Inactivation Rates. Coupled Enzyme Assay. Reaction mixtures consisted of: ADH, 15 units; NADH, 0.20 mM; diol dehydratase, 0.1 unit; bovine serum albumin 0.06 mg; potassium phosphate buffer, pH 8.0, 30 mM; AdoCbl, 0.02 mM; and the desired amount of 1,2-propanediol and/or inactivator. Total volume was 2.50 mL. The reaction mixture was incubated at 37 °C with stirring. The inactivation reaction was started by the addition of AdoCbl and stopped by addition of 50 μl of a 6.0 M 1,2-propanediol solution. Enzyme activity (measured by the rate of production of propionaldehyde) was determined at a minimum of four different inactivation times; the slope of the corresponding semilog plot represents the observed inactivation rate constant ($k_{i,obsd}$) for the given concentration of inactivator and 1,2-propanediol.

For rate determinations under anaerobic conditions, both the reaction mixture and the AdoCbl solution were thoroughly deoxygenated under argon. AdoCbl was transferred to the reaction mixtures using a gas tight syringe.

DNP Method. Reaction mixtures consisted of the following: diol dehydratase ~0.06 unit; potassium phosphate buffer, pH 8.0, 30 mM; AdoCbl 0.013 mM; bovine serum albumin 0.4 mg; and the desired amount of substrate and/or inactivator. Total volume was 2 mL, 37 °C. The reaction was started by addition of AdoCbl and the inactivation was quenched with 50 μL of 6 M 1,2-propanediol. The amount of enzyme remaining active as a function of time after the addition of AdoCbl was determined by measuring the production of propionaldehyde at 1.0-min intervals for 8 min after the addition of 1,2-propanediol. The rate of propionaldehyde production was linear for at least 8 min after the addition of 1,2-propanediol.

Determination of β-Hydroxypropionaldehyde. The production of β-hydroxypropionaldehyde was determined by the acrolein-specific assay of Circle et al. (1945). Reaction mixtures consisted of the following: diol dehydratase, ~5.0 units; potassium phosphate buffer, pH 8.0, 30 mM; bovine serum albumin, 0.4 mg; AdoCbl, 0.013 mM. Total volume was 2.0 mL, 37 °C. The reaction was started by the addition of AdoCbl and quenched by the addition of 0.1 mL of 2.0 N HCl.

β-Hydroxypropionaldehyde can also be determined satisfactorily using the DNP assay as descrbed for propionaldehyde. The reaction mixtures used were the same as above with 0.6 unit of dioldehydrase giving the best results. Absorbance was measured at 475 nm. The extinction coefficient at 475 nm from the dinitrophenylhydrazone of β-hydroxypropionaldehyde is somewhat smaller than that from the dinitrophenylhydrazone of propionaldehyde.

[^{14}C]*Glycerol Labeling of Dioldehydrase.* Reaction mixtures consisted of the following: dioldehydrase, 70 units; [^{14}C]glycerol (1.33×10^9 cpm/mmol), 0.02 mmol; potassium phosphate buffer, pH 8.0, 30 mM; AdoCbl, 3.2 μM; total volume was 2.0 mL. Two controls were performed, one in which AdoCbl was omitted from the reaction mixture and the other in which OH-Cbl was substituted for AdoCbl. The reaction mixtures were incubated at 37 °C for 20 min. Two basic procedures were used for removing glycerol, Cl_3CCOOH precipitation of the protein or dialysis against guanidine hydrochloride.

Following the 20-min incubation period, the protein was precipitated by the addition of 0.2 mL of 20% Cl_3CCOOH solution and centrifuged, and the supernatant was removed. The protein pellet was redissolved in 2.0 mL of H_2O and the sequence was repeated. After the fifth precipitation, the supernatant contained only background activity. The pellet was resuspended in 1.0 mL of H_2O (an aliquot was removed at this point for the determination of protein), added to 10 mL of scintisol complete, and assayed for radioactivity.

Alternatively, a 1.0-mL aliquot was removed from each reaction solution after the incubation and dialyzed against three successive 100-mL volumes of 5 M guanidine hydrochloride, each for 24 h, followed by dialysis against H_2O. When the radioactivity in the dialysis solution diminished to background, the 1.0-mL sample was added to 10 mL of scintisol complete and assayed for radioactivity.

Substrates. Perdeuterioglycerol (glycerol-d_5) was purchased from Merk, Sharp, and Dohme, Ltd., and used without further purification.

[*1*-^{14}C]*Glycerol* was purchased from Amersham.

(R)-1,1-Dideuterioglycerol ((*R*)-glycerol-d_2) was synthesized from D-mannitol via the intermediate preparation of the 1,2,5,6-diisopropylidene derivative (Baer, 1952), and of D-2,3-isopropylidene glycerol (Ghangas and Fondy, 1971). The experimental details of the following steps are given by Eagar et al. (1975). Potassium D-2,3-isopropylidene glycerate was prepared by alkaline potassium permanganate oxidation of D-2,3-isopropylidene glycerol and was esterfied with methyl iodide in hexamethylphosphoramide. The methyl ester was then reduced with lithium aluminum deuteride (99% ^2H, Stohler Isotope Chemicals). The ketal was hydrolyzed in ethanol–sulfuric acid and the product was distilled to give (*R*)-1,1-dideuterioglycerol with $[\alpha]^{25}_D + 0.014°$.[2] Deuterium content was at least 98% as determined by ^1H NMR.

(RS)-1,1-Dideuterioglycerol ((*RS*)-glycerol-d_2) was prepared from (*RS*)-1,2-isopropylidene glycerol (Ghangas and Fondy, 1971) by a method analogous to that described above. Deuterium content was at least 98% as determined by ^1H NMR.

(RS)-1,1-Dideuterio-1,2-propanediol was prepared by lithium aluminum deuteride reduction of *dl*-ethyl lactate (Fieser and Fieser, 1967). Deuterium content was at least 98%, as determined by ^1H NMR. The isotope effect on catalysis was 13, somewhat greater than the value of 10–12 reported by Abeles (1972). The UV-visible spectrum of the holoenzyme–dideuteriopropanediol complex was identical to those reported for 1,2-propanediol (Wagner et al., 1966) and 3-fluoro-1,2-propanediol (Eagar et al., 1975).

3,3,3-Trifluoro-1,2-propanediol was prepared from 3,3,3-trifluoro-1-bromacetone by lithium aluminum hydride reduction to the corresponding alcohol (McBee and Burton, 1952), which was collected by fraction distillation at 124–124.5

[2] Optical rotations are uncorrected for water content (Huff, 1961).

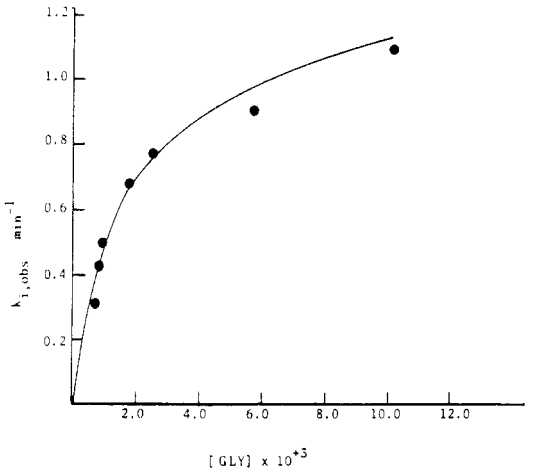


FIGURE 1: Dependence of the observed rate of inactivation, $k_{i,obsd}$, on glycerol concentration under anaerobic conditions and in the absence of 1,2-propanediol. Values of $k_{i,obsd}$ were obtained from the least-squares line of the linear plot of ln% enzyme activity remaining vs. inactivation time for each concentration of glycerol. The corresponding double-reciprocal plot is linear and gives a value of 1.3 min^{-1} for k_i and 1.6×10^{-3} M for K_M for glycerol.

