THE MECHANISM OF ACTION OF ADENOSYLCOBALAMIN-DEPENDENT DIOL DEHYDRATASE

I. GLYCEROL AND OTHER SUBSTRATE ANALOGS AS SUBSTRATES AND INACTIVATORS OF DIOL DEHYDRATASE: KINETICS, STEREOSPECIFICITY, AND MECHANISM.

II. HYDROGEN TRANSFER IN THE INACTIVATION OF DIOL DEHYDRATASE BY GLYCEROL.

III. HYDROGEN TRANSFER IN CATALYSIS BY DIOL DEHYDRATASE.

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To 'Toots'

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Chapter I

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, most 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. Experiments with isotopically substituted glycerols indicate that diol dehydratase distinguishes between "R" and "S" binding conformations, the enzyme-(R)-glycerol complex being predominantly responsible for the product-forming reaction, while the enzyme-(S)-

glycerol complex results primarily in the inactivation reaction. A comparative study of the reaction of meso- and dl-2,3-butanediols with diol dehydratase shows that the stereospecificity of hydrogen abstraction during inactivation is the same as that of catalysis, suggests that hydrogen abstraction from C-1 of substrate may be concerted with cleavage of the carbon-cobalt bond, and further suggests that formation of a carbon-cobalt bond between coenzyme and substrate is not obligatory for catalysis. 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.

Chapter II

ABSTRACT

We have investigated the kinetic characteristics of the inactivation of the adenosylcobalamin-dependent enzyme propanediol dehydratase by glycerol, (RS)-1,1-dideuterioglycerol, (R)-1,1-dideuterioglycerol, (S)-1,1-dideuterioglycerol, and perdeuterioglycerol in the absence of substrate and in the presence of 1,2-propanediol and 1,1-dideuteriopropanediol. Substitution of deuterium for hydrogen at C-1 of 1,2-propanediol or at the pro-(R) hydroxymethyl group of glycerol has no effect on the apparent dissociation constants K_I and K_G, respectively, of the holoenzyme-substrate complexes. In contrast, the same substitution at the pro-(S) hydroxymethyl group of glycerol increases the value of the apparent K_G. The results suggest that the binding of only the pro-(S) conformation is perturbed by this latter substitution. In the presence of a competing substrate, this K_G is observed to vary as a function of glycerol concentration in a manner indicating that kinetic terms involving steps of the reaction subsequent to binding of substrate contribute to determining its value. The inactivation kinetics obtained with these glycerols in the presence of 1,2-propanediol and 1,1-dideuterio-1,2-propanediol imply that hydrogen (or deuterium) attached to C-1 of 1,2-propanediol participates in the inactivation

process and contributes to the expression of a kinetic isotope effect on the rate of inactivation. The mechanism for this inactivation must involve the cofactor as an intermediate hydrogen carrier, presumably in the form of 5'-deoxyadenosine. Moreover, a mechanism involving a rate-determining transfer of hydrogen from an intermediate containing three equivalent hydrogens accounts for the results. When diol dehydratase holoenzyme is inactivated by [1-^{3}H]-glycerol, 5'-deoxyadenosine which is enriched in tritium by a factor of 2.1 over that in glycerol can be isolated from the reaction mixture.

Chapter III

ABSTRACT

Studies (Chapter II; Bachovchin et al. (1978) Biochemistry 17, 2218) of the mechanism of inactivation of adenosylcobalamin-dependent diol dehydratase have led to the development of a general method to describe the kinetics of a reaction pathway containing a reservoir of mobile hydrogen. Analysis by this method of catalytic rate measurements for mixtures of 1,2-propanediol and 1,1-dideuterio-1,2-propanediol supports a mechanism involving an intermediate with three equivalent hydrogens, in which hydrogen transfer from this intermediate to product is the major rate-contributing step. The results of this analysis predict that diol dehydratase should exhibit a tritium isotope effect of 4.5, a prediction which was verified experimentally. Other results using tritium as a trace label (Essenberg et al. (1971) J. Am. Chem. Soc. 93, 1242) are discussed in light of these deuterium isotope studies. Results of analogous experiments employing mixtures of glycerol and perdeuterioglycerol as substrates suggest that during catalysis with this substrate, neither hydrogen abstraction from C-1 of glycerol nor from the intermediate is strictly rate determining.

ABSTRACTS OF PROPOSITIONS

Proposition I

In light of the studies reported in the body of this thesis, further investigations on the mechanism of adenosylcobalamin-dependent diol dehydratase are proposed.

Proposition II

Metastasis of cancer cells can be inhibited by introduction into the cancer host of microorganisms such as Bacillus Calmette-Guerin and Corynebacterium parvum. Experiments are proposed to study the possible interactions of these agents with the immune system in inhibiting metastasis.

Proposition III

Complement fixation via the classical pathway is initiated by interaction of the complement protein Cl_q with the heavy chain constant (C_H) region of immunoglobulin M and certain classes of immunoglobulin G. Certain C_H amino acids, in particular tryptophan and tyrosine, have been implicated in this interaction. A study of the pH-dependence of complement fixation by immunoglobulins, and of inhibition of complement fixation by peptides, containing 3-nitrotyrosine is proposed.

Proposition IV

A recombinant DNA study of the structure of genes coding for immunoglobulin heavy chains is proposed. In particular, sequence analysis of the "introns", untranslated segments of DNA which separate coding regions, may prove enlightening in relation to antibody gene evolution.

Proposition V

Release into the environment of large amounts of synthetic, nonbiological organic compounds is a major concern. The use of bacterial mutagenesis techniques for creation of mutant microorganisms with enzymes capable of converting these compounds into biodegradable products is proposed.

INTRODUCTION

Background

The red, crystalline substance known as vitamin B_{12} was purified in 1948 at the Merck (Rickes et al., 1948) and Glaxo Laboratories (Smith & Parker, 1948) after a twenty-year search for the anti-pernicious anemia factor present in liver. While this success ended a protracted period of intensive effort, it also marked the birth of a singularly fascinating area of research, which has made important contributions to both the biological and chemical sciences.

The structure of vitamin B_{12} was announced by Hodgkin et al. (1955) (Fig. 1). The molecule contains a cobalt atom (Co^{3+}, low spin d_6) coordinated to the four nitrogen atoms of corrin, a tetrapyrrole macrocycle. Corrin is superficially similar to porphyrin, but lacks the latter's completely conjugated π-electron system and strictly planar configuration, and has a methine instead of the usual methylene bridge between two of the rings ("A" and "D"). The lower (α) ligand site is occupied by an unusual nucleotide, 5,6-dimethylbenzimidazole, while the β-ligand site may be occupied by a ligand such as those shown. Of the β-ligands indicated, all but cyanide occur naturally; the latter is introduced as an artifact of the purification procedure. The species with the nucleoside (adenosylcobalamin, AdoCbl, coenzyme B_{12}) and the methyl group (methylcobalamin, MeCbl) as β-ligands are unique in their

Figure 1

(a) Derivatives of cobalamin. R = CH_2CONH_2, R' = $CH_2CH_2CONH_2$

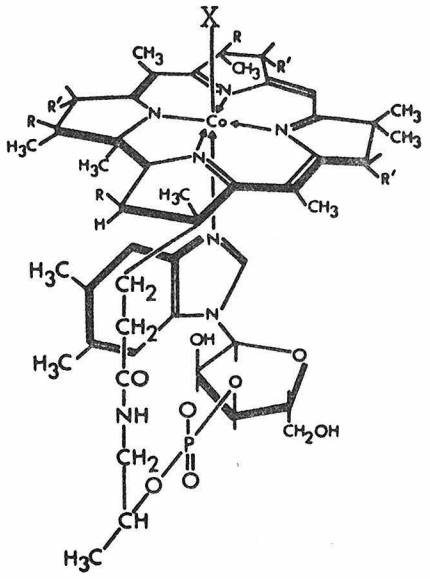
X = OH (hydroxycobalamin, OH-Cbl)

CN (cyanocobalamin, CN-Cbl)

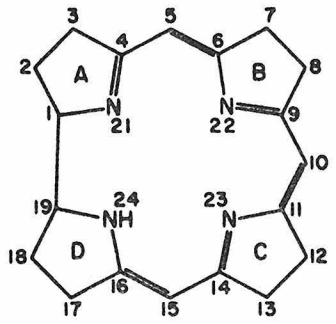
CH_3 (methylcobalamin, Me-Cbl)

5'-deoxy-5'adenosyl (adenosylcobalamin, AdoCbl)

(b) Corrin. Letters A-D identify the individual pyrroline rings. The numbering system corresponds to that of the porphyrin ring; hence the number 20 has been omitted (Hogenkamp, 1975).


X
R
R'
CH3
H3C
N
Co
H
CH2
CO
NH
OH
O
P
CH2OH

(a)


A
B
C
D
NH
3
4
5
6
7
2
8
1
21
22
9
10
19
24
23
11
18
12
16
14
17
15
13

(b)

Figure 1

being the only known biologically occurring compounds (besides the alkyl mercurys) with a carbon-metal bond. The structure of adenosylcobalamin was announced by Lenhert & Hodgkin (1961).

The structural complexity of the cobalamins is mirrored in the roles which they play in biology. In man and other mammals, the importance of B_{12} is evinced by the symptoms which occur in its absence. The most common of these are pernicious (megaloblastic) anemia, and neurological abnormalities associated with the appearance of lesions on and the demyelination of the spinal cord. As little as 1 μg/day of cobalamin is required by man to avert these symptoms. While the precise metabolic role played by cobalamins in ameliorating these conditions remains unclear, evidence has been obtained implicating their direct or indirect involvement in the biosynthesis of deoxyribonucleotides and metabolism of fatty acids (Beck, 1975; Kishimoto, et al., 1973; Cardinale et al., 1970; Barley et al., 1972; Green et al., 1975). In addition, the methyl derivative acts as a methyl transfer agent in the conversion of homocysteine to methionine, and may also participate in nucleotide biosynthesis.

Mammals do not synthesize cobalamin, and have developed an elaborate absorption and transport system to extract it from their diet (Ellenbogen, 1975). Cobalamin which has been freed by the digestive process is bound to a protein

termed intrinsic factor. The intrinsic factor-cobalamin complex then binds to the microvilli of the distal ileum in a process which requires Ca^{2+} and pH above 6.4. Once the complex is bound, cobalamin is released into the cell in an energy-requiring step, from which it enters the blood and binds to globulins called transcobalamins, for transport and storage.

All cobalamin is synthesized exclusively by microorganisms, which also use this molecule to perform a variety of chemical reactions. These reactions encompass such diverse processes as isomerizations and synthesis of deoxyribonucleotides. The involvement of cobalamin in the fundamental process of nucleic acid biosynthesis bespeaks an evolutionary antiquity which constitutes an apparently marked contrast to its structural complexity. Clearly, the evolutionary history of cobalamin, if and when elucidated, will present a fascinating picture.

Adenosylcobalamin-Dependent Enzymes

Study of the cobalamin-dependent reactions which occur in microorganisms, and include analogs of those known or suspected to occur in mammalian metabolism, has provided the bulk of current knowledge about the mechanism of action of cobalamin as a prosthetic group. Perhaps the most intriguing of these reactions are those illustrated in

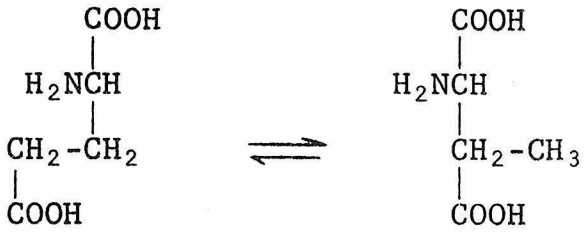
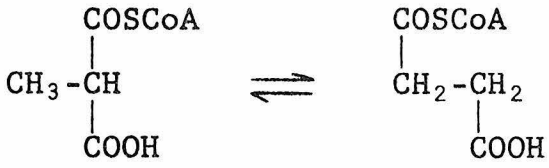
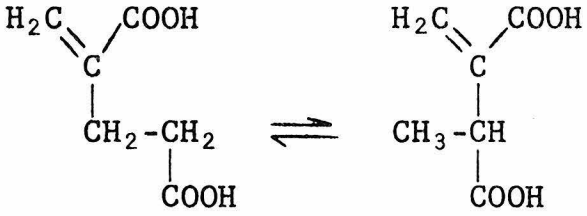
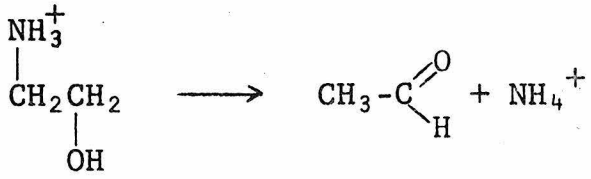
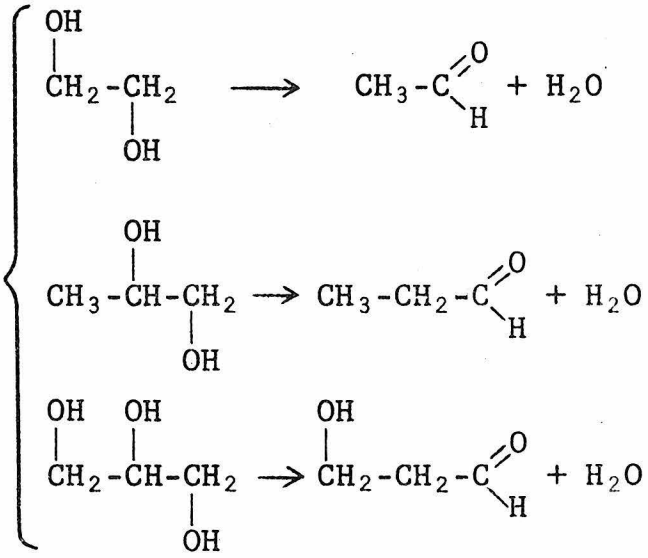
Fig. 2: a family of eleven AdoCbl-dependent reactions, ten rearrangments and a reduction. The apparent variety of the rearrangements is somewhat illusory, and all may be generalized by the following scheme:

a vicinal interchange of hydrogen and a group "X". Variation of the latter, which may be an -OH, $-NH_2$, alkyl, or acyl group, is responsible for the observed diversity of the reactions. Mechanistic studies have shown that, unlike most enzyme-catalyzed reactions, these rearrangements have essentially no counterpart in laboratory chemistry.