°C. The alcohol (12.8 g, 0.0674 mol) was then added to a solution of 4 g of NaOH in 10 mL of water, and 3,3,3-trifluoro-1,2-epoxypropane distilled out of the reaction mixture in 95% yield. The epoxide was refluxed overnight in 1% H_2SO_4, the solution was extracted five times with ethyl ether, and the ether was evaporated to give the diol.

Isobutylene glycol was prepared by acid hydrolysis of isobutylene oxide (Columbia Organic Chemical Co.) (Pattison and Norman, 1957).

1,2-Butanediol was prepared by acid hydrolysis of 1-butylene oxide.

1-Chloro-2,3-propanediol was purchased from Calbiochem and distilled before use.

Thioglycerol (3-mercapto-1,2-propanediol) was purchased from Aldrich Chemical Co. and distilled before use.

2,3-Butanediol was purchased as a mixture of the meso and d isomers from Aldrich Chemical Co. and was distilled before use.

1,2-Propanediol, glycerol, and ethylene glycol were purchased as reagent grade chemicals and distilled before use. (*R*)- and (*S*)-1,2-Propanediols were synthesized by a method previously described (Eagar et al., 1975). Concentrations of aqueous solutions of these glycols were determined by bichromate oxidation using a method adapted from Englis and Wollerman (1952).

NMR spectra were obtained at 60 MHz in the Fourier transform mode on a Varian T-60 spectrometer in deuteriochloroform or ^2H_2O.

UV-visible spectra of holoenzyme–substrate complexes were obtained between 300 and 600 nm at 27 and 37 °C using a Beckman Acta III spectrophotometer. The sample solution was assayed for residual enzyme activity to confirm that apoenzyme was present in excess of cofactor.

Results

Kinetics of Glycerol Inactivation in the Absence of 1,2-Propanediol. In the presence of glycerol and in the absence of 1,2-propanediol and oxygen, diol dehydratase holoenzyme undergoes rapid, irreversible, inactivation such that catalytic activity cannot be regenerated by addition of either 1,2-propanediol or AdoCbl or by extensive dialysis. Moreover, addition of saturating amounts of 1,2-propanediol will instantly stop the inactivation reaction. This provides a method for quantitatively measuring the kinetics of the inactivation reaction. Saturating amounts of 1,2-propanediol are added after variable periods of time to a holoenzyme–glycerol mixture, thus quenching the inactivation; the amount of enzyme remaining catalytically active can then be determined by measuring the subsequent rate of propionaldehyde production.

TABLE I: Kinetic Parameters of Substrates and Inactivators of Diol Dehydratase.

Substrate	$K_M \times 10^{+4}$	k_p(s^{-1})[f]	k_i (min^{-1})
(*S*)-1,2-Propanediol	0.186[a]	191	~0
(*RS*)-1,2-Propanediol	0.30[a]	250	~0
(*R*)-1,2-Propanediol	0.60[a]	340	~0
Glycerol	16.0[b]	412[d]	1.30
1-Chloro-2,3-propanediol	4.1	>0[c]	0.36
Trifluoropropanediol	1.24 (K_i)	>0[c]	0.016
2,3-Butanediol (meso + D)	2.0 (K_i)	4.0[d]	0.068
1,2-Butanediol	5.9 (K_i)	>0[c]	0.054
Isobutylene glycol	8.8	18	0.47
Ethylene glycol	6.6	150[d,e]	0.098
Thioglycerol	0.1	~0	1.74

[a] Values of K_M (K_i) for the natural substrates were determined in competition with glycerol with the natural substrates serving as "inhibitors" of inactivation as described in the Results. Preliminary values of K_M for the 1,2-propanediols obtained by this method were previously published (Eagar et al., 1975). The values given here are slightly different and represent a refinement in technique. [b] A publication has recently appeared (Toraya et al., 1976) also reporting glycerol to be a substrate and inactivator of diol dehydratase. A value of K_M for glycerol was given that agrees with the value we have determined. [c] Product formation does occur but rate was not quantitated. [d] Determined from amount of product formed before complete inactivation. [e] Direct comparison of initial rate to that of 1,2-propanediol. [f] Based on a molecular weight of 250 000 and specific activity of 60 units/mg, with 1,2-propanediol as substrate.

The rate of inactivation follows a first-order rate law through at least three half-lives

$$[E_a] = [E_0]e^{-(k_{i,obsd})t} \quad (1)$$

where E_a is the amount of active enzyme remaining at time t, E_0 is the initial amount of enzyme present, and $k_{i,obsd}$ is the observed first-order rate constant for inactivation. Thus, a plot of ln[E_a] vs. time is linear and the slope gives a value of $k_{i,obsd}$ for a given glycerol concentration. A plot of $k_{i,obsd}$ vs. glycerol concentration (Figure 1) shows Michaelis–Menten type saturation behavior. The inactivation process can, accordingly, be described in terms of reversible association between enzyme and glycerol, followed by irreversible inactivation

$$E + G \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EG \xrightarrow{k_i} I \quad (2)$$

where I represents irreversibly inactivated enzyme. The rate of inactivation is

$$\frac{d[E_a]}{dt} = -k_i[EG] \quad (3)$$

This differs from the classical situation because [EG] itself does not remain constant. However, if one considers only that enzyme which is still active

$$[E_a] = [E] + [EG]$$

where E is active enzyme free in solution, with

$$[EG] = \frac{[E][G]}{K_G}$$

the rate of inactivation (eq 3) becomes

$$\frac{-d[E_a]}{dt} = \frac{k_i[E_a]}{1 + \frac{K_G}{[G]}} \quad (4)$$

Integration gives

$$[E_a] = [E_a]_0 e^{-k_{i,obsd}t} \quad (5)$$

where

$$k_{i,obsd} = \frac{k_i}{1 + \frac{K_G}{[G]}} \quad (6)$$

Thus, a plot of $1/k_{i,obsd}$ vs. $1/[G]$ should, and does, give a straight line (Figure 2); the y intercept gives $1/k_i$; the slope or the x intercept determines K_G. The values thus determined appear in Table I.

Kinetics of Glycerol Inactivation in the Presence of 1,2-Propanediol. 1,2-Propanediol protects against glycerol inactivation in a manner indicative of simple competition between glycerol and 1,2-propanediol for the active site. The kinetics which obtain are analogous to those of simple competitive inhibition, with 1,2-propanediol as the "inhibitor" of inactivation. Consider:

$$E + G \rightleftharpoons EG \xrightarrow{k_i} I$$

$$E + S \rightleftharpoons ES \rightleftharpoons E + P$$

where G = glycerol, S = 1,2-propanediol, I = inactive enzyme, and k_i is the first-order rate constant for inactivation. Using the same treatment just described for the substrate-free case to allow for the change of [EG] with time, one can show that

$$[EG] = \frac{[E_a]}{1 + \frac{K_G}{[G]}\left(1 + \frac{[S]}{K_S}\right)}$$

The inactivation kinetics remain first order in the presence of 1,2-propanediol and the amount of active enzyme at any time is given by

$$[E_a] = [E_a]_0 e^{-k_{i,obsd}t}$$

where

$$k_{i,obsd} = \frac{k_i}{1 + \frac{K_G}{[G]}\left(1 + \frac{[S]}{K_S}\right)} \quad (7)$$

Values of $k_{i,obsd}$ in the presence of 1,2-propanediol were determined experimentally at various concentrations of glycerol and 1,2-propanediol as previously described. The resulting double-reciprocal plots ($1/k_{i,obsd}$ vs. $1/[G]$) at each fixed 1,2-propanediol concentration are linear with a common y intercept, illustrating the purely competitive nature of the "inhibition" of glycerol inactivation by 1,2-propanediol (Figure 2). Furthermore, Figure 2 also demonstrates that eq 7 is a valid treatment of the results obtained over a wide range of substrate and glycerol concentrations. The value of k_i obtained from the y intercept of Figure 2 agrees well with the experimentally determined value (Table I) and lends further validity to this treatment.

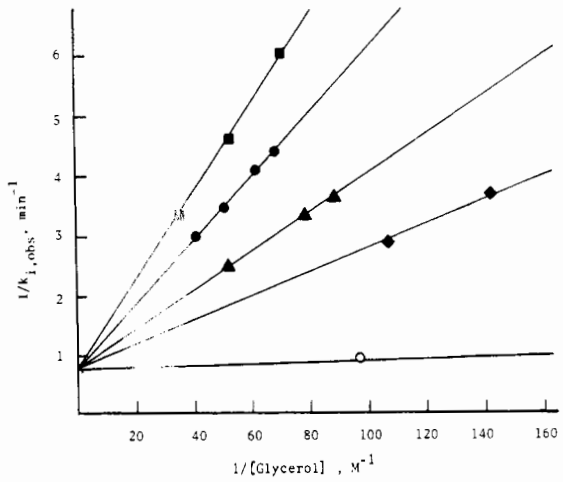


FIGURE 2: Plot of the reciprocal of the observed inactivation rate constant vs. the reciprocal of glycerol concentration in the presence of the following concentrations of (*RS*)-1,2-propanediol: (■) 1.9×10^{-3} M; (●) 1.34×10^{-3} M; (▲) 8.6×10^{-4} M; (♦) 4.75×10^{-4} M; (○) no (*RS*)-1,2-propanediol. Although only one point can be depicted on the above graph for the substrate-free case, a straight line is obtained with the illustrated slope and intercept.

Thus, the above equation allows what we believe to be the first reliable determination of the Michaelis constant for 1,2-propanediol and diol dehydratase (Table I). The S isomer of 1,2-propanediol is more effective at inhibiting inactivation than is the RS mixture, while (*R*)-1,2-propanediol is a much less effective inhibitor than the RS mixture. That the R and S isomers differ in their ability to protect diol dehydratase from glycerol inactivation reflects a difference in their respective values of K_M (Table I). The values of K_M obtained in this way are really K_i's. However, the ratio of K_i values for (*R*)- and (*S*)-1,2-propanediol determined by this method agrees well with the value of 3.2 reported by Jensen et al. (1975) and suggests that in this case $K_i = K_M$.

Kinetics of Inactivation for Other Substrates. All substrate analogues listed in Table I exhibit inactivation behavior, which can be described by the above equations developed for inactivation by glycerol in both the presence and absence of 1,2-propanediol. Values of k_i and K_M for each of these inactivators were determined for reasons of convenience by measurement of the rate of inactivation in the presence of 1,2-propanediol and are listed in Table I.

Deuterium Isotope Effects on the Rate of Inactivation by Glycerol. The rates of enzyme inactivation at saturating concentrations of (*R*)-glycerol-d_2, (*RS*)-glycerol-d_2, and glycerol-d_5 were measured by the method just described and were considerably slower than for the undeuterated substrate. Substitution of deuterium in glycerol has little or no measurable effect on K_M (manuscript in preparation) and the ratio of the rate of inactivation observed for glycerol to that observed for the same concentration of one of the deuterated glycerols accordingly reflects the isotope effect on k_i. Thus, when measurements are made at large concentrations of glycerol and its deuterated derivatives

$$\frac{(k_{i,obsd})_H}{(k_{i,obsd})_D} = \left(\frac{k_H}{k_D}\right)_i$$

Table II lists the various kinetic parameters obtained in this way.

The $k_{i,obsd}$ values determined in these experiments should be only slightly smaller than the true k_i values (because at 0.04 M inactivator with $K_M = 1.6 \times 10^{-3}$ M, $K_M/[G] \sim 4 \times 10^{-2}$

TABLE II: Catalytic and Inactivation Rate Constants for Glycerol and its Deuterated Analogues.

Substrate	Competitive[a] Turnover No. (T = 60 min)	k_p(s^{-1})[b]	$\left(\frac{k_H}{k_D}\right)_p$	$k_{i,obsd}$ (min^{-1}) 0.04 M Inactivator	$\left(\frac{k_H}{k_D}\right)_i$
Glycerol	19 000	412	1	1.25	1
Glycerol-d_5	33 000	52	8.0	0.0893	14
(*RS*)-Glycerol-d_5	16 340	190	2.2	0.658	1.9
(*R*)-Glycerol-d_2	5 320	61	6.8	0.694	1.8

[a] Competitive turnover numbers are based on a specific activity of 60 units/mg and a molecular weight of 250 000 (Essenberg et al., 1971). [b] Calculated from eq 9 using k_i and P_∞/E_0.

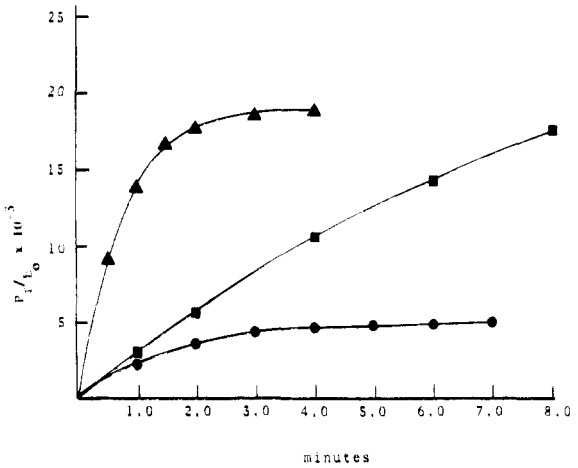


FIGURE 3: Production of β-hydroxypropionaldehyde as a function of time for: (▲) glycerol; (■) glycerol-d_4; (●) (*R*)-glycerol-d_2. β-hydroxypropionaldehyde was determined by the DNP assay as described under Experimental Procedures and is depicted here as the competitive turnover number, P_τ/E_0, for uniformity. [E_0] based on 60 units/mg and a molecular weight of 250 000 g/mol.

≪ 1, see eq 6) and can therefore represent experimentally determined values of k_i for each deuterated glycerol.

With glycerol-d_5, we observe a large isotope effect (14) on the rate of inactivation. We observe smaller, nearly equal values for (*R*)-glycerol-d_2 (1.8) and (RS)-glycerol-d_2 (1.9).

Product Formation from Glycerol. Diol dehydratase holoenzyme also catalyzes the dehydration of glycerol to β-hydroxypropionaldehyde in addition to undergoing irreversible inactivation. β-Hydroxypropionaldehyde was identified (1) by the ^{1}H NMR of its 2,4-dinitrophenylhydrazone derivative, (2) by comparative silica gel thin-layer chromatography of the 2,4-dinitrophenylhydrazone with an authentic sample prepared by the method of Hall and Stern (1950), and (3) by conversion to acrolein, which was then determined by the acrolein-specific assay of Circle et al. (1945).

Turnover Number. The total amount of β-hydroxypropionaldehyde formed relative to the amount of enzyme initially present was determined for glycerol, (*R*)-glycerol-d_2, (*RS*)-glycerol-d_2, and glycerol-d_5 as substrates. A known amount of diol dehydratase was allowed to react for 60 min with saturating amounts of each of the above glycerols at 37 °C. No further β-hydroxypropionaldehyde was produced during an additional 60-min period, indicating that complete inactivation of the enzyme had occurred during the initial 60-min period. The total amount of β-hydroxypropionaldehyde formed was determined by the acrolein-specific assay and the results are presented in Table II as "competitive turnover numbers", the average number of molecules of product formed by each molecule of holoenzyme before its inactivation.

Although both (*R*)-glycerol-d_2 and (*RS*)-glycerol-d_2 yield less product than glycerol itself before inactivating the enzyme, glycerol-d_5 forms nearly twice as much product as glycerol.

Rate of Formation of β-Hydroxypropionaldehyde. The production of β-hydroxypropionaldehyde was followed for several minutes with glycerol, glycerol-d_5, and (*R*)-glycerol-d_2 each serving as substrate. The results in Figure 3 show that, although the initial rate of product formation is much greater for glycerol than glycerol-d_5, the rate at which glycerol inactivates the enzyme relative to inactivation by glycerol-d_5 is greater by an even larger factor, thus resulting in a net enhancement of product formation from glycerol-d_5.

Because of competition from irreversible inactivation, the rate constant for production formation (k_p) cannot be obtained with any degree of confidence by extrapolation of the results in Figure 3; the rate changes too drastically in the region near $t = 0$. However, these values can be calculated if one recognizes that inactivation is a first-order process in [EG], as previously shown.