Figure 3 summarizes the current, generally accepted mechanism of adenosylcobalamin-dependent rearrangements. The first step is homolytic cleavage of the carbon-cobalt bond, a reaction which appears to be facilitated by binding of cofactor to enzyme, even in the absence of substrate. Subsequently, a hydrogen atom is transferred from C-1 of substrate to C-5' of the nucleoside moiety. This hydrogen atom becomes stereochemically equivalent to those initially present at C-5' by rotation about the C-4'-C-5' bond. The migration of group X from C_1 to C_2 is accomplished by a process which is not understood. (Some mechanisms suggested for this rearrangement are listed in Fig. 4. As discussed in Chapter III we currently favor the third one listed.)

Figure 2

Adenosylcobalamin-Dependent Reactions. †These enzymes have been found in mammalian tissues.

Enzyme	Reaction
1. Glutamate mutase	
2. Methylmalonyl CoA mutase†	
3. α-Methyleneglutarate mutase	
4. Ethanolamine deaminase	
5. Diol dehydratase	
6. Glycerol dehydratase	

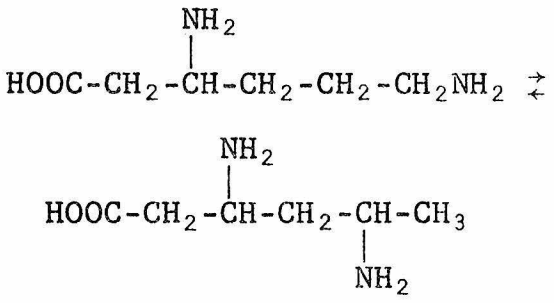
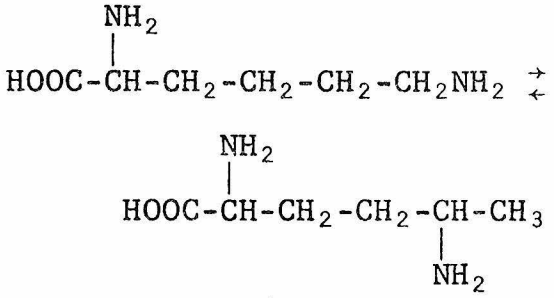
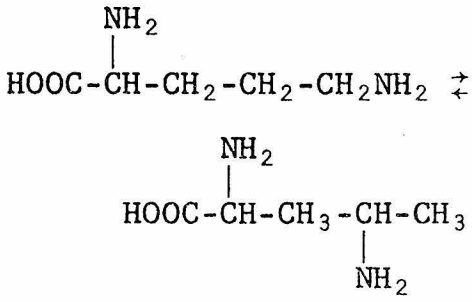
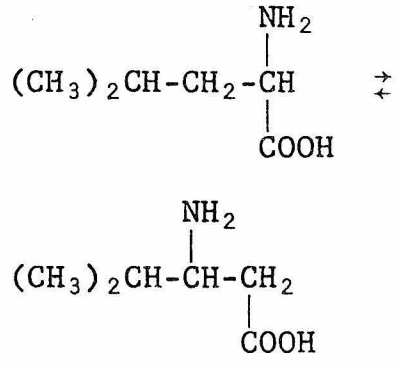
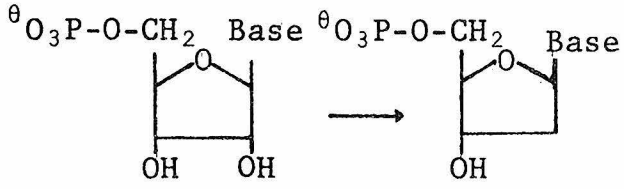
Enzyme	Reaction
7. l-β-Lysine mutase	
8. d-α-Lysine mutase	
9. d-Ornithine mutase	
10. Leucine 2,3-Aminomutase (Poston, 1976; 1977).†	
11. Ribonucleotide reductase	

Figure 3

Mechanism of Adenosylcobalamin-Dependent Rearrangements.

Adenosylcobalamin is represented schematically as $>Co(CH_2Ad)<$, substrate as S, and product as P.

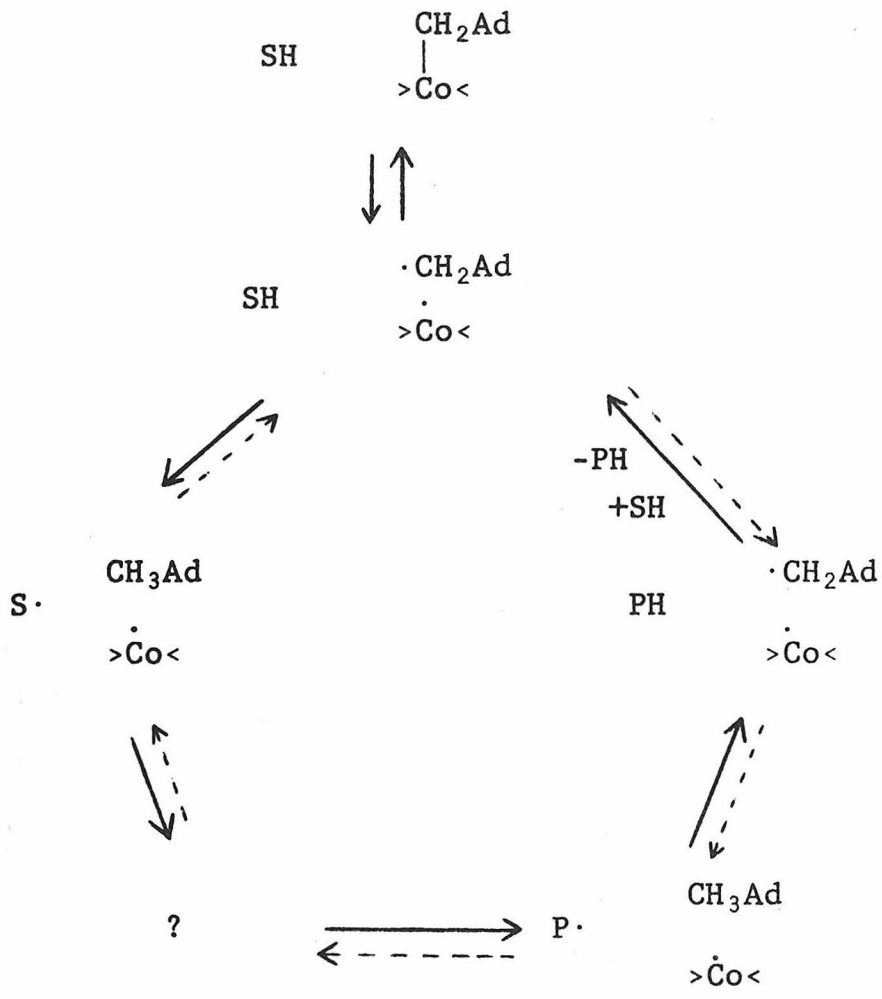


Figure 3

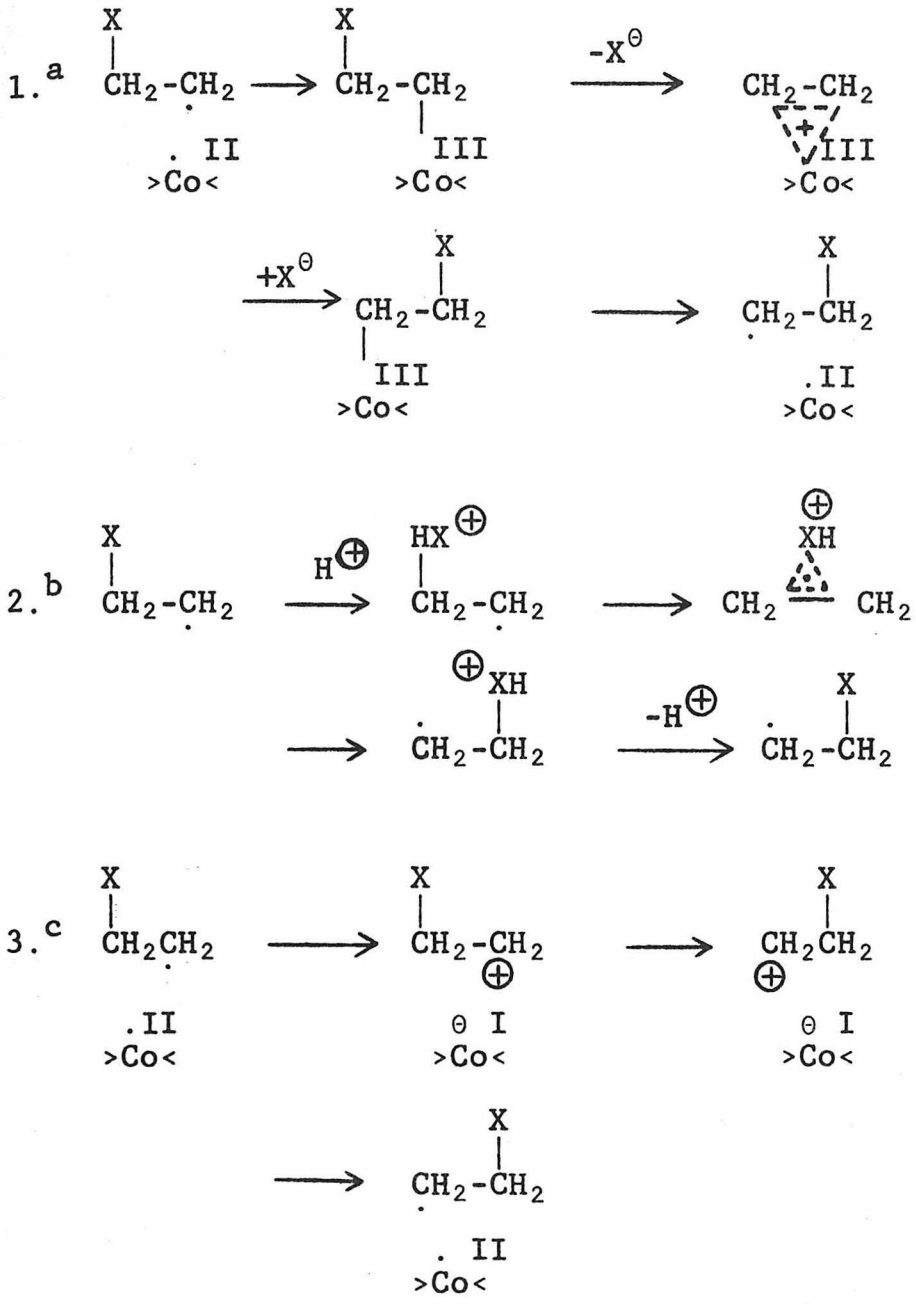
To complete the rearrangement, one of the three hydrogens at C-5' of the cofactor is transferred to C-2 of substrate. With the enzymes diol dehydratase, glycerol dehydratase, and ethanolamine deaminase, an HX group is then eliminated stereospecifically from C-1 prior to release of product.

Hydrogen transfer. Because hydrogen transfer reactions lend themselves to studies involving isotopic substitution, this aspect of the catalytic mechanism has been examined thoroughly. The migration of hydrogen occurs without exchange with solvent in these rearrangements (Brownstein & Abeles, 1961; Babior, 1969; Somack & Costilow, 1973; Erfle et al., 1964; Iodice & Barker, 1963; Kung & Tsai, 1971; Wood et al., 1964). All of these reactions exhibit a primary isotope effect when hydrogen-isotope labeled substrates are used, indicating that cleavage of a C-H bond is an important rate-contributing step.

The fate of the hydrogen derived from C-1 of substrate has been studied in detail. Early observations with diol dehydratase (Brownstein & Abeles, 1961) showed that hydrogen removed from C-1 appeared at C-2 of the product, thus seeming to indicate that the rearrangement is intramolecular However, the key experiment which showed otherwise was carried out by Abeles & Zagalak (1966). Diol dehydratase was allowed to react with mixtures of [1-^{3}H]-1,2-propanediol and unlabeled ethylene glycol, and tritium was found

Figure 4

Possible Mechanisms for Migration of Group X.



a) Silverman & Dolphin (1976).

b) Golding & Radom (1976).

c) Eagar et al. (1972); Halpern (1974).

at C-2 of acetaldehyde, the product derived from ethylene glycol. This result implicated the holoenzyme as an intermediate hydrogen carrier. In later work, the 5'-carbon of AdoCbl was found to play this role in the diol dehydratase (Frey & Abeles, 1966), ethanolamine deaminase (Babior, 1968), and several other rearrangements. During catalysis with tritiated substrate, AdoCbl containing tritium at C-5' can be isolated by denaturing the holoenzyme complex with trichloroacetic acid. Furthermore, both this biosynthetically labeled coenzyme and the synthetically labeled species, when added to apoenzyme transfer all of their tritium to product during catalysis (Frey et al., 1967a).

Particularly interesting is the observation that both C-5' hydrogens participate in this reaction (Frey et al., 1967b), an unexpected result in view of the observed stereospecificity of enzymatic reactions. Presumably, this would only occur if the two C-5' hydrogen atoms become stereochemically equivalent during the course of catalysis. Futhermore, experiments carried out with mixtures of [1-^{3}H]-1,2-propanediol and unlabeled ethylene glycol as substrate indicated (Frey et al., 1967a) that even with infinite amounts of the latter, tritium would still be found at C-2 of propionaldehyde product. Thus, there is a finite probability for hydrogen to be returned to C-2 of the same molecule from which it was abstracted. These

observations suggest the intervention of an intermediate containing at least three stereochemically equivalent hydrogens, one of which is transferred to C-2 of the product molecule.

The presence of this three-hydrogen intermediate along the catalytic pathway has been inferred in a number of studies. Use of ^{3}H as a trace label in the diol dehydratase (Frey et al., 1967a; Essenberg et al., 1971) and ethanolamine deaminase (Babior, 1969; Weisblat & Babior, 1971) reactions has supplied such evidence. The methylmalonyl CoA mutase (Miller & Richards, 1969) and glutamate mutase (Eagar et al., 1972) reactions were studied by allowing mixtures of unlabeled and deuterated substrates to proceed partially toward equilibrium, after which the distribution of deuterated substrate and product molecules was determined by mass spectroscopy for comparison to distributions calculated for various models of the reaction. The results of these and other studies point to the existence of an intermediate in which the hydrogen derived from substrate becomes equivalent to the two hydrogens present at C-5' of AdoCbl.