The rate constant for product formation (k_p) is the analogue of the rate constant for inactivation k_i and $d[P]/dt = k_p[EG]$. Under conditions where the enzyme is saturated with substrate

$$[EG] = [E_a]$$

As before, [E_a] is the concentration of total active enzyme at any time, a quantity which continuously decreases because of the inactivation process. From eq 5

$$[E_a] = [E_0]e^{-k_i t}$$

whence $d[P]/dt = k_p[E_0]e^{-k_i t}$. The total amount of product formed at any time is given by the integrated form of this equation

$$[P_\tau]/[E_0] = \frac{k_p}{k_i}(1 - e^{-k_i t}) \qquad (8)$$

where $[P_\tau]/[E_0]$ is the "competitive turnover number" at any time. At large t, the expression reduces to

$$\frac{[P_\infty]}{[E_0]} \approx \frac{k_p}{k_i}$$

where $[P_\infty]/[E_0]$ is the total amount of product formed relative to the initial amount of enzyme after complete inactivation of the enzyme.

The values of $[P_\infty]/[E_0]$ have been determined as have those of k_i for glycerol and each of its deuterated derivatives (Table II) so that values of k_p can be calculated; they are also listed in Table II. Indeed, one can plot $[P_\tau]/[E_0]$ as a function of time

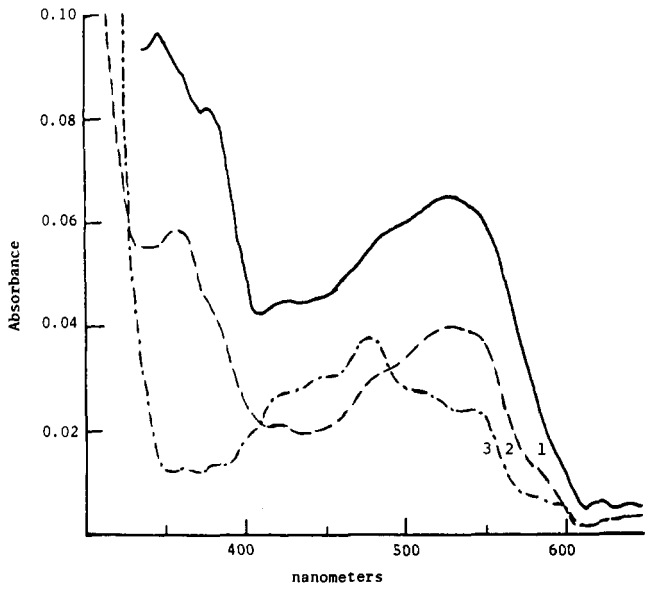


FIGURE 4: Comparison of the effect of glycerol to the effect of 1,2-propanediol on the spectrum of enzyme-adenosylcobalamin. (1) Diol dehydratase, 37 units; AdoCbl, 2.3×10^{-3} μmol; K_2HPO_4, 3 μmol in 0.3 mL. The reference cell was identical to sample but lacked AdoCbl. (2) Glycerol, 0.06 mmol, was added in 0.03 mL to both sample and reference of (1). Total volume 0.33 mL. (3) 1,2-Propanediol, 18 μmol was added to sample and reference of (1). Total volume 0.33 mL, 27 °C.

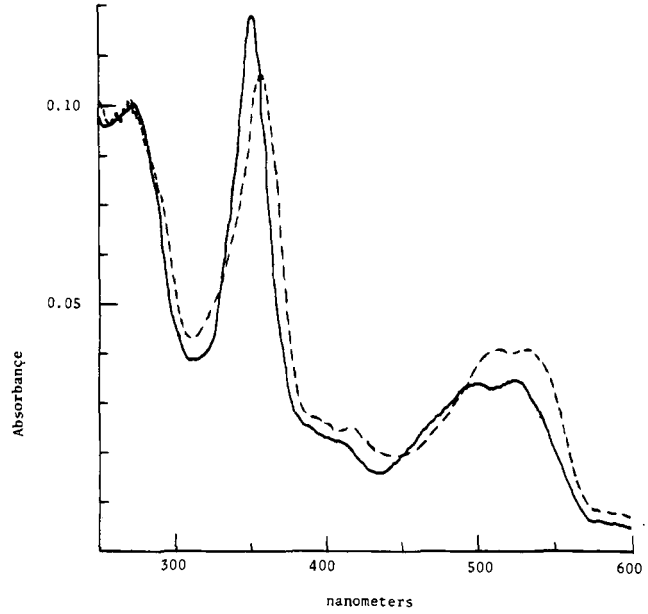


FIGURE 5: Comparison of the absorption spectrum of OH-Cbl free in solution with that bound to dioldehydrase. (—) OH-Cbl, 1.4×10^{-3} μmol; K_2HPO_4 buffer, pH 8.0, 0.3 μmol. Total volume 0.32 mL. (- - -) Enzyme, 30 units; OH-Cbl, 1.4×10^{-3} μmol; and K_2HPO_4 in 0.32 mL. The reference cell was identical to the sample cell but lacked OH-Cbl, 27 °C.

using the values of k_i and k_p in Table II and accurately reproduce the experimental graphs of Figure 3.

Deuterium Distribution in Products. The 2,4-dinitrophenylhydrazones of β-hydroxypropionaldehydes formed from the variously deuterated glycerols were purified by thin-layer chromatography on silica gel with chloroform as the eluting solvent. The ^1H NMR spectra of these derivatives showed that (*R*)-glycerol-d_2 gives product with essentially no deuterium at C-3, while (*RS*)-glycerol-d_2 gives product with approximately 60% deuterium at C-3.

Product Formation—Other Substrates. With the exception of 1,2-butanediol and thioglycerol, all of the substrate analogues listed in Table I gave easily detectable amounts of product. Products from isobutylene glycol and 2,3-butanediol were identified as isobutyraldehyde and 2-butanone, respectively, by thin-layer chromatography of the 2,4-dinitrophenylhydrazones on silica gel with chloroform as the eluting solvent. Aldehydes from 3,3,3-trifluoro-1,2-propanediol and 3-chloro-1,2-propanediol were detected by the DNP assay but were not rigorously characterized.

Spectral Observation of the Diol Dehydratase-Adenosylcobalamin-Glycerol Complex. Figure 4 compares the visible spectra of AdoCbl bound to diol dehydratase in the presence of glycerol to that obtained in the presence of 1,2-propanediol. Incubation of 1,2-propanediol and holoenzyme produces a Cbl^{II}-like spectrum. In contrast, incubation of holoenzyme and glycerol produces an alkylcobalamin-like spectrum very similar to that of AdoCbl itself bound to diol dehydratase in the absence of substrate. This alkylcobalamin-like spectrum, which is obtained after complete inactivation by glycerol, remains unchanged for up to 24 h at room temperature in the dark; after this time, the slow formation of OH-Cbl can be detected. As the half-life for inactivation of the enzyme by glycerol under these conditions is about 30 s, single wavelengths at 485 nm (λ_{max} for Cbl^{II}) or 525 nm (λ_{max} for AdoCbl and other alkylcobalamins) were monitored immediately following addition of glycerol; no changes were detected.

Even though 1,1-dideuterio-1,2-propanediol reacts more slowly by a factor of 13 than 1,2-propanediol itself, use of 1,1-dideuterio-1,2-propanediol in place of 1,2-propanediol does not measurably affect the amount of enzyme-bound AdoCbl observable as Cbl^{II}. Because glycerol-d_5 is a reasonably good "substrate" but inactivates much more slowly than glycerol, we looked for spectral changes when glycerol-d_5, instead of glycerol, was added to holoenzyme; none were observed. Thus, with either form of glycerol, we could see no evidence for Cbl^{II}.

However, Cbl^{II} can be generated by addition of 1,2-propanediol after the addition of glycerol. If the addition of 1,2-propanediol almost immediately follows that of glycerol, a large fraction of the enzyme-bound AdoCbl is observed as Cbl^{II}. If, however, the glycerol-enzyme mixture is allowed to stand for 4 min, addition of 1,2-propanediol does not generate any Cbl^{II} in the spectrum.