A likely candidate for this intermediate is 5'-deoxyadenosine, as first suggested by Ingraham (1964). A plausible mode of its formation would be rupture of the carbon-cobalt bond of AdoCbl, followed by hydrogen transfer

to the 5'-carbon atom of the resulting nucleoside. Stereochemical equivalence of the three hydrogens could be effected by rapid rotation of the C-5' methyl group, as has been observed by ^{1}H NMR for nucleosides bound to ethanolamine deaminase (Hull et al., 1975). In fact, formation of 5'-deoxyadenosine has been demonstrated in the diol dehydratase (Wagner et al., 1966; Finlay et al., 1972), methylmalonyl CoA mutase (Babior et al., 1973), l-β-lysine mutase (Baker et al., 1973), and ethanolamine deaminase (Babior, 1970a) reactions. However, in these cases 5'-deoxyadenosine was formed during reaction with substrate analogs which irreversibly inactivate holoenzyme; hence, 5'-deoxyadenosine could be construed as the product of a destructive side reaction, rather than of catalysis. In contrast, results obtained from further studies of ethanolamine deaminase (Babior, 1970b; Babior et al., 1974a), in which 5'-deoxyadenosine was detected upon interruption of catalysis, showed that the amount of 5'-deoxyadenosine recovered varied with the denaturing agent used. This result was interpreted to indicate reversible formation of this moiety during catalysis. Thus, the role of 5'-deoxyadenosine as an intermediate hydrogen carrier in AdoCbl-dependent rearrangements is fairly well established.

Cleavage of the Carbon-Cobalt Bond. In principle, the carbon-cobalt bond of AdoCbl may be cleaved in three ways to produce a 5'-deoxyadenosyl fragment:

$\oplus$ CH_2Ad $\cdot CH_2Ad$ $\ominus$ CH_2Ad

$>\ddot{C}o<$ $>\dot{C}o<$ $>Co<$

(Cbl^{I}) (Cbl^{II}) (Cbl^{III})

Each type of cleavage would produce a cobalt atom of a different, stable electronic structure (Co^{I}, d_8; Co^{II}, d_7; or Co^{III}, d_6). In addition, this reaction presumably determines the manner in which hydrogen is transferred, i.e., as hydride, atom, or proton. Thus, the nature of this reaction has been the subject of considerable effort. Current evidence, mostly spectroscopic, favors the second of these, homolytic cleavage to yield a Cbl^{II} species and 5'-deoxyadenosyl radical. When diol dehydratase (Wagner et al., 1966; Eagar et al., 1975) and ethanolamine deaminase (Joblin et al., 1975) holoenzymes are allowed to react with their respective substrates, the uv-visible absorption spectrum of the holoenzyme complex changes from an AdoCbl spectrum to one very similar to that of Cbl^{II} (low spin d_7), at a rate kinetically competent with catalysis. Upon depletion of added substrate, the spectrum changes to one characteristic of OH-Cbl (Fig. 1, Cbl^{III}) in an oxygen-dependent process which results in destruction of enzyme-bound cofactor (Wagner et al., 1966) and inactivation of holoenzyme. A similar reaction occurs in the presence of oxygen with Cbl^{II} free in solution.

In efforts to detect and characterize unpaired electrons

in these systems, electron spin resonance experiments have been carried out with ethanolamine deaminase (Babior et al., 1972; 1974b), diol dehydratase (Finlay et al., 1973), and glycerol dehydratase (Cockle et al., 1972). When solutions of these holoenzymes are frozen during reaction with substrates or substrate analogs, spectra are observed with a signal at $g = 2.2$ (assigned to Cbl^{II}), and a narrower doublet signal at $g \sim 2$, attributed to a free radical. The appearance of these signals requires the presence of both holoenzyme and substrate or analog, and occurs on a time scale sufficiently rapid to represent actual catalytic intermediates (Valinsky et al., 1974). These signals have been simulated in terms of a weak electrostatic exchange interaction between two unpaired electrons (Schepler et al., 1975).

Thus, a variety of evidence for homolysis of the cobalt-carbon bond of AdoCbl has been accumulated; the participation of this species as a catalytic intermediate seems to be on solid ground. Recently, a paper has appeared (Toraya et al., 1979) which indicates that cleavage of this bond is effected by interaction of the peripheral amide side chains of the corrin ring with the protein in the diol dehydratase reaction.

Migration of Group X. While the above aspects of the mechanism of AdoCbl-dependent rearrangements (Fig. 3) are fairly well established, very little is known about the

nature of group X migration. Elegant stereochemical studies employing ^{18}O-labelled substrates enabled Retey et al. (1966b) to infer the existence in the diol dehydratase reaction of a 1,1-propanediol intermediate which is dehydrated stereospecifically to aldehyde by the enzyme. The carbon from which group X migrates undergoes a net inversion of configuration in the diol dehydratase (Retey et al., 1966a; Zagalak et al., 1966) and glutamate mutase (Sprecher & Sprinson, 1964) reactions, while retention was observed with methylmalonyl CoA mutase (Sprecher et al., 1964; Retey & Zagalak, 1973) and racemization with ethanolamine deaminase (Retey et al., 1974).

A standard enzymological technique used in approaching such problems is to demonstrate an enzyme-catalyzed exchange between the reaction products and compounds thought to represent the form of the intermediate in question. These experiments have enjoyed no success in studies of AdoCbl-dependent enzymes. Moreover, attempts to generate active holoenzyme by allowing apoenzyme to interact with 5'-deoxyadenosine and several cobalamin derivatives postulated to represent catalytic intermediates (Carty, 1973; Eagar, 1974; Krouwer & Babior, 1977; Krouwer et al., 1978) have also failed.

In view of the lack of information concerning group X migration which is available from study of the enzyme mechanisms, chemists have examined model reactions in

efforts to explain this aspect of the reaction. Some of these studies and the proposed mechanisms arising from them (Fig. 4) have been reviewed (Schrauzer, 1971, 1976; Brown, 1973; Abeles & Dolphin, 1976). More recently, Golding & Radom (1976) have suggested that 1,2-rearrangements of radicals can be facilitated by protonation of the migrating group. Corey et al. (1977) have suggested a novel mechanism involving oxidative addition and reductive elimination with substrate and Cbl^{I} in which the corrin ring undergoes electrocyclic cleavage; however, several features of the enzymatic reactions are inconsistent with this mechanism. For example, no evidence has been found for the existence of an intermediate in which substrate becomes covalently attached to cobalt; the Corey mechanism does not account for the observation of ESR spectra exhibiting Cbl^{II} and organic radical resonances; the acetamide group at C-2 of corrin, postulated to initiate electrocyclic cleavage, is at best a questionable nucleophile. At present, no genuinely satisfactory picture of the mechanism of these rearrangements has arisen from model reactions studied thus far.

The work presented in this thesis describes studies on a new class of reactions of diol dehydratase. Diol dehydratase catalyzes the conversion of a number of vicinal diols other than 1,2-propanediol to the corresponding carbonyl compounds. In addition, in a concomitant and

competing reaction, these substrates irreversibly inactivate holoenzyme. We have carried out a detailed mechanistic study of the inactivation reaction (Chapters I and II). The results of these experiments suggested further experiments on the mechanism of catalysis, which are reported in Chapter III. The results, which indicate that the mechanism of inactivation is strikingly similar to that of catalysis, and suggest an important role for the protein in this rearrangement, are of value in enhancing our understanding of the mechanism of action of adenosylcobalamin as a cofactor in enzymatic processes. Some of the work described has already appeared (Bachovchin et al., 1977, 1978; Moore et al., 1979; Moore & Richards, 1979).

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CHAPTER I

GLYCEROL AND OTHER SUBSTRATE ANALOGS AS SUBSTRATES AND INACTIVATORS OF DIOL DEHYDRATASE: KINETICS, STEREOSPECIFICITY, AND MECHANISM.

INTRODUCTION

Propanediol dehydratase ((RS)-1,2-propanediol hydrolyase; EC 4.2.1.28) from Klebsiella pneumoniae (ATCC 8724) is one of ten known enzymes which utilize adenosylcobalamin as a cofactor to catalyze 1,2-rearrangements of hydrogen and some group X, where X can be an alkyl, acyl, amino, or hydroxyl substituent (Hogenkamp, 1968; Babior, 1975a,b; Abeles & 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 C-2 hydroxyl oxygen of the two isomeric substrates (Retey et al., 1966)), relatively minor structural modifications can abolish catalytic or inhibitory activity (Lee & Abeles, 1963). Toraya & 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. Toraya et al. (1976) also reported that glycerol functions both as a substrate and an inactivator of diol dehydratase.

This chapter reports results with a number of modified vicinal diols including glycerol, thioglycerol, 3-chloro-1,2-propanediol, 1,2-butanediol, isobutylene glycol,

3,3,3-trifluoro-1,2-propanediol, and meso-, d-, and dl-2,3-butanediols. In general, these substances act as substrates (leading to product formation by the usual rearrangement), and also bring about irreversible inactivation of holoenzyme. 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 the 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 a component 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 & 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 rate constant for product formation exceeds that observed for the noninactivating substrate 1,2-propanediol.

EXPERIMENTAL

Enzyme Preparations. Diol dehydratase was obtained from Klebsiella pneumoniae by a procedure similar to that

of Lee & Abeles (1963). Fraction E-8 with a specific activity of between 25 and 60 was used for all determinations. 1,2-Propanediol-free enzyme was prepared by dialysis at 4° C against several changes of 10 mM K_2HPO_4 buffer (Frey et al., 1967).

Adenosylcobalamin. Adenosylcobalamin (AdoCbl) was purchased from Sigma Chemical Co.

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) and β-nicotinamide adenine dinucleotide (reduced form, NADH, Sigma) reduce propionaldehyde to 1-propanol. The maximum velocity of this reaction is 30 % of that for acetaldehyde reduction (Bruemmer & Roe, 1971). The production of propionaldehyde by diol dehydratase can be measured by monitoring the decrease of absorbance at 340 nm, due to 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, 0.2 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 or inactivator. Total volume 2.3 ml. The reaction mixture was incubated at 37° C with stirring. The inactivation reaction was started by addition of AdoCbl and stopped by addition of 0.05 ml 6 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 ($k_{i,obs}$) for the given concentration of inactivator and 1,2-propanediol. The reproducibility of $k_{i,obs}$ as measured by this method is quite reliable, usually within three to five percent. However, when $k_{i,obs}$ is quite small (less than about 0.06 min^{-1}) or large (greater than about 1.2 min^{-1}) the uncertainty can in some cases approach eight or nine percent.

For rate determinations under anaerobic conditions, both the reaction mixture and 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, about 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 2.0 ml, 37° C. The reaction was started by addition of AdoCbl and the inactivation quenched with 0.05 ml 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 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 diol dehydratase, ~3 units, potassium phosphate buffer, pH 8.0, 30 mM; bovine serum albumin, 0.4 mg; AdoCbl, 0.013 mM; and glycerol, 70 mM. Volume 2.0 ml, 37° C. The reaction was started by the addition of AdoCbl and quenched by the addition of 0.1 ml 2 N HCl.

β-Hydroxypropionaldehyde can also be determined satisfactorily using the DNP assay as described for propionaldehyde. The reaction mixtures used were the same as above with 0.6 unit of diol dehydratase 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 derivative of propionaldehyde.

[1-^{14}C]-Glycerol Labeling of Diol Dehydratase. Reaction mixtures consisted of the following: apoenzyme, 40 units; [1-^{14}C]-glycerol (0.47 mCi/mmole), 0.14 M; potassium phosphate buffer, pH 8.0, 30 mM; AdoCbl, 0.03 mM; volume 0.5 ml. Two controls were performed, one in which AdoCbl was omitted from the reaction and one in which OH-Cbl was substituted for AdoCbl. The reaction mixtures were incubated at 37° C for 30 min. Two basic procedures were used for removing glycerol, trichloroacetic acid (TCA) precipitation of the protein, or gel chromatography, in each case followed by dialysis. Following the 30 min incubation period, 0.04 ml 20 % TCA was added. After centrifugation, the supernatant was removed and the protein pellet was washed in 0.5 ml H_2O and centrifuged. The resulting protein pellet was suspended in 1.0 ml H_2O and dialyzed successively against 0.13 l 10 mM K_2HPO_4, 3 x 0.13 l of a solution containing 8 M urea; 50 mM Tris citrate, pH 4; 1 mM disodium EDTA; and 0.1 % sodium dodecyl sulfate, and finally against 3 x 0.13 l 10 mM K_2HPO_4. When the radioactivity of the dialysis solution had diminished to background, 0.45 ml of the sample and 0.45 ml H_2O were added to 10 ml PCS:xylene (2:1) and assayed for ^{14}C. A 0.1 ml aliquot was assayed for protein content by the method of Lowry et al. (1951).

Alternatively, the 0.5 ml sample was applied to a 2.6 x 15 cm BioGel P-150 column and eluted with a buffer

containing 10 mM K_2HPO_4, 0.1 M glycerol, and 0.01 % NaN_3. The protein-containing fractions were pooled and dialyzed as described above. A 0.9 ml aliquot of the dialysate was assayed for radioactivity and a 0.4 ml aliquot for protein.

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

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

(R)-1,1-Dideuterioglycerol ((R)-gly-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 & 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 esterified with methyl iodide in hexamethylphosphoramide. The methyl ester was then reduced with lithium aluminum deuteride (99 % D, Stohler Isotope Chemicals). The ketal was hydrolyzed in ethanol-sulfuric acid and the product distilled to give (R)-1,1-dideuterioglycerol with $[\alpha]_D^{25}$-0.14°[1]. Deuterium content was at least 98 % as determined by proton NMR.

(RS)-1,1-Dideuterioglycerol ((RS)-gly-d_2) was prepared

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1. Optical rotations are uncorrected for water content (Huff, 1961).

from (RS)-1,2-isopropylidene glycerol (Ghangas & Fondy, 1971) by a method analogous to that described above.