Cl_3CCOOH precipitation of the protein from a solution containing holoenzyme and glycerol leads to recovery of only OH-Cbl. Addition of Cl_3CCOOH to an identical reaction mixture containing 1,2-propanediol (instead of glycerol) results in nearly complete recovery of AdoCbl.

Exposure of a solution of holoenzyme and glycerol to a 100-W bulb at 18 in. for 30 min resulted in nearly complete formation of enzyme-bound OH-Cbl.

Spectral Observations of Enzyme-Bound OH-Cbl. Although the general character of the OH-Cbl spectrum remains essentially unchanged on binding to diol dehydratase, small bathochromic shifts of the bands above 300 nm do occur and serve to distinguish OH-Cbl free in solution from that bound to diol dehydratase (Figure 5). OH-Cbl also binds to bovine serum albumin, for example, but the resulting electronic spectrum is identical to that of free OH-Cbl. For OH-Cbl in 0.01 M K_2HPO_4 buffer at pH 8.0, we observe λ_{max} at 525, 510, and 352 nm. For OH-Cbl bound to diol dehydratase in K_2HPO_4 buffer at pH 8.0, the corresponding λ_{max} are 540, 515, and 362, respectively.

Spectral Observation of Cbl^{II} Intermediates. UV-visible

TABLE III: Reactivation of Several Forms of Inactive Diol Dehydratase.

Reaction[a] Solution	Method of[b] Inactivation	Enzyme Activity Units: Before Inactivation	After Inactivation	After[c] Treatment A	After[d] Treatment B
1	OH-Cbl	17	0	13	9
2	O_2	17	0	2.8	4.6
3	Glycerol	17	0	2.0	4.0
4	Control	17	17	15	11

[a] Reaction mixture no. 3 contained 80 μmol of glycerol, otherwise the four reaction solutions were identical and contained: K_2HPO_4 buffer, pH 8.0, 80 μmol; bovine serum albumin, 0.02 mg; and diol dehydratase, 17 units. Total volume 2.1 mL. Enzyme activity was determined by assaying a 10-μL aliquot for activity by a method described under Experimental Procedures. [b] Inactivation was carried out by addition of: 40 μg of OH-Cbl to solution no. 1; 40 μg of adenosylcobalamin to solutions no. 2 and 3; and 40 μL of H_2O to solution no. 4, followed by 15-min incubation at 37 °C in the dark. An aliquot was removed for the determination of activity. [c] Reactivation: treatment A. Each of the four solutions, after inactivation, were subjected to dialysis for 12-h periods against each of two changes of 2 L of solution containing: K_2SO_3, 100 mmol; $Mg(CH_3CO_2)_2$, 60 mmol; 1,2-propanediol, 200 mmol; and K_2HPO_4 buffer, pH 8.0, 20 mmol. This was followed by dialysis for 3-h periods against two changes of 2 L of solution containing: 1,2-propanediol, 200 mmol; and K_2HPO_4 buffer, pH 8.0, 20 mmol. [d] Treatment B refers to a repetition of treatment A.

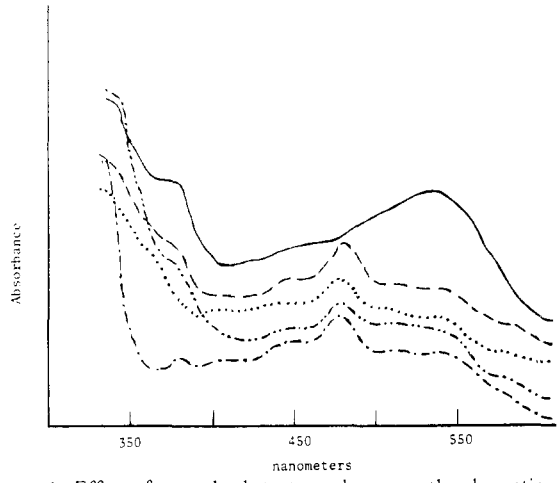


FIGURE 6: Effect of several substrate analogues on the absorption spectrum of enzyme-bound adenosylcobalamin. (—) Enzyme, 30 units; K_2HPO_4, 3.5 μmol; AdoCbl, 1.8×10^{-3} μmol; total volume 0.35 mL. Reference cell was identical minus AdoCbl. To the above sample and reference cuvettes, 25 μmol of either ethylene glycol (- - -), thioglycerol (· · ·), trifluoropropanediol (- · · -), or isobutylene glycol (- · -) was added in a 0.05-mL aliquot. Total volume 0.40 mL, 27 °C.

absorption spectra of holoenzyme–substrate complexes with ethylene glycol, trifluoropropanediol, isobutylene glycol, and thioglycerol are nearly indistinguishable from the spectrum of holoenzyme with 1,2-propanediol (Figure 6). In the case of ethylene glycol, after about seven half-lives, the spectrum was observed to undergo a rapid change, indicating formation of enzyme-bound OH-Cbl; at this time, only about 0.8% of the original activity is present. With trifluoropropanediol, isobutylene glycol, and thioglycerol, the Cbl^{II} spectrum persists for hours after complete inactivation of the holoenzyme. With 2,3-butanediol, no Cbl^{II} spectrum is observed and conversion of enzyme-bound AdoCbl to OH-Cbl is kinetically competent with inactivation.

Recovery of Active Apoenzyme from Inactive Apoenzyme–OH-Cbl Complexes. Dialysis of an enzyme–OH-Cbl complex against a solution containing Mg^{2+} and SO_3^{2-} leads to recovery of the OH-Cbl in the dialysate. Subsequent dialysis to remove the SO_3^{2-} (which appears to be a potent inhibitor of diol dehydratase) leaves apoenzyme, which on addition of AdoCbl is catalytically active (up to 80% of the original activity can be recovered). Treatment with either reagent alone or both reagents sequentially does not remove enzyme-bound OH-Cbl. Table III gives details of this procedure, which is a modification of a procedure for exchanging OH-Cbl and AdoCbl with glycerol dehydratase (Schneider et al., 1970), and some representative results.

Reactivation of O_2-Inactivated Holoenzyme. Exposure of diol dehydratase holoenzyme to oxygen in the absence of 1,2-propanediol leads to inactivation and formation of enzyme-bound OH-Cbl, which can be followed spectrophotometrically.

Hydroxycobalamin can be removed from oxygen-inactivated holoenzyme by the above method; however, only very little activity is regenerated. Continued dialysis of the resulting apoenzyme does lead to recovery of up to 30% activity (Table III).

Reactivation of Glycerol-Inactivated Holoenzyme. In the presence of light, the cobalamin of the inactivated holoenzyme is converted to OH-Cbl, which, as in the case above for oxygen inactivation, can be removed without simultaneous recovery of enzymatic activity. In the absence of light, much longer periods of dialysis are required even to remove the enzyme-bound cobalamin; again, significant enzymatic activity is not regenerated.

Inactivation by [^{14}C]Glycerol. Preliminary results using radioactive glycerol indicate that when diol dehydratase holoenzyme is inactivated with glycerol several molecules of glycerol become strongly associated with each molecule of enzyme in an AdoCbl-dependent process. The results are complicated by the finding that control experiments using apoenzyme alone or OH-Cbl inactivated enzyme also give evidence of labeling, although to a lesser extent.

The radioactive label in the above experiments could not be separated from the protein by the Cl_3CCOOH procedure described in the experimental, or by extensive dialysis vs. 5 M guanidine hydrochloride or 0.5 M hydroxylamine. The use of 0.5 M glycerol or 0.5 M 1,2-propanediol in conjunction with the above techniques in an effort to displace [^{14}C]glycerol did not affect the results; neither did a combination of all the above techniques on an individual sample.

Thus, although several molecules of glycerol appear to become very tightly associated with diol dehydratase apoenzyme, the acquisition of more than one molecule of glycerol is strictly

AdoCbl dependent. Further investigation on this subject is under way.

Discussion

Mechanism of Inactivation and Catalysis. The following mechanistic scheme has evolved for the conversion of 1,2-propanediol to propionaldehyde by diol dehydratase (Abeles and Dolphin, 1976):

$$\underset{}{\mathrm{SH} + \mathrm{AdoCbl\text{-}E} \rightleftharpoons \mathrm{SH\text{-}AdoCbl\text{-}E} \longrightarrow \mathrm{S}\cdot \overset{\mathrm{HAdo}}{\underset{\cdot\mathrm{Cbl}}{}}}$$
$$\underset{\mathrm{(I)}}{} \qquad \underset{\mathrm{(II)}}{} \qquad (9)$$
$$\rightleftharpoons ? \rightleftharpoons \mathrm{PH}\ \overset{\cdot\mathrm{Ado}}{\underset{\cdot\mathrm{Cbl}}{}} \rightleftharpoons \mathrm{PH} + \mathrm{AdoCbl\text{-}E}$$
$$\mathrm{(III)} \qquad \mathrm{(IV)}$$

Intermediate **I** represents the reversibly associated enzyme–substrate complex **II**; an intermediate in which hydrogen has been removed from substrate to yield 5′-deoxyadenosine (HAdo); **III** represents one or more intermediates involving rearrangement; **IV** is a species in which hydrogen has been returned to substrate from the 5′ carbon of 5′-deoxyadenosine, presumably completing formation of product. The conversion of **I** to **II** is thought to be irreversible; attempts to demonstrate hydrogen exchange between cofactor and unreacted substrate have been unsuccessful (Carty et al., 1971; Frey and Abeles, 1966).

Four areas of experimental results of this work can be used to assess possible molecular mechanisms of inactivation and catalysis: (1) kinetics of catalysis and inactivation, (2) kinetic isotope effects, (3) spectral observations of enzyme-bound cofactor before and after inactivation, (4) stereospecificity of catalysis and inactivation. These four aspects were studied in considerably greater detail with glycerol as substrate inactivator. However, results obtained with this substrate may also apply to the others, in view of their similar kinetic behavior.

Kinetics of Catalysis and Inactivation. Equations of the Michaelis–Menten type, modified to account for decreasing enzyme activity with time due to irreversible inactivation, accurately describe all catalysis and inactivation kinetics presented here. The kinetics of inactivation for all substrates studied are similar in several respects: (1) the rate of inactivation is first order with respect to enzyme, (2) saturation behavior with respect to substrate is observed, and (3) 1,2-propanediol acts as a purely competitive inhibitor of inactivation. These similarities suggest that the mechanism of inactivation may be the same or very similar for each inactivator. Furthermore, they indicate that inactivation is a phenomenon associated with the active site.

Kinetic Isotope Effect. Results obtained with glycerol-d_5 ($V_{max}H/V_{max}D \sim 8$) demonstrate that cleavage of a carbon–hydrogen bond is one of the important rate-contributing steps in the enzyme-catalyzed formation of product from glycerol. This suggests that the mechanism of catalysis is probably very similar to that of the normal substrate 1,2-propanediol. The isotope effect of 14 on inactivation suggests that the mechanism of inactivation may in part be very similar to catalysis. However, we have verified that β-hydroxypropionaldehyde neither inhibits nor inactivates diol dehydratase holoenzyme. Inactivation, then, must occur somewhere between the initial binding of glycerol and the release of product.

Spectral Observation of Enzyme-Bound Cofactor. Substrate-induced transformation of enzyme-bound AdoCbl to a species spectrophotometrically indistinguishable from Cbl^{II} has been observed to occur with both diol dehydratase and ethanolamine ammonia-lyase (Wagner et al., 1966; Abeles and Lee, 1964; Babior, 1969). This transformation is associated with catalysis and presumably involves homolytic cleavage of the carbon–cobalt bond.

A number of the modified substrate inactivators we have examined can induce the transformation described above in the visible spectrum of enzyme-bound AdoCbl, suggesting the presence, in these cases, of intermediate **II** (Cbl^{II}). The absence of a detectable Cbl^{II} species with glycerol and 2,3-butanediol as substrates, therefore, more likely represents a change in the rate-limiting step, resulting in the predominance of an intermediate which is not Cbl^{II} rather than a change in the mechanism of catalysis (i.e., a Cbl^{II} species still intervenes but in a concentration which is too low to be detected by the techniques used).

The inactivation reaction may be the result of the formation of a highly reactive intermediate, such as **II** or **III** which, due to the modification of the substrate, lacks the proper steric or electronic configuration necessary for normal catalysis. Destruction of cofactor and/or enzyme by some noncatalytic reaction of the above intermediate may be responsible for inactivation.

Destruction of enzyme-bound cofactor is commonly associated with inactivation of AdoCbl-dependent enzymes and is usually characterized by the formation of OH-Cbl. For example, when enzyme and AdoCbl are incubated together in the absence of substrate, formation of enzyme-bound OH-Cbl from enzyme-bound AdoCbl occurs at a rate apparently equal to that of inactivation (Wagner et al., 1966), probably by reaction of O_2 with Cbl^{II}. The formation of Cbl^{II} in this case cannot be observed spectrophotometrically, but the formation of a small amount of some paramagnetic species has been demonstrated to occur even in the absence of substrate (Finlay et al., 1973).

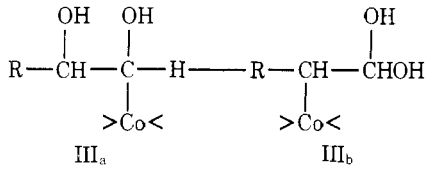
Of the inactivators listed in Table I, only with 2,3-butanediol is inactivation accompanied by the transformation of enzyme-bound cofactor to enzyme-bound OH-Cbl at a rate similar to that of inactivation. With this inactivator, as in the case of oxygen inactivation, the formation of Cbl^{II} cannot be detected spectrophotometrically.

However, of the inactivators that do induce formation of Cbl^{II}, none exhibit a transformation of the Cbl^{II} spectrum to that of OH-Cbl concomitantly with inactivation. The formation of enzyme-bound OH-Cbl follows more rapidly after inactivation with ethylene glycol as substrate than with any of the other inactivators that induce Cbl^{II} formation. This may be the result of a diminished ability of ethylene glycol, the smallest of these substrates, to protect the inactive enzyme·Cbl^{II} complex from oxygen.

Glycerol is unique in that no significant change in the visible spectrum of enzyme-bound AdoCbl can be detected, yet glycerol is both a very good substrate and powerful inactivator. (A slight change in the spectrum from that observed for AdoCbl bound to enzyme in the absence of substrate or inactivator appears in Figure 4. The shoulder at 370 nm characteristic of alkylcobalamin is diminished slightly, while a new peak at 363 nm appears. This latter peak is undoubtedly due to a small amount of enzyme-bound OH-Cbl formed prior to addition of glycerol.) Initially, the visible spectrum probably represents enzyme-bound AdoCbl. Following inactivation, however, the visible spectrum must represent either a "trapped" intermediate or a new alkyl or thiocobalamin not normally formed during catalysis.

AdoCbl-dependent rearrangements have been postulated to take place via a transalkylation reaction (intermediate $\mathbf{III}_a$

is presumably formed from intermediate **II**.), although no concrete evidence has yet been obtained for such a reaction occurring with any AdoCbl-dependent enzyme. The visible spectrum of glycerol-inactivated AdoCbl·enzyme may be due to a "trapped" intermediate, such as **III**$_a$ or **III**$_b$. Intermediate



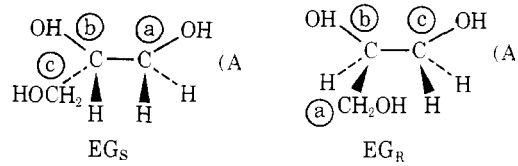
III$_b$ is more likely in view of the observed lability of the intermediate, secondary alkylcobalamins being generally less stable than primary alkyl cobalamins (Hogenkamp, 1975).