(S)-1,1-Dideuterioglycerol ((S)-gly-d_2) was prepared from 1,1-dideuterio-(d)-2,3-isopropylidene glycerol (Eagar et al., 1975). The alcohol (47g, 0.351 mole) was dissolved in 250 ml H_2O and a few crystals of p-toluene sulfonic acid added. Water and acetone were removed by evaporation at ∿ 50°C under reduced pressure. To the resulting syrup was added a 10% molar excess of distilled benzaldehyde and 250 ml benzene. Water was removed by repeated azeotropic distillation with benzene under reduced pressure. The syrup was dissolved in 150 ml benzene and washed twice with 2% K_2CO_3. The combined aqueous washings were extracted with benzene, which was added to the organic fraction. The benzene solution was dried and evaporated to a syrup, which contained a mixture of 1,2- and 1,3-benzylidene ketals of (R)-gly-d_2 (Sedarevich, 1966; Cambie et al., 1972). Several bubbles of dry HCl were passed through the syrup, which was then dissolved in ∿ 500 ml of 4:3 ligroin: benzene. The solution was allowed to cool to 4°C. Addition of a tiny seed crystal initiated crystallization of cis-1,3-benzylidene-(R)-1,1-dideuterioglycerol, which was collected by filtration and washed with cold solvent. Several repetitions of this procedure, followed by recrystallization of the collected material, yielded 26.7g,

(0.147 mole) of the cis ketal, mp 83° (Cambie et al., 1972).

The ketal was inverted to trans-1,3-benzylidene-(S)-1,1-dideuterioglycerol-2-benzoate by the following procedure, adapted from Mitsunobu & Eguchi (1971) and Alfredsson & Garegg (1973). The ketal (26g) and triphenylphosphine (75g) were dissolved in 250 ml dry tetrahydrofuran (THF) in a flame-dried flask under argon while cooling in an ice bath. A solution of 35g benzoic acid and 50g diethylazodicarboxylate, in dry THF, was added dropwise with stirring. After stirring overnight, the solution was washed with 5% K_2CO_3, dried over Na_2SO_4, and evaporated to dryness under reduced pressure. The solid was dissolved in a minimum amount of 4:1 toluene:ethyl acetate and chromatographed on silica gel in this solvent. The desired product eluted first, and was evaporated to dryness. The solid was dissolved in a minimum amount of acetone and allowed to dry slowly. The resulting crystalline mass was washed with methanol and dried in vacuum over KOH. Yield 22.8g (80 mmole), mp 103-104°.

The benzoate ketal was refluxed for 3 hr in 1 N HCl. The acid solution was cooled and extracted with chloroform and ethyl acetate. The aqueous layer was concentrated under reduced pressure. Distillation under vacuum at a bath temperature of 155-170° gave 4.6g (49 mmol) (S)-1,1-dideuterioglycerol, $[\alpha]_D^{25}$ +0.14.°

(RS)-1,1-Dideuterio-1,2-propanediol was prepared by lithium aluminum deuteride reduction of dl-ethyl lactate (Fieser & 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 & Burton, 1952), which was collected by fractional distillation at 124-124.5°C. The alcohol (12.8g, 0.0674 mole) was then added to a solution of 4g 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 & Norman, 1957).

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

3-Chloro-1,2-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.

meso-2,3-Butanediol was purchased as a mixture of the meso and d isomers from Aldrich and distilled before use. A 1 M solution showed virtually no optical rotation, so little or no d isomer is present. The l isomer is not a natural product; as the Aldrich material is prepared biologically, it was assumed to contain no l isomer.

d-2,3-Butanediol was purchased from Burdick & Jackson and had $[\alpha]_D^{25}$ - 11.25° (c = 1 M). This compound contained approximately 1.6 mole % 1,2-propanediol as an impurity, as determined by kinetic and chemical analysis with diol dehydratase holoenzyme. This impurity was removed enzymatically prior to use in kinetic experiments.

dl-2,3-Butanediol was prepared by oxidation of cis-2-butene with performic acid. 22g cis-2-Butene was condensed in a 1-l 3-neck flask at -10°C. Chloroform (125 ml) was added with stirring. A solution was prepared by mixing 41g 90% formic acid and 44.5g 30% H_2O_2 and was added dropwise with stirring, with the temperature kept between -5° and -10° C. After 15 hr, the solution was concentrated to ∿ 70 ml under reduced pressure, and neutralized carefully with 6 N NaOH. The pH was adjusted to 7-7.2, and the solution was evaporated to near dryness. The

sticky solid was extracted with chloroform. The chloroform was dried over Na_2SO_4, decolorized with charcoal, filtered, and evaporated under reduced pressure. From the resulting brown syrup was distilled 12g dl-2,3-butanediol under vacuum at 51° C.

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 & 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.

Sodium dodecyl sulfate (SDS) gel electrophoresis was carried out with materials purchased from Bio-Rad. 12% and 7.5% crosslinked gels were employed.

Isoelectric focussing was carried out on pH 3.5-9 PAG plates from LKB. Diol dehydratase apoenzyme and O_2-

and glycerol-inactivated holoenzyme were not denatured before application to the focussing gel.

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.

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

$$[E_a] = [E_o]e^{-k_{i,obs}(t)} \qquad (1)$$

where E_a is the amount of active enzyme remaining at time t, E_o is the initial amount of enzyme present, and $k_{i,obs}$ is the observed first-order rate constant for inactivation.

Thus, a plot of $\ln[E_a]$ as a function of time is linear and the slope gives a value of $k_{i,obs}$ for a given glycerol concentration. A plot of $k_{i,obs}$ as a function of glycerol concentration (Fig. 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:

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

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

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

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

Figure 1

Dependence of the observed rate of inactivation, $k_{i,obs}$, on [G] under anaerobic conditions in the absence of 1,2-propanediol. Values of $k_{i,obs}$ were obtained from the linear least squares plot of ln% residual enzyme activity vs time for each glycerol concentration. The corresponding double reciprocal plot is linear and gives k_i = 1.3 min^{-1} and K_G = 1.6 mM.

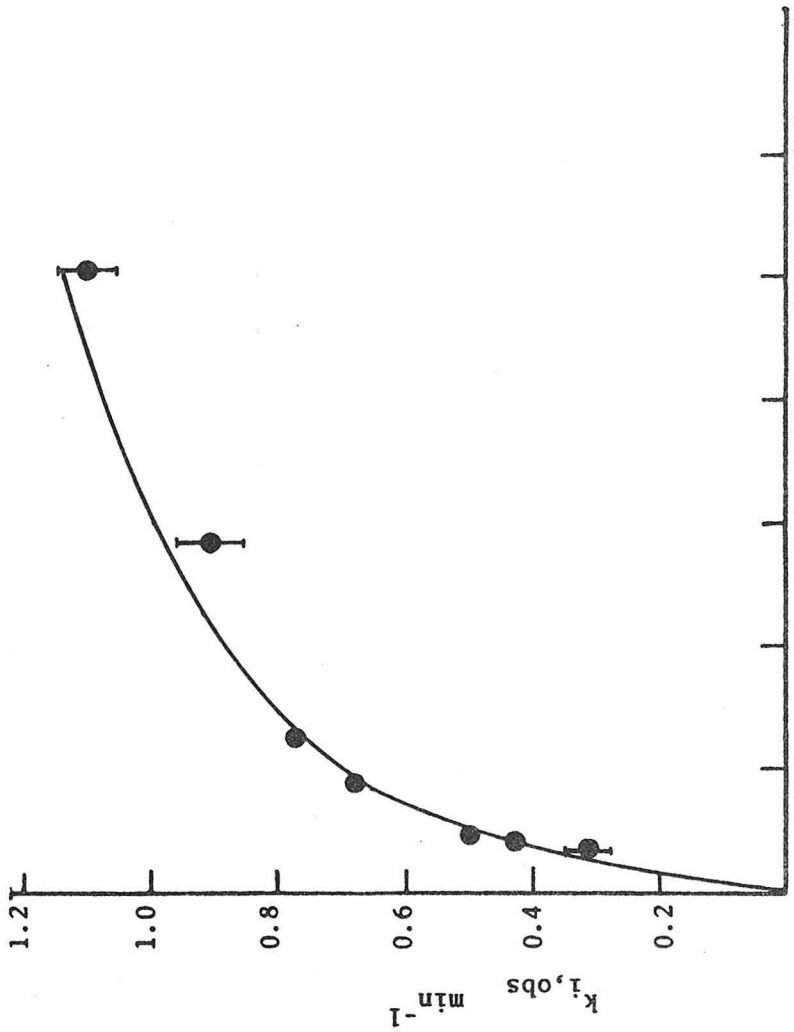


Figure 1

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

Integration gives

$$[E_a] = [E_a]_o e^{-k_{i,obs}(t)} \qquad (5)$$

where

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

Thus, a plot of $1/k_{i,obs}$ vs $1/[G]$ should, and does give a straight line (Fig. 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.

Thermodynamic Parameters. The rate constants k_i for glycerol inactivation and k_p for catalysis with 1,2-propanediol were measured as a function of temperature over the range 12^o - 38^o C at saturating concentrations of these substrates. The results for glycerol are shown in Fig. 3 as a plot of $\ln(k_i)$ vs $1/T$. The resulting activation energies and entropies (Moore, 1962) are $E_a = 9.2 \pm 1$ and 16 ± 1 kcal/mole and (at 37^o C) $\Delta S^+ = -17.8$ and -16.4 e.u. for propanediol and glycerol, respectively.

The value of k_i for glycerol was independent of pH in the range 6.0 - 8.6.

Kinetics of Glycerol Inactivation in the Presence of

Figure 2

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 mM; ●, 1.34 mM; ▲, 0.86 mM; ◆, 0.48 mM; ○, no (RS)-1,2-propanediol. Although only one point can be depicted for the substrate-free case, a straight line is obtained with the illustrated slope and intercept.

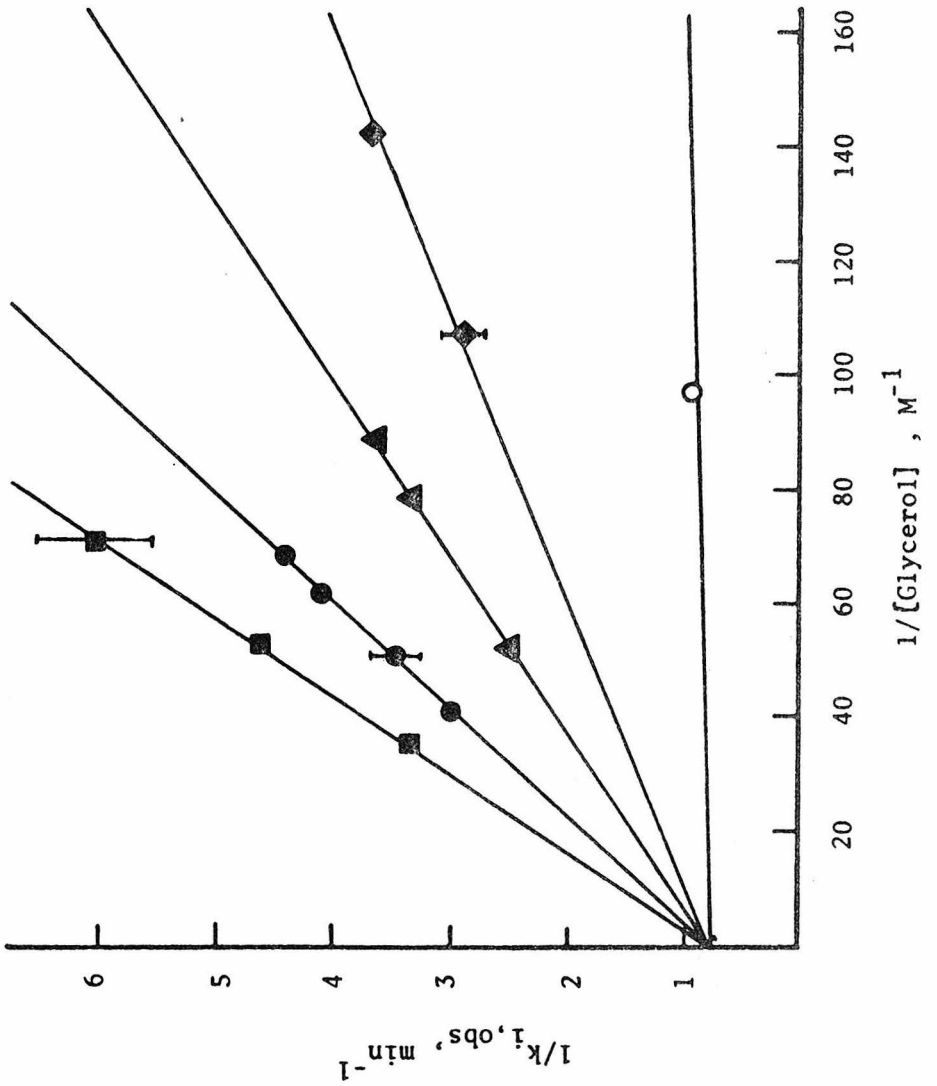


Figure 2

Figure 3

A plot of $\ln(k_{i,obs})$ as a function of 1/T for inactivation of diol dehydratase by glycerol. Unless otherwise indicated, the error bars are approximately the same size as the illustrated data points.

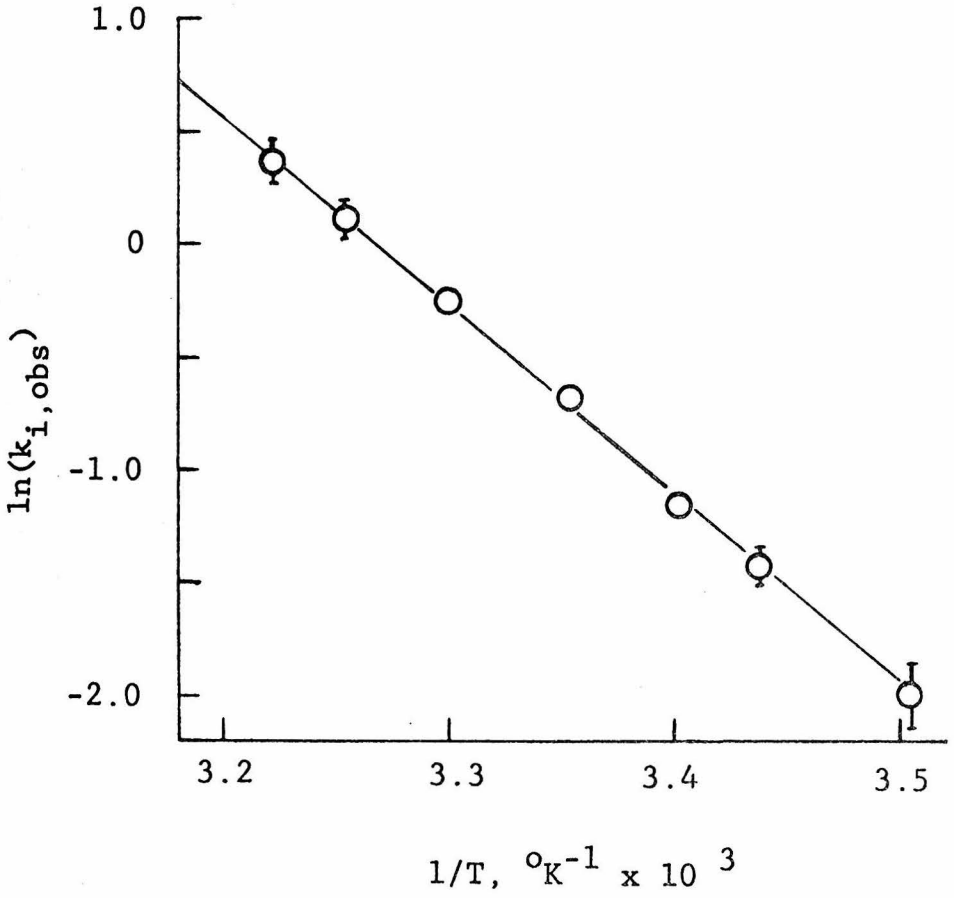


Figure 3

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:

$$E + G \underset{\leftarrow}{\rightarrow} EG \overset{k_i}{\rightarrow} I$$

$$E + S \underset{\leftarrow}{\rightarrow} ES \rightarrow E + P$$

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

$$[E_a] = [E_a]_o e^{-k_{i,obs}(t)}$$

where

$$k_{i,obs} = \frac{k_i}{1 + \frac{K_G}{[G]}(1 + \frac{[S]}{K_S})} \qquad (7)$$

Values of $k_{i,obs}$ 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 ($1/k_{i,obs}$ 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 (Fig. 2). Furthermore, Fig. 2 also demonstrates that eq 7 is a valid description of the results obtained over a wide range of substrate and glycerol concentrations. The value of k_i obtained from the y-intercept of Fig. 2 agrees well with the experimentally determined value (Table I) and lends further validity to this treatment.

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. 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 & Neese (1975) and suggests that in this case $K_I = K_m$.

Kinetics of Inactivation — Other Substrates. All substrate analogs listed in Table I, except d- and

dl-2,3-butanediols, 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_I or 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. d- and dl-2,3-butanediols act as purely competitive inhibitors of inactivation and catalysis by other substrates.

Deuterium Isotope Effects on the Rate of Inactivation by Glycerol. The rates of enzyme inactivation at saturating concentrations of (R)-gly-d_2, (RS)-gly-d_2, (S)-gly-d_2, and gly-d_5 were measured by the method just described, and were considerably slower than that of the undeuterated substrate. The ratio of the rate of inactivation observed for glycerol to that observed for a saturating concentration of one of the deuterated glycerols reflects an isotope effect on k_i. Thus, for rates measured at large concentrations of glycerol and its deuterated derivatives,

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

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

The $k_{i,obs}$ values determined in these experiments should be within 10 % of the true k_i values (because at

Table I

Kinetic parameters of substrates and inactivators of diol dehydratase.

[a]Values of K_I for the natural substrates were determined in competition with glycerol with the natural substrates serving as "inhibitors" of inactivation as described in Results. [b]Toraya et al. (1976) have also reported glycerol to be a substrate and inactivator of diol dehydratase. A value of K_G for glycerol was given that agrees with this value. [c]Product formation does occur, but the rate was not quantitated. [d]Determined from the amount of product formed before complete inactivation. [e]Direct comparison of the initial rate to that of 1,2-propanediol. [f]Based on a molecular weight of 250,000 and a specific activity of 60 unit/mg with 1,2-propanediol as substrate. [g]Calculated from the K_I values of the d- and dl isomers.

Substrate	$K_m \times 10^4$ M	$k_p (sec^{-1})$[f]	$k_i (min^{-1})$
(S)-1,2-Propanediol	0.19 (K_I)[a]	191	0
(RS)-1,2-Propanediol	0.30 (K_I)[a]	250	0
(R)-1,2-Propanediol	0.60 (K_I)[a]	340	0
Glycerol	16[b]	412[d]	1.30
3-Chloro-1,2-propanediol	4.1	>0[c]	0.36
Trifluoropropanediol	1.24 (K_I)	>0[c]	0.016
Thioglycerol	0.1	∿0	1.74
Ethylene glycol	6.6	150[d,e]	0.098
1,2-Butanediol	5.9 (K_I)	>0[c]	0.054
Isobutylene glycol	8.8	18[d]	0.47
meso-2,3-Butanediol	2.0 (K_I)	4[d]	0.068
d-2,3-Butanediol	5.5 (K_I)	0	0
dl-2,3-Butanediol	1.07 (K_I)	0	0
l-2,3-Butanediol	0.59 (K_I)[g]	0	0

Table I

Table II. Catalytic and Inactivation Rate Constants for Glycerol and its Deuterated Analogs.

Substrate	Competitive Turnover No. (T = 60 min)[a]	k_p (sec^{-1})[b]	$(\frac{k_H}{k_D})_p$	$k_{i,obs}$ (min^{-1})[c]	$(\frac{k_H}{k_D})_i$
Glycerol	19,000	412	1	1.25	1
Glycerol-d_5	33,200	52	8	0.089	14
(RS)-Glycerol-d_2	16,300	190	2.2	0.66	1.9
(R)-Glycerol-d_2	5,300	61	6.8	0.69	1.8
(S)-Glycerol-d_2	51,000	720	0.6	0.77	1.6

[a]Competitive turnover numbers based on a specific activity of 60 unit/mg and a molecular weight of 250,000 (Essenberg et al., 1971).

[b]Calculated from eq 8 using k_i and P_∞/E_o.

[c][G] = 0.04 M.

0.04 M inactivator with K_G = 1.6 mM, $K_G/[G] \sim 0.04 << 1$, see eq 6) and can therefore represent experimentally determined values of k_i for each deuterated glycerol.

With gly-d_5, we observe a large isotope effect (14) on k_i, and smaller, nearly equal values for (R)-gly-d_2 (1.8), (RS)-gly-d_2 (1.9), and (S)-gly-d_2 (1.6).

Product Formation from Glycerol. Diol dehydratase holoenzyme also catalyzes the dehydration of glycerol to β-hydroxypropionaldehyde in addition to effecting irreversible inactivation. β-Hydroxypropionaldehyde was identified (1) by the ^1H 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 & 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)-gly-d_2, (RS)-gly-d_2, (S)-gly-d_2, and gly-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 aldehyde 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)-gly-d_2 and (RS)-gly-d_2 yield less product than glycerol itself before inactivating holoenzyme, gly-d_5 and (S)-gly-d_2 form considerably more product than glycerol.

Rate of Formation of β-Hydroxypropionaldehyde. The production of β-hydroxypropionaldehyde was followed for several minutes with glycerol, gly-d_5, and (R)-gly-d_2 each serving as substrate. The results in Fig. 4 show that, although the initial rate of product formation is much greater for glycerol than for gly-d_5, the rate at which glycerol inactivates the enzyme relative to inactivation by gly-d_5 is greater by an even larger factor, thus resulting in a net enhancement of product formation from gly-d_5.

Because of competition from irreversible inactivation, the rate constant for product formation (k_p) cannot be obtained with any degree of confidence by extrapolation of the results in Fig. 4; the rate changes too drastically 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

Figure 4

Production of β-hydroxypropionaldehyde as a function of time for: ▲, glycerol; ■, gly-d_5; ●, (R)-gly-d_2. β-Hydroxypropionaldehyde was determined by the DNP assay as described and is depicted here as the competitive turnover number, P_T/E_o, for uniformity. E_o based on 60 unit/mg and a molecular weight of 250,000 g/mole (Essenberg et al., 1971). The uncertainty in the values of $P_T/E_o \times 10^{-3}$ is approximately $\pm$ 1.5.

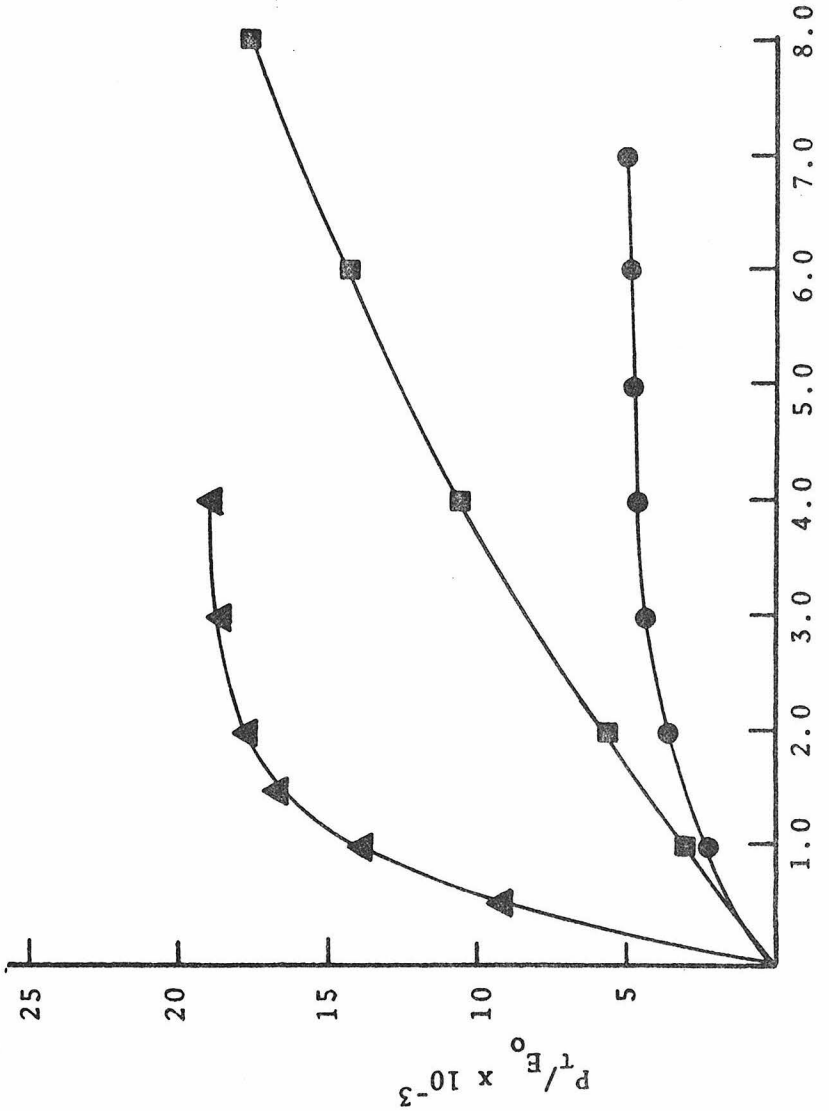


Figure 4

analog of the inactivation rate constant 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,

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

whence $d[P]/dt = k_p[E_o]\exp(-k_i t)$. The total amount of product formed at any time is given by the integrated form of this equation:

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

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

$$\frac{[P_\infty]}{[E_o]} = \frac{k_p}{k_i} \qquad (8)$$

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

The values of $[P_\infty]/[E_o]$ 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

$[P_T]/[E_o]$ as a function of time using the values of k_i and k_p in Table II and accurately reproduce the experimental graphs of Fig. 4.

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)-gly-d_2 gives product with essentially no deuterium at C-3, while (RS)-gly-d_2 and (S)-gly-d_2 give product with approximately 60% and at least 90% deuterium at C-3, respectively.

Product Formation - Other Substrates. With the exception of 1,2-butanediol, thioglycerol, and d- and dl-2,3-butanediols, the substrate analogs listed in Table I gave easily detectable amounts of product. Products from isobutylene glycol and meso-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 Holoenzyme-Glycerol Complex. Figure 5 compares the visible spectrum 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 enzyme-bound AdoCbl in the absence of substrate. This spectrum, which is obtained after complete inactivation by glycerol, remains unchanged for up to 24 hr in the dark at room temperature; after this time, 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 sec, 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, use of the deuterated substrate does not measurably affect the amount of enzyme-bound AdoCbl observable as Cbl^{II}. Because gly-d_5 is a reasonably good "substrate" but inactivates much more slowly than glycerol, we looked for spectral changes when gly-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 addition of glycerol. If the addition of 1,2-propanediol almost immediately follows that of

Figure 5

The effects of glycerol and 1,2-propanediol on the uv-visible spectrum of enzyme-bound AdoCbl. (1) Diol dehydratase, 37 units; AdoCbl, 2.3 nmole; K_2HPO_4, 10 mM; volume 0.3 ml. (2) 60 μmole glycerol added in a 0.03 ml aliquot to sample and reference of (1); volume 0.33 ml. (3) 18 μmole 1,2-propanediol added to sample and reference of (1); volume 0.33 ml. T = 27° C. Spectrum (1) has been displaced vertically somewhat for the sake of clarity.