However, the above hypothesis does not account for the observed inactivity of apoenzyme upon removal of cobalamin and the apparent covalent labeling of apoenzyme by glycerol. In view of these results, a more likely scenario is attack of a reactive substrate moiety, such as pictured in intermediate **II**, on the enzyme, followed by, or concomitant with, a reaction between Cbl^{II} and an amino acid residue at the active site. This would account for the inability to remove the light-sensitive cobalamin intact from the active site, the inactivity of apoenzyme upon removal of cobalamin, and the observed covalent labeling of the enzyme by glycerol. However, this still does not account for the observed labeling of several molecules of glycerol/active site, a result for which at present we have no explanation.

The presence of a reactive apoenzyme moiety at the active site is also indicated by the observation that removal of OH-Cbl from oxygen-inactivated holoenzyme does not regenerate active apoenzyme. (This substrate-free inactivation has previously been attributed only to destruction of enzyme-bound cofactor which then remains irreversibly bound to the enzyme (Wagner et al., 1966).) In this respect, the mechanisms of inactivation by oxygen and glycerol may be somewhat similar. A further similarity between glycerol and oxygen-inactivated holoenzyme is seen in Table III. Continued dialysis vs. the Mg^{2+} and SO_3^{2-} solution past that required to remove enzyme-bound cobalamin restores some activity. Furthermore, the rate of the restoration appears to be about the same for both the glycerol- and oxygen-inactivated apoenzymes. This reactivation after prolonged dialysis is perhaps due to slow reduction of some oxidized species on the enzyme by SO_3^{2-} and is the subject of continuing investigation.

The above results indicate that inactivation almost certainly involves the breakdown of catalysis at some intermediate step. Destruction of cofactor would first be suspected as a likely cause of inactivation; however, in most cases, the enzyme-bound cobalamin species we observe (alkyl-cobalamin or Cbl^{II}) is one either observed during normal catalysis or associated with active holoenzyme. Furthermore, removal of the cobalamin species does not regenerate active apoenzyme. Although enzyme-bound OH-Cbl does eventually result with all inactivated holoenzyme complexes, such formation of OH-Cbl is probably the result, rather than the cause, of inactivation. This indicates that the protein must play an active role in inactivation, and, by implication, an active role in catalysis.

Stereospecificity of Catalysis and Inactivation. The two hydroxymethyl groups attached to the prochiral (C-2) carbon of glycerol are enantiotopic-paired substituents. This makes possible two chemically distinct diastereomeric combinations of enzyme with glycerol, designated for purpose of discussion as EG$_S$ and EG$_R$.

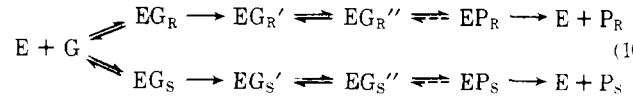


EG$_S$ EG$_R$

EG$_S$ represents glycerol bound to enzyme with the (pro-*S*)-hydroxymethyl group at the site involved in hydrogen abstraction ("A"), while EG$_R$ represents the corresponding enzyme-glycerol complex with the (pro-*R*)-hydroxymethyl group so oriented.

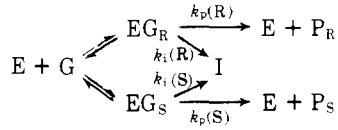
In the following discussion, we shall use results with variously deuterated glycerols to argue that product formation occurs mainly via a pathway through EG$_R$, while inactivation occurs via a pathway through EG$_S$.

If one assumes the mechanism of the enzyme-catalyzed reaction with glycerol to be the same as that with 1,2-propanediol, the minimum kinetic scheme that must be constructed is



(10)

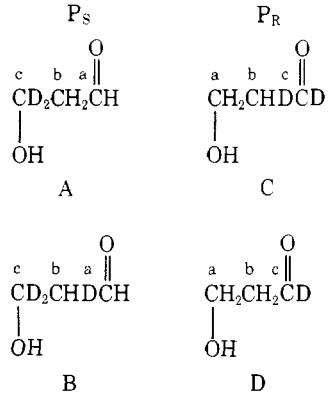
EG$_x$, EG$_x'$, EG$_x'''$, and EP$_x$ are analogous to intermediates **I–IV** in eq 9. To present the following largely qualitative argument, we shall further abbreviate the kinetic scheme above to



(11)

recognizing that $k_p(R)$ and $k_p(S)$ are composite constants representing many steps, including at least two steps involving hydrogen transfer (**I–II** and **III–IV**, eq 9). Each of these two steps can contribute to the expression of an isotope effect on $k_p(R)$ or $k_p(S)$. $k_i(S)$ and $k_i(R)$ are the rate constants for inactivation of the respective enzyme–glycerol complexes.

Consider first the products formed from (*R*)-glycerol-d_2; four are possible:



Products A and B are derived from EG$_S$, while C and D are derived from EG$_R$. (Products B and D result from intermolecular hydrogen transfer. That this occurs has been demonstrated by Essenberg et al. (1971) and involves C-5′ of cofactor as the intermediate hydrogen carrier.) ^{1}H NMR of the 2,4-dinitrophenylhydrazones of the above products indicates that better than 90% of products C and D are formed. This result is particularly striking, since products C and D should be disfavored relative to A and B due to kinetic isotope effects on

product formation. This is strong evidence that product aldehyde originates almost entirely from EG_R.

The observed kinetic isotope effects further support this conclusion. For example (*R*)-glycerol-d_2 and (*RS*)-glycerol-d_2 contain the same amount of deuterium. (*R*)-glycerol-d_2, however, exhibits a large isotope effect on product formation (6.8), while a much smaller effect (2.1) is observed with (*RS*)-glycerol-d_2. This clearly indicates that deuterium on carbon "c" has a greater effect on product formation than deuterium on carbon "a".

The isotope effect of 8.0 observed with glycerol-d_5 must represent the full extent to which an isotope effect can be expressed on catalysis, or more specifically, in light of the above arguments, on k_p(R). The reduction of the isotope effect from 8.0 to 6.8 for (*R*)-glycerol-d_2, however, must be due to some contribution from hydrogen located on carbon "a". The contribution may be in the form of a small amount of product formation from the EG_S form or possibly some very slow exchange between the deuterium of enzyme-bound coenzyme and hydrogen on the "a" carbon. Only a very small contribution (2%) from hydrogen on the "a" carbon is needed to account for the decrease of the isotope effect from 8.0 to 6.8.

Analogous arguments support the conclusion that inactivation occurs mainly via EG_S. For inactivation, glycerol-d_5 shows an isotope effect of 14; this must also represent the full extent to which an isotope effect can be expressed. Were hydrogen at carbon "c" as important to inactivation as to catalysis, an isotope effect on inactivation much larger than the observed 1.8 would be exhibited by (*R*)-glycerol-d_2. Accordingly, hydrogen at carbon "a" must make a significant contribution to inactivation.

Does the isotope effect of 1.8 for (*R*)-glycerol-d_2 reflect some inactivation via EG_R? There is an alternative explanation. When holoenzyme is inactivated by glycerol (no deuterium) in the presence of 1,1-dideuterio-1,2-propanediol, an isotope effect is expressed on the inactivation rate (Bachovchin et al., manuscript in preparation). Thus, deuterium located on 1,2-propanediol can participate in the inactivation reaction and contribute to the expression of an isotope effect on k_i. The mechanism of this effect most likely involves the 5′ carbon of cofactor as the intermediate hydrogen carrier. The importance of this result to the present discussion is that a similar effect must occur with (*R*)-glycerol-d_2. The EG_R binding conformation can behave as a deuterated substrate contributing deuterium to the coenzyme during catalysis. Moreover, that the coenzyme should be rich in deuterium follows from the much greater reactivity of the "c" carbon deuteriums in product formation and the much greater rate of catalysis compared to that of inactivation. Thus the small isotope effect of 1.8 with (*R*)-glycerol-d_2 as inactivator indicates inactivation predominantly via EG_S; little, if any, inactivation occurs via EG_R.