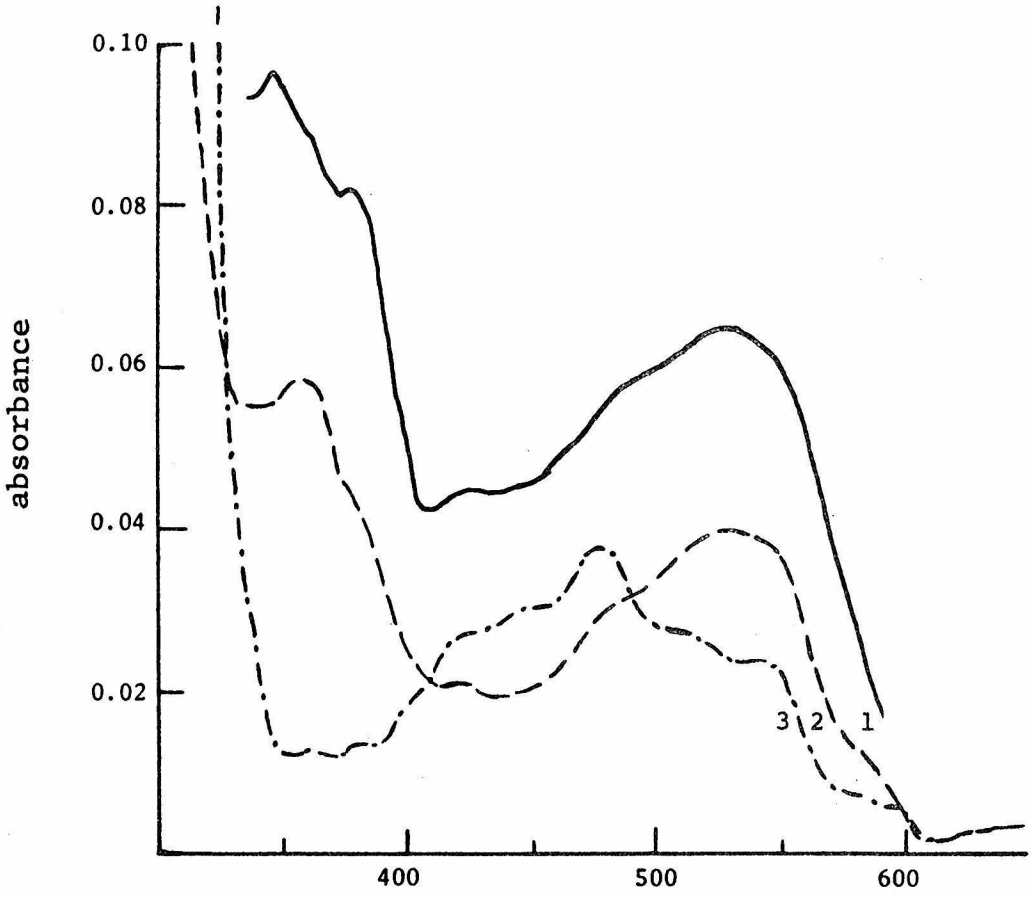


Figure 5

glycerol, a large fraction of enzyme-bound AdoCbl is observed as Cbl^{II}. If, however, the enzyme-glycerol mixture is allowed to stand for 4 min, the addition of 1,2-propanediol does not generate any Cbl^{II} in the spectrum.

TCA precipitation of the protein from a solution containing holoenzyme and glycerol leads to recovery of only OH-Cbl. Addition of TCA to an identical reaction mixture containing 1,2-propanediol instead of glycerol results in nearly complete recovery of AdoCbl. In further attempts to isolate and characterize the cobalamin species formed during inactivation by glycerol, inactivated holoenzyme was denatured by heat; 5 M guanidinium chloride, pH 8; 8 M urea solution, pH 4; or digested with α-lytic protease. In each case, only OH-Cbl was recovered.

Exposure of a holoenzyme-glycerol solution to a 100 watt 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 unchanged on binding to diol dehydratase, small bathochromic shifts of the bands above 300 nm do occur and serve to distinguish free OH-Cbl from the enzyme-bound species (Fig. 6). 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 10 mM K_2HPO_4 buffer at pH 8, we observe λ_{max} at 525, 510, and 352 nm, while

Figure 6

The absorption spectra of OH-Cbl in solution and bound to diol dehydratase. (——): OH-Cbl, 1.4 nmole; K_2HPO_4, 10 mM; volume 0.32 ml. (----): apoenzyme, 30 units; OH-Cbl, 1.4 nmole; K_2HPO_4, 10 mM; volume 0.32 ml. The reference cell was identical to the sample cell but lacked OH-Cbl. T = 27° C.

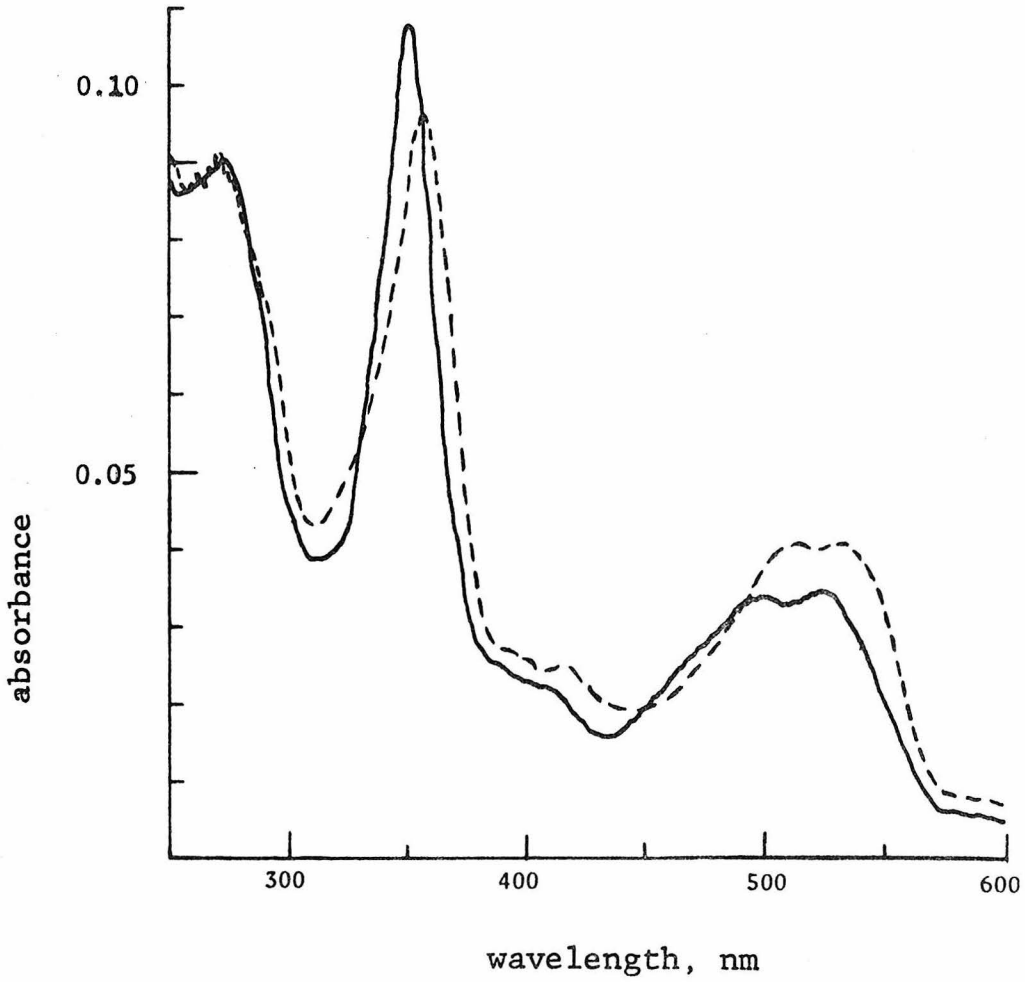


Figure 6

for OH-Cbl bound to diol dehydratase under identical conditions, the corresponding values are 540, 515, and 362 nm, respectively.

Spectral Observations - Other Substrates. UV-Visible absorption spectra of holoenzyme-substrate complexes with ethylene glycol, trifluoropropanediol, isobutylene glycol, and thioglycerol are nearly indistinguishable from that observed in the presence of 1,2-propanediol (Fig. 7a). In the case of ethylene glycol, after about seven half-lives the spectrum undergoes a rapid change indicating formation of 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 holoenzyme.

The uv-visible spectra of holoenzyme complexes with meso- and dl-2,3-butanediols are shown in Fig. 7b. With meso-2,3-butanediol no Cbl^{II} spectrum is observed; this substrate induces conversion of an AdoCbl spectrum to one resembling that of OH-Cbl at a rate comparable to that of inactivation. dl- and d-2,3-butanediols, in contrast, elicit no change in the spectrum. Furthermore, exposure of a solution containing the holoenzyme-dl-2,3-butanediol complex to a 60 watt bulb at 10 cm for 15 min causes less than a 15 % increase in A_{360} due to OH-Cbl formation, compared to a ~40 % increase observed in the absence of substrate. Addition of 1,2-propanediol to a solution

Figures 7a and 7b

7a: The effect of several substrate analogs on the absorption spectrum of enzyme-bound AdoCbl. (—): apoenzyme, 30 units; K_2HPO_4, 10 mM; AdoCbl, 1.8 nmole; volume 0.35 ml. Reference cell was identical but lacked AdoCbl. To the sample and reference cuvets was added 0.05 ml of 0.5 M solutions of ethylene glycol (--); thioglycerol (··); trifluoropropanediol (—··); or isobutylene glycol (— · —) Volume 0.4 ml, T = 27° C.

7b: Absorption spectra of holoenzyme-2,3-butanediol complexes. (a) dl-2,3-butanediol; apoenzyme, 100 units. (b) meso-2,3-butanediol; apoenzyme, 80 units. The sample and reference solutions were identical except for AdoCbl, and contained 20 mM K_2HPO_4, pH 8. Spectrum (b) has been displaced vertically for the sake of clarity. The d isomer gave the same results as the dl. Volume 1.1 ml, 37° C.

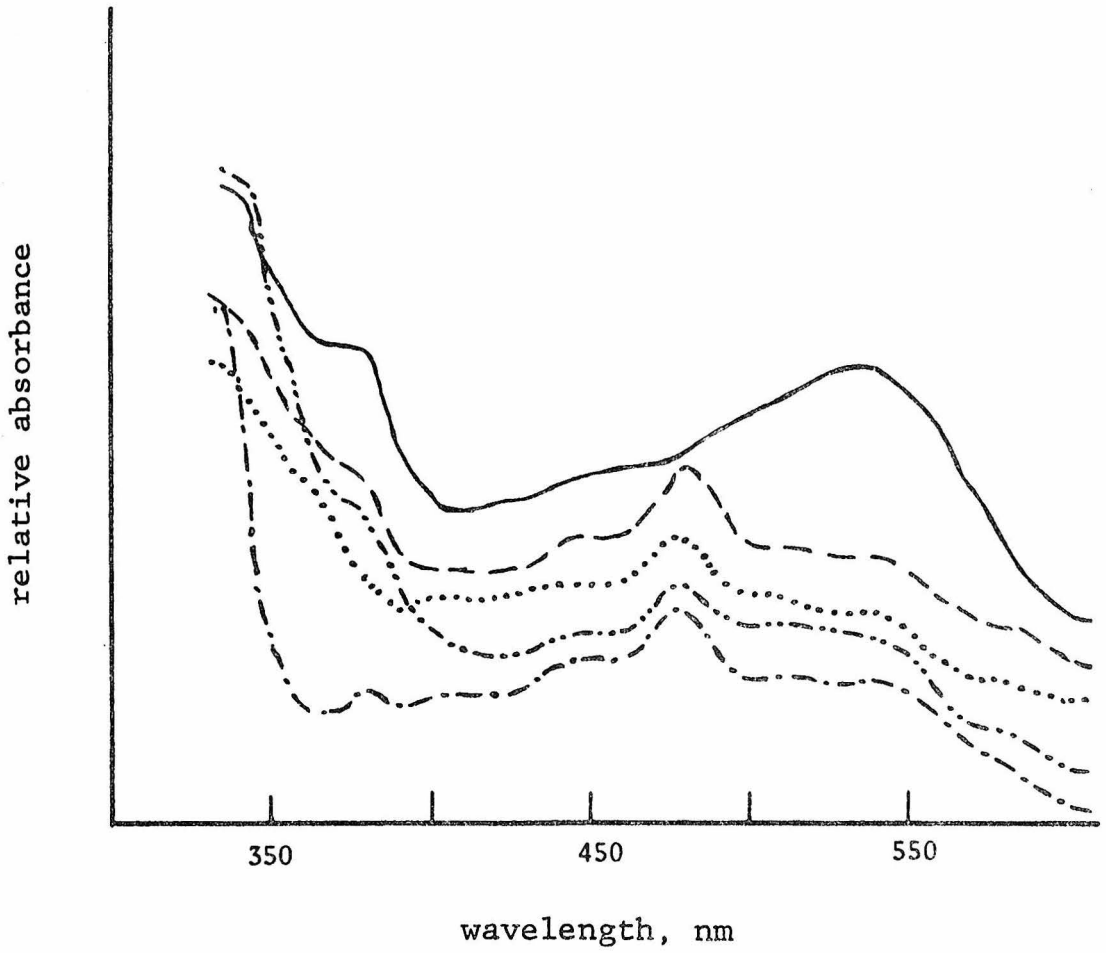


Figure 7a

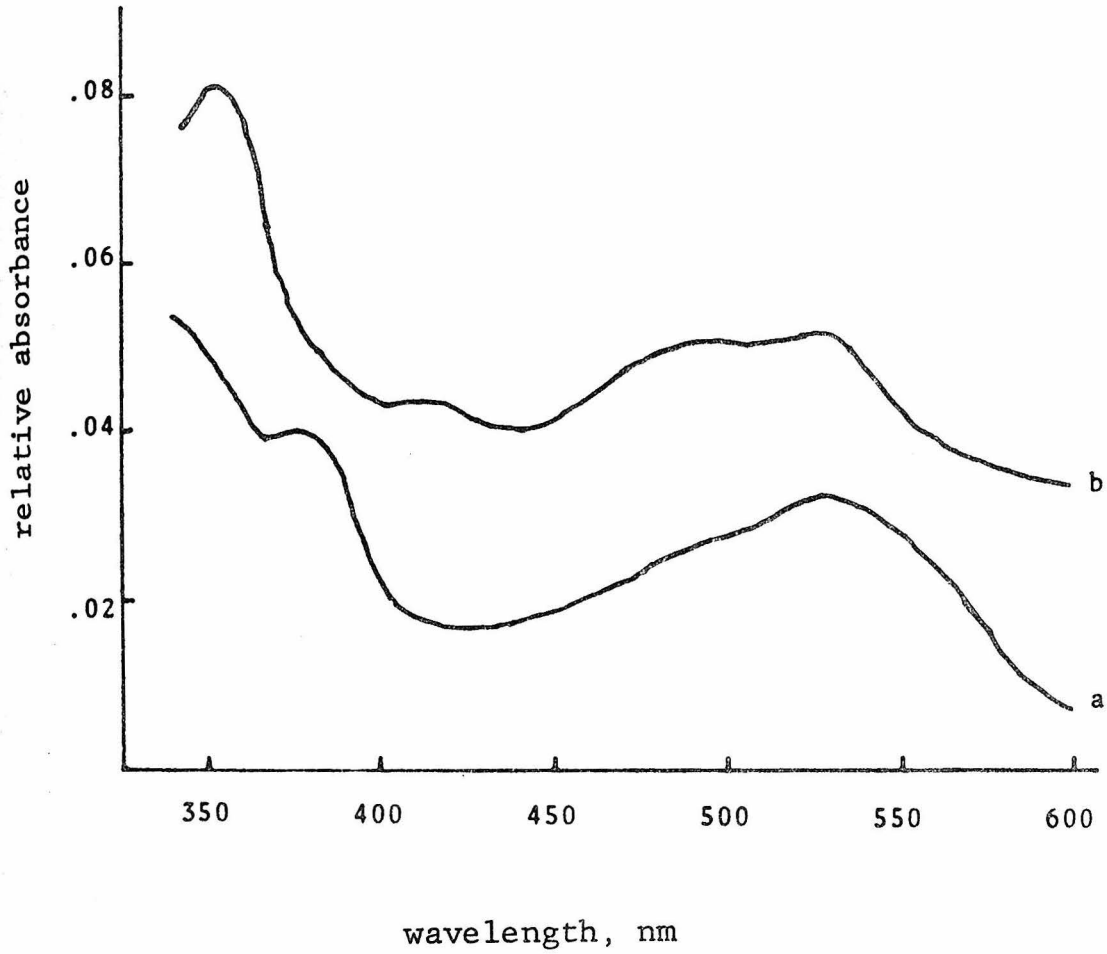

relative absorbance
.08
.06
.04
.02
350
400
450
500
550
600
b
a
wavelength, nm

Figure 7b

containing holoenzyme and d- or dl-2,3-butanediol produces a peak at 480 nm characteristic of Cbl^{II}, while identical treatment of a solution containing holoenzyme and the meso substrate does not detectably alter the observed OH-Cbl spectrum.

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 OH-Cbl in the dialysate. Subsequent dialysis to remove SO_3^{2-} (which is apparently a potent inhibitor of diol dehydratase) leaves apoenzyme, which upon addition of AdoCbl is catalytically active (up to 90 % of the original activity can be recovered). Treatment with either reagent alone or both 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; this reaction can be followed spectrophotometrically.

OH-Cbl can be removed from oxygen-inactivated holoenzyme by the above method; however, only very little activity is regenerated. Continued dialysis of the resulting apoenzyme

Table III

Reactivation of several forms of inactivated diol dehydratase.

[a]Reaction solution #3 contained 0.04 M glycerol, otherwise the four reaction solutions were identical and contained: K_2HPO_4 buffer, pH 8, 40 mM; bovine serum albumin, 0.02 mg; diol dehydratase, 17 units. Volume 2.1 ml. Enzyme activity was determined by assaying a 0.01 ml aliquot for activity as described in Experimental.

[b]Inactivation was carried out by addition of: 40 µg OH-Cbl to solution #1; 40 µg AdoCbl to solutions #2 and #3, and 0.04 ml H_2O to solution #4, followed by 15 min incubation in the dark at 37° C. An aliquot was removed for determination of activity.

[c]Reactivation: Treatment A. After inactivation, each of the four solutions was subjected to dialysis for 12 hr against each of two changes of 2 l of solution to which had been added: K_2SO_3, 100 mmole; $Mg(CH_3CO_2)_2$, 60 mmole; 1,2-propanediol, 200 mmole; and K_2HPO_4, pH 8.0, 20 mmole. This was followed by dialysis for 3 hr against 2 x 2 l of a buffer containing 0.1 M 1,2-propanediol and 10 mM K_2HPO_4 buffer, pH 8.0.

[d]Treatment B refers to repetition of treatment A.

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

Table III

does lead to recovery of up to 30 % activity (Table III). The recovery of active apoenzyme was not enhanced by allowing O_2-inactivation to occur in the presence of sodium dithionite, dithiothreitol, or potassium borohydride, nor by the presence of these agents in the dialysis solution at concentrations of 2 mM.

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 of 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 recovered. The presence of the three reducing agents listed above had no effect in this case, either.

Inactivation by [1-^{14}C]-Glycerol. When diol dehydratase holoenzyme is inactivated by [1-^{14}C]-glycerol, up to 40 or 50 radioactive molecules become strongly associated with each protein molecule in an AdoCbl-dependent process. Control experiments using apoenzyme alone or OH-Cbl-inactivated enzyme also give evidence of labeling, although only to the extent of about one or two labels per enzyme molecule. Experiments in which the number of enzyme active sites exceeded the amount of AdoCbl by factors of two to seven also yielded approximately 40 - 50 radioactive

labels per protein molecule. Accordingly, we conclude that virtually all of these labels are due to β-hydroxypropionaldehyde, the product of catalysis, and may arise via Schiff's base formation involving nucleophilic groups on the protein.

The [^{14}C] labels could not be removed from the protein by gel chromatography; dialysis against 5 M guanidinium chloride, pH 8; 8 M urea solution, pH 4; or 0.5 M hydroxylamine, or by TCA precipitation of the protein. In attempts to reduce the number of labels, several strategies were tried. The inactivation reaction was carried out in the presence of nucleophiles such as imidazole or dithiothreitol (these do not significantly affect the catalytic properties of diol dehydratase). The reaction was carried out in the presence of KBH_4 in an attempt to reduce β-hydroxypropionaldehyde immediately upon its release from the active site. Alternatively, apoenzyme was preincubated with propionaldehyde or acetaldehyde prior to addition of AdoCbl and radioactive glycerol.

Unlabeled β-hydroxypropionaldehyde was also generated <u>in situ</u> by adding a substoichiometric amount of AdoCbl to an apoenzyme-glycerol solution; following completion of this reaction, [1-^{14}C]-glycerol and excess AdoCbl were added. We have observed that the presence of these labels does not detectably alter the catalytic properties of residual, active diol dehydratase. This approach was most successful,

resulting in up to a ten-fold reduction in the amount of label associated with protein.

SDS-Gel Electrophoresis of diol dehydratase which has been inactivated by oxygen and glycerol does not reveal changes in the four-band pattern which has been described by Poznanskaya et al. (1977). Preliminary isoelectric focussing data suggest that the pI of diol dehydratase, in the neighborhood of 4 - 4.5, is not measurably changed by inactivation by oxygen or glycerol.

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 & Dolphin, 1976):

$$\underset{}{SH + AdoCbl\text{-}E \rightleftharpoons SH\text{-}AdoCbl\text{-}E \rightarrow S\cdot \begin{matrix} HAd \\ \cdot Cbl \end{matrix}}$$

$$\underset{}{\qquad\qquad\qquad\qquad\qquad\qquad (I) \qquad\quad (II)}$$

$$\underset{}{\rightleftharpoons \; ? \rightleftharpoons PH \begin{matrix} \cdot Ad \\ \cdot Cbl \end{matrix} \rightleftharpoons PH + AdoCbl\text{-}E}$$

$$\underset{}{\qquad\quad (III) \quad (IV)} \qquad\qquad (9)$$

Intermediate I represents the reversibly associated enzyme-substrate complex; II, an intermediate in which hydrogen has been removed from substrate to yield 5'-deoxyadenosine (HAd); 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 & 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 gly-d_5 ($V_{max}H/V_{max}D \sim 8$) demonstrate that cleavage of a carbon-hydrogen bond is an important rate-contributing step 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 that of catalysis. However, we have verified that β-hydroxypropionaldehyde neither inhibits nor inactivates diol dehydratase holoenzyme. Inactivation, then, must occur at some point between initial binding of glycerol and the release of product.

Spectral Observations 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 deaminase (Wagner et al., 1966; Abeles & 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 uv-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 meso-2,3-butanediol as substrates therefore more likely represents a change of the rate-limiting step, resulting in the predominance of an intermediate which is not Cbl^{II}, rather than a change in the catalytic mechanism (i.e., a Cbl^{II} species still intervenes, but in a concentration 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 modification of the substrate, lacks the proper electronic or steric 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 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 presence of a small amount of some paramagnetic species has been demonstrated even in the absence of substrate (Finlay et al., 1973).

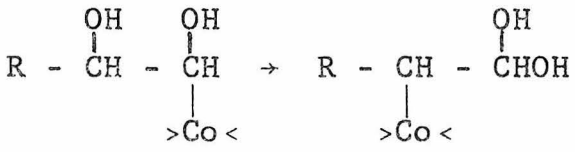
Of the inactivators listed in Table I, only with meso-2,3-butanediol is inactivation accompanied by transformation of enzyme-bound cofactor to enzyme-bound OH-Cbl at a rate similar to that of inactivation. With this inactivator, as in the case of oxygen inactivation, formation of Cbl^{II} cannot be detected spectrophotometrically. (In contrast, the d and dl isomers (Fig. 7b) do not alter the spectrum of enzyme-bound AdoCbl. The mechanistic significance of this result will be discussed in the next section.)

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 little change in the visible spectrum of enzyme-bound AdoCbl can be detected, yet glycerol is both a very good substrate and powerful

inactivator. (Slight changes in the spectrum from that observed for AdoCbl bound to enzyme in the absence of substrate or inactivator appear in Fig. 5. The shoulder at 370 nm characteristic of an alkylcobalamin is diminished slightly, while a new peak at 363 nm appears.) Initially, the spectrum probably represents enzyme-bound AdoCbl. Following inactivation, however, the uv-visible spectrum must represent either a "trapped" intermediate or a new alkyl- or thiocobalamin not normally observed during catalysis.

AdoCbl-dependent rearrangements have been postulated to take place via a transalkylation reaction (intermediate IIIa 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 uv-visible spectrum of glycerol-inactivated holoenzyme may be due to a trapped intermediate such as IIIa or IIIb.



IIIa IIIb

IIIb is more likely in view of the observed lability of the intermediate, secondary alkylcobalamins being generally less stable than primary alkylcobalamins (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 the enzyme by

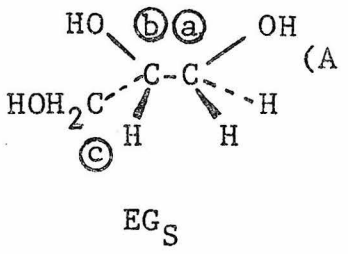
glycerol. In view of these results, a more likely scenario is attack of a reactive intermediate derived from substrate (such as II) on the enzyme, followed by or concomitant with a reaction between Cbl^{II} and an amino acid residue at the active site. This would account for the failure to remove the light-sensitive cobalamin intact from the active site, the inactivity of apoenzyme upon removal of cobalamin, and a small amount of the observed covalent labeling of the enzyme by glycerol.

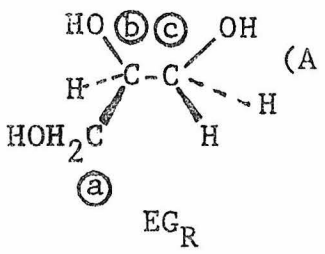
The presence of a reactive apoenzyme moiety at the active site is also indicated by the observation that removal of OH-Cbl from oxygen-inactivated holoenzyme does not regenerate active apoenzyme. This substrate-free inactivation has previously been attributed only to destruction of enzyme-bound cofactor which then remains irreversibly bound to 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 against the Mg^{2+}/SO_3^{2-} solution beyond that required to remove enzyme-bound cobalamin restores some activity. Moreover, the rate of 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-}. However, the presence of

dithiothreitol, sodium dithionite, or potassium borohydride did not enhance this reactivation.

The above results indicate that inactivation almost certainly involves the breakdown of catalysis at some intermediate step. Destruction of enzyme-bound cofactor would first be suspected as a likely cause of inactivation; however, in most cases the enzyme-bound cobalamin species we observe (alkylcobalamin 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. (i) Glycerol. The two hydroxymethyl groups attaced 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.



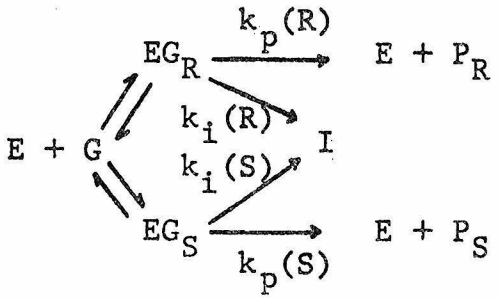


EG_S represents glycerol bound to enzyme with the (pro-S)-hydroxymethyl group at the site involved in hydrogen abstration ("A"), while EG_R represents the corresponding enzyme-glycerol complex with the (pro-R)-hydroxymethyl group so oriented. In the following discussion, we shall use results obtained with the 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

$$E + G \rightleftharpoons \begin{matrix} EG_R \rightarrow EG_{R'} \rightleftarrows EG_{R''} \rightleftarrows EP_R \rightarrow E + P_R \\ EG_S \rightarrow EG_{S'} \rightleftarrows EG_{S''} \rightleftarrows EP_S \rightarrow E + P_S \end{matrix} \qquad (10)$$

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

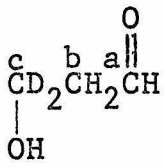


(11)

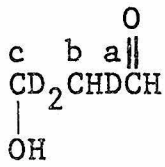
recognizing that $k_p(R)$ and $k_p(S)$ are composite constants representing many steps, including at least two steps involving hydrogen transfer (I-II and III-IV, eq 9). Each of these two steps can contribute to the expression of an isotope effect on $k_p(R)$ or $k_p(S)$. $k_i(S)$ and $k_i(R)$ are the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

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

P_S

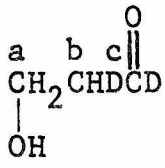


A

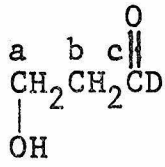


B

P_R



C



D

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.) ^1H 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. Moreover, ^1H NMR of the 2,4-dinitrophenylhydrazones of product derived from (S)-gly-d_2 (deuterium at carbon "a") shows virtually no hydrogen attached to the hydroxyl-bearing carbon. These results constitute strong evidence that product aldehyde originates almost entirely from EG_R.

The observed kinetic isotope effects further support this conclusion. For example (R)-gly-d_2 and (RS)-gly-d_2 contain the same amount of deuterium. (R)-gly-d_2, however, exhibits a large isotope effect on product formation (6.8), while a much smaller effect (2.2) is observed with (RS)-gly-d_2. This result indicates that deuterium on carbon "c" has a greater effect on the rate of product formation than deuterium on carbon "a".

The inverse isotope effect on k_p obtained with (S)-gly-d_2 ($k_H/k_D = 0.6$) is actually due to a perturbation of K_G caused by substitution of deuterium for hydrogen at the

pro-(S) hydroxymethyl group (Chapter II). The apparent result is a weakening of the binding of the EG_S conformation of (S)-gly-d_2, with little concomitant effect on that of the EG_R conformation. Thus, the catalytic EG_R conformation competes more favorably for the active site of diol dehydratase. The same effect occurs with gly-d_5, and the observed $(k_H/k_D)_p = 8$ is in reality a combination of two effects: reduction of k_p due to participation of heavy isotope, and enhancement of the rate due to more favorable competition of the EG_R conformation for the catalytic site. Hence, the actual isotope effect on catalysis is probably about 12. Accordingly, the reduction of this isotope effect to 6.8 for (R)-gly-d_2 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 (2-8%) from hydrogen on the "a" carbon is needed to account for the decrease of the isotope effect from 8-12 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.