These conclusions are in stereochemical agreement with results obtained with 1,2-propanediol and 3-fluoro-1,2-propanediol (Eagar et al., 1975). With both of these substrates, we have found that the "R" binding conformation is more favorable for catalysis than is the "S" binding conformation. Furthermore, substitution of fluorine at C-3 of (*S*)-1,2-propanediol reduces catalysis by a factor of two, while the same modification of the R isomer results in almost no change in the catalytic rate. The present observation is that a hydroxyl group substituted at C-3 of (*R*)-1,2-propanediol has no detrimental effect on catalysis, while the same modification of the "S" isomer is very deleterious to catalysis.

Thus, a pattern is revealed indicating that the properties of the holoenzyme–substrate complex are such that a substituent on the methyl group directed away from the site of hydrogen abstraction is more readily tolerated for EG_R than for EG_S.

Substrate Specificity. Although the results presented here demonstrate that diol dehydratase holoenzyme binds and reacts with a wider array of substrates than previously thought, the specificity for 1,2-propanediol is still rather high. Inspection of the data in Table I reveals that any modification of the normal substrate results, as a general rule, in a decreased k_p, increased K_M, and the appearance of k_i. Glycerol is somewhat unusual in having an increased value of k_p relative to that of 1,2-propanediol, though this is more than offset by its large values of K_M and k_i.

Replacement of the C-3 methyl group by hydrogen (ethylene glycol) results in a 20-fold increase in K_M, as well as in irreversible inactivation. Thus, the methyl group plays an important role in both binding and in preventing inactivation. Since the rate of inactivation obtained with ethylene glycol is independent of the presence of oxygen (as it is with all inactivators listed in Table I), the methyl group does not prevent inactivation by protecting the active site from exposure to oxygen. The methyl group must somehow play a role in the proper positioning or stabilization of the substrate for catalysis. Comparison of the effects on K_M, k_p, and k_i observed with ethylene glycol, isobutylene glycol, 2,3-butanediol, and 1,2-butanediol indicates that these parameters are independently affected by the addition or deletion of a methyl group to the normal substrate skeleton, suggesting that steric factors important for binding are different from those that influence catalysis and inactivation. Surprisingly, substitution of a methyl group at C-1 (2,3-butanediol) does not entirely preclude catalysis, which must in this case involve abstraction of hydrogen from a secondary carbon atom, followed by rearrangement to give the corresponding ketone.

Substitution for hydrogen at C-3 of 1,2-propanediol by hydroxyl, thiol, fluorine, chlorine, methyl, or three fluorines substantially affects binding, catalysis, and inactivation. A quantitatively consistent picture relating these effects to the size or chemical nature of the substituent, however, does not emerge. A possible reason for this may be that each of the above analogues is, in reality, a mixture of two substrates, the R and S isomers. A given substituent may have an entirely different effect on the binding, catalysis, and inactivation behavior of each isomer, as we have shown for 3-fluoro-1,2-propanediol (Eagar et al., 1975) and glycerol.

Relation of Diol Dehydratase to Glycerol Dehydratase. Glycerol dehydratase is an AdoCbl-dependent enzyme isolated from a different strain of the same bacterium that produces diol dehydratase (Zagalak and Pawelkiewicz, 1962), and there are many similarities between these two enzymes.

These results reveal additional similarities. Like glycerol dehydratase, diol dehydratase also converts glycerol to β-hydroxypropionaldehyde and undergoes simultaneous inactivation. Moreover, values of K_M for glycerol and ethylene glycol reported with glycerol dehydratase (1.5×10^{-3} M and 0.66×10^{-3} M, respectively (Yakusheva et al., 1974)) are similar to those obtained with diol dehydratase (Table I). However, the rate constant for inactivation of diol dehydratase by glycerol (1.3 min^{-1}) differs significantly from that for glycerol dehydratase (0.35 min^{-1}) (Poznanskaya et al., 1972). Thus, the main kinetic difference between the two enzymes may be a greater rate for the inactivation of diol dehydratase by glycerol than for the inactivation of glycerol dehydratase; in other respects, these two enzymes are even more similar than previously thought.

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References

Abeles, R. H. (1972), *Proc. Robert A. Welch Found. Conf. Chem. Res. 15.*

Abeles, R. H., and Dolphin, D. (1976), *Acc. Chem. Res. 9,* 114.

Abeles, R. H., and Lee, H. A., Jr. (1964). *Ann. N.Y. Acad. Sci. 112,* 695–702.

Babior, B. M. (1969), *Biochim. Biophys. Acta 178,* 406.

Babior, B. M. (1975a), *Acc. Chem. Res. 8,* 376.

Babior, B. M. (1975b), Cobalamin, New York, N.Y., Wiley, Chapter 4.

Baer, E. G. (1952), *Biochem. Prep. 2,* 31.

Bruemmer, J. H., and Roe, B. (1971), *J. Agric. Food Chem. 19,* 266.

Carty, T. J., Babior, B. M., and Abeles, R. H. (1971), *J. Biol. Chem. 246,* 6313.

Circle, S. J., Stone, L., and Boruff, C. S. (1945), *Ind. Eng. Chem., Anal. Ed., 17,* 259.

Eagar, R. G., Jr., Bachovchin, W. W., and Richards, J. H. (1975), *Biochemistry 14,* 5523.

Englis, D. T., and Wollerman, L. A. (1952), *Anal. Chem. 24,* 1983.

Essenberg, M. K., Frey, P. A., and Abeles, R. H. (1971), *J. Am. Chem. Soc. 93,* 1242.

Fieser, L. E., and Fieser, M. (1967), Reagents for Organic Synthesis, New York, N.Y., Wiley, p 384.

Finlay, T. H., Valinsky, J., Mildvan, A. S., and Abeles, R. H. (1973), *J. Biol. Chem. 248,* 1285.

Frey, P. A., and Abeles, R. H. (1966), *J. Biol. Chem. 241,* 2732.

Frey, P. A., Essenberg, M. K., and Abeles, R. H. (1967), *J. Biol. Chem. 242,* 5369.

Ghangas, G. S., and Fondy, T. P. (1971), *Biochemistry 10,* 3204.

Hall, R. H., and Stern, E. S. (1950), *J. Chem. Soc.,* 490.

Hogenkamp, H. P. C. (1968), *Annu. Rev. Biochem. 37,* 225.

Hogenkamp, H. P. C. (1975), Cobalamin, Babior, B. M., Ed., New York, N.Y., Wiley, Chapter 1.

Huff, E. (1961), *Biochim. Biophys. Acta 48,* 506.

Jensen, R. F., and Neese, R. A. (1975), *Biochem. Biophys. Res. Commun. 62,* 816.

Lee, H. A., Jr., and Abeles, R. H. (1963), *J. Biol. Chem. 238,* 2367.

McBee, F. T., and Burton, T. M. (1952), *J. Am. Chem. Soc. 74,* 3022.

Pattison, F. L. M., and Norman, J. J. (1957), *J. Am. Chem. Soc. 79,* 2515.

Poznanskaya, A. A., Korsova, T. L., Malakhov, A. A., and Yakovlev (1972), *Vopr. Med. Khim. 2,* 145.

Retey, J., Umani-Rochi, A., Seibl, J., and Arigoni, D. (1966), *Experientia 22,* 502.

Schneider, Z., Larsen, E. G., Jacobsen, G., Johnson, B. C., and Pawelkiewicz, J. (1970), *J. Biol. Chem. 245,* 3388.

Schneider, Z., and Pawelkiewicz, J. (1966), *Annu. Rev. Biochem. 12,* 207.

Toraya, T., and Fukui, S. (1972), *Biochim. Biophys. Acta 284,* 536.

Toraya, T., Shirakashi, T., Kosoga, T., and Fukui, S. (1976), *Biochem. Biophys. Res. Commun. 69,* 475.

Wagner, O. W., Lee, H. A., Jr., Frey, P. A., and Abeles, R. H. (1966), *J. Biol. Chem. 241,* 1751.

Yakusheva, M. I., Malakhov, A. A., Poznanskaya, A. A., and Yakovlev, V. A. (1974), Anal. Biochem. 60, 293.

Zagalak, B., and Pawelkiewicz, J. (1962), *Life Sci. 8,* 395.