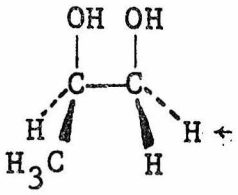
Were hydrogen at carbon "c" as important to inactivation as to catalysis, an isotope effect on inactivation much larger than the observed 1.8 would be exhibited by (R)-gly-d_2. Accordingly, hydrogen at carbon "a" must make a significant contribution to inactivation.

Does the isotope effect of 1.8 for (R)-gly-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., 1978; Chapter II). 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)-gly-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)-gly-d_2 as inactivator indicates inactivation predominantly via EG_S; little, if any, inactivation occurs

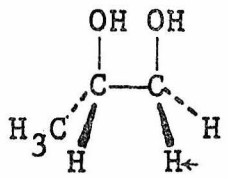
via EG_R.

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, it was 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 changes 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.

(ii) 2,3-Butanediols. The hydrogen abstracted from C-1 of 1,2-propanediol during catalysis is that located "opposite" the C-3 methyl groups in the following diagram (Zagalak et al., 1966) (arrows):

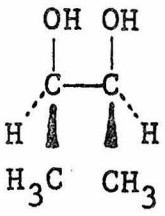


(R)-1,2-propanediol

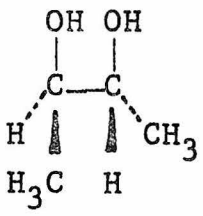


(S)-1,2-propanediol

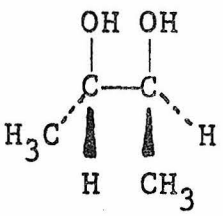
Meso, d, and l-2,3-butanediols have the following configurations (Rubin et al., 1952).



meso



d



l

The configurations of the d and l isomers correspond to replacement of the reactive hydrogen at C-1 of (R)- and (S)-1,2-propanediol, respectively, by a methyl group. The kinetic data reported here indicate that this modification precludes both catalysis and inactivation, which are known to require abstraction of hydrogen from this carbon atom. Furthermore, the reactivity of the meso substrate indicates that the mere steric bulk of a methyl group does not prevent catalysis. Accordingly, we conclude that the catalysis and inactivation reactions with the 2,3-butanediols, and presumably other substrate analogs, both exhibit a high degree of stereochemical discrimination with respect to hydrogen attached to C-1 of substrate.

These results illustrate the remarkable stereospecificity of the diol dehydratase reaction, and serve as additional evidence for the assertion that the catalysis and inactivation mechanisms of these and other substrates are similar.

d and dl-2,3-butanediols form Michaelis-Menten complexes with holoenzyme, but do not alter the uv-visible spectrum of enzyme-bound AdoCbl. In contrast, binding of 1,2-propanediol and other substrate analogs to diol dehydratase is commonly associated with cleavage of the carbon-cobalt bond of AdoCbl to generate a species with an absorption spectrum characteristic of Cbl^{II} (in a few cases, this spectrum rapidly converts to one characteristic of OH-Cbl) (Wagner et al., 1966; Eagar et al., 1975). Other substances less structurally similar to 1,2-propanediol, such as glycolaldehyde (Wagner et al., 1966) and chloroacetaldehyde (Finlay et al., 1972) behave similarly. In this respect, the behavior of dl-2,3-butanediol, with no hydrogen stereochemically available for abstraction from C-1, constitutes a notable departure from that of other diol dehydratase substrates, all of which present hydrogen for transfer to C-5' of AdoCbl. These results imply that simple binding of substrate to holoenzyme is not sufficent to effect cleavage of the carbon-cobalt bond. On this basis, we suggest that hydrogen abstraction from C-1 of substrate may be concerted with homolysis of the carbon-cobalt bond of AdoCbl.

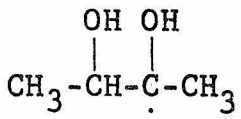
Substrate Specificity. Although the results presented here demonstrate that diol dehydratase holoenzyme binds and reacts with a wider array of substrates than previously thought, the specificity for 1,2-propanediol is still rather high. Inspection of the data in Table I reveals that any modification of the normal substrate results, generally, 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 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-butanediols, 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.

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 analogs 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 was shown for 3-fluoro-1,2-propanediol (Eagar et al., 1975) and glycerol.

Surprisingly, substitution of a methyl group at C-1 (meso-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. With this substrate, initial transfer of hydrogen to C-5' of AdoCbl would generate a tertiary radical:



Subsequent formation of a covalent bond by this species and Cbl^{II}, as described by Babior (1970) and Essenberg et al. (1971) and supported by model studies (Silverman & Dolphin,

1976), would result in a tertiary alkylcobalamin, an exceedingly unstable molecule whose formation has not been observed (Hogenkamp, 1975). Thus, the ability of meso-2,3-butanediol to function as a substrate for diol dehydratase suggests that an intermediate with a covalent carbon-cobalt bond between Cbl and substrate is not obligatory for catalysis. Some results for the ethanolamine deaminase reaction have been similarly interpreted (Krouwer & Babior, 1977: Krouwer et al., 1978).

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 & Pawelkiewicz, 1962); 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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CHAPTER II

HYDROGEN TRANSFER IN THE INACTIVATION OF DIOL DEHYDRATASE BY GLYCEROL

INTRODUCTION

Until recently, propanediol dehydratase, which catalyzes the rearrangement of both (R)- and (S)-1,2-propanediol to propionaldehyde, was thought to be inactive toward glycerol (Toraya et al., 1976; Bachovchin et al., 1977; Chapter I). This behavior distinguished diol dehydratase from another very similar AdoCbl-dependent enzyme, glycerol dehydratase, which converts glycerol to β-hydroxypropionaldehyde as well as acting upon propanediol and ethylene glycol. In fact, diol dehydratase holoenzyme does catalyze the conversion of glycerol to β-hydroxypropionaldehyde. However, glycerol also irreversibly inactivates holoenzyme, thus explaining why catalytic activity toward glycerol was not detected previously (Lee & Abeles, 1963). Both the inactivation and catalysis reactions exhibit large primary deuterium isotope effects (k_H/k_D = 14 and 8, respectively); thus hydrogen transfer is an important rate-contributing step in each case. Experiments with various isotopically substituted glycerols demonstrated that two diastereomeric combinations are possible between the enzyme and glycerol; the enzyme-glycerol complexes have been designated "EG_R" and "EG_S." When glycerol is bound in the EG_R complex, a hydrogen is abstracted from the pro-(R) carbon and the catalytic rate actually exceeds that observed with 1,2-propanediol as substrate. When glycerol is bound in the EG_S complex, hydrogen is abstracted from

the pro-(S) carbon and inactivation, rather than catalysis, follows as the dominant event (Chapter I). 1,2-Propanediol acts as a purely competitive inhibitor of inactivation over a wide range of both glycerol and propanediol concentrations.

Irreversible inactivation has been observed with ethylene glycol and diol dehydratase and with glycerol and glycerol dehydratase, although the inactivation rates are considerably slower than that of glycerol inactivation of diol dehydratase (Lee & Abeles, 1963; Poznanskaya et al., 1972). Thus, irreversible inactivation by substrate appears to be a common phenomenon associated with these enzymes and a study of its mechanism may advance our understanding of the normal catalytic mechanism.

Accordingly, a detailed study of the mechanism of inactivation was undertaken. The kinetics of glycerol inactivation of diol dehydratase holoenzyme in the presence of 1,2-propanediol and 1,1-dideuterio-1,2-propanediol were investigated. The results and their bearing on the inactivation and catalysis mechanisms are discussed.

EXPERIMENTAL

Enzyme Preparations. Propanediol dehydratase was obtained from Klebsiella pneumoniae (ATCC 8724) as described in Chapter I and by Lee & Abeles (1963). 1,2-Propanediol-free enzyme was prepared as previously reported in

Chapter I.

Adenosylcobalamin (AdoCbl) was purchased from Sigma Chemical Co.

5'-Deoxyadenosine was prepared by the intermediate synthesis of N-acetyl-5'-iodo-2',3'-isopropylidene adenosine by a previously reported method (Jahn, 1965; McCarthy et al., 1968). 5'-Deoxyadenosine was also prepared directly from 5'-deoxy-5'-iodoadenosine (Aldrich) by catalytic hydrogenation as described by McCarthy et al. (1968). The materials obtained by both methods were identical.

Substrates. 1,2-Propanediol, 1,1-dideuterio-1,2-propanediol, glycerol, perdeuterioglycerol, (RS)-1,1-dideuterioglycerol, (R)-1,1-dideuterioglycerol, (S)-1,1-dideuterioglycerol, and [1-^{14}C]-glycerol were obtained as described (Bachovchin et al., 1977; Chapter I). [1-^{3}H]-glycerol was purchased from Amersham.

Determination of Inactivation Rates. Inactivation rates were determined using the coupled enzyme assay (Chapter I). Reaction mixtures in general consisted of: yeast alcohol dehydrogenase (ADH, Sigma), 15 units; NADH, 0.2 mM; apodiol dehydratase, 0.05 - 0.25 unit; bovine serum albumin, 0.12 mg; potassium phosphate buffer, pH 8, 30 mM; AdoCbl, 0.02 mM; and the desired amount of 1,2-propanediol and/or glycerol. The total volume was 2.3 ml. The reaction mixture was incubated at 37^{o} C with stirring. The inactivation reaction was started by the addition of

AdoCbl and stopped by addition of 0.05 ml of a 6 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 was taken as the observed inactivation rate constant ($k_{i,obs}$) for the given concentration of glycerol and 1,2-propanediol. As in Chapter I, uncertainties in the measured inactivation rates were in the range 3 - 8 %.

Determination of Michaelis Constants K_G. The values of the Michaelis constants K_G for the various glycerols were determined from the variation of $k_{i,obs}$, the observed inactivation rate, with glycerol concentration in the absence of competing inhibitor or substrate and in the presence of the competitive inhibitors d- or dl-2,3-butanediol. For data obtained in the presence of the inhibitors K_G was obtained from the slope of the double reciprocal plot. K_G was calculated from rate data obtained in the absence of competing substrate or inhibitor as described in Results.

Inactivation by [1 -^{3}H]-Glycerol. A typical solution was composed of the following: apoenzyme, 40 units; [1 -^{3}H]-glycerol, 0.49 mCi/mmole, 0.142 M; K_2HPO_4 buffer, pH 8, 10 mM; AdoCbl, 0.025 mM; total volume 0.5 ml, 37° C. In control experiments the apoenzyme solution was either boiled for 2 min or inactivated by the addition of 0.01 ml

of 6.4 mM CN-Cbl prior to addition of AdoCbl. The holoenzyme-[1 -^{3}H]-glycerol solution was allowed to react for 1 hr in the dark. 5'-Deoxyadenosine (0.15 ml of a 2 mg/ml solution) was added as carrier, and 0.05 ml 6.4 mM CN-Cbl was added to visualize subsequent chromatography. The solution was immersed in boiling water for 2 min and then applied to a 2.6 x 16 cm column of BioGel P-2 (200-400 mesh) and eluted in 4 ml fractions with 15 mM NH_3/0.02 % NaN_3 buffer in the dark. The nucleoside-containing fractions were pooled, acidified to pH 3.0 with 2 N HCl, and applied to a 1 x 9 cm column of AG50W-X2 (200-400 mesh) previously equilibrated with 1 mM HCl. The column was washed with 100 ml water to remove radioactive glycerol. The nucleoside was eluted with 0.1 N NH_3, lyophilized, dissolved in a small amount of methanol, and applied to strips of Whatman 3MM paper or to silica gel plates. Paper chromatograms were developed by the descending method in the solvent systems given in Table I; silica gel plates were developed by the ascending method.

The nucleoside was visualized by its ultraviolet extinction and eluted from the chromatogram with methanol. The methanol was removed under vacuum and the residue was taken up in 1 ml water and added to 10 ml scintisol complete or PCS:xylene (2:1) for radioactivity assay. Standardization was accomplished by using [α-^{3}H]- and [α-^{14}C]-toluene of known specific activities.

Table I: Chromatography of 5'-Deoxyadenosine.

Method/solvent	R_f
Paper	
n-BuOH:H_2O (43:7)	0.34
n-BuOH:HOAc:H_2O (12:3:5)	0.60
n-BuOH:HOAc:H_2O (4:1:5)	0.77
1 M NH_4OAc:95 % EtOH:H_2O (9:20:1)	0.75
Silica gel	
H_2O-saturated n-BuOH:1 % NH_4OH	0.35
sec-BuOH:H_2O:NH_4OH (50:18:7)	0.27

RESULTS

Kinetics of Inactivation by Perdeuterioglycerol. The rate of inactivation of diol dehydratase by perdeuterioglycerol (gly-d_5) was determined as a function of the concentration of gly-d_5 in the absence of 1,2-propanediol under anaerobic conditions at 37° C. As with glycerol, the inactivation rates are always first-order in active holoenzyme. The concentration of gly-d_5 was assumed to be essentially constant throughout the inactivation reaction, as only a negligible fraction of the initial gly-d_5 was converted to β-hydroxypropionaldehyde. Accordingly, we can apply simple Michaelis-Menten equations which have been suitably modified to account for a decreasing amount of catalytically active holoenzyme (Chapter I). Thus:

$$[E_a] = [E_a]_o e^{-k_{i,obs}(t)} \qquad (1)$$

where $[E_a]_o$ is the concentration of active enzyme present initially, $[E_a]$ is the concentration of enzyme, both free and with inactivator bound in a Michaelis-Menten complex, which remains active at any time t, $k_{i,obs}$ is the observed first-order inactivation rate constant, given by

$$k_{i,obs} = \frac{k_i}{1 + \frac{K_G}{[G]}} \qquad (2)$$

where k_i is the true first-order rate constant for

inactivation and K_G is the Michaelis constant for the enzyme-glycerol complex. A plot of $1/k_{i,obs}$ vs $1/[gly\text{-}d_5]$ is linear; from the slope and intercept values, K_G and k_i were obtained. The K_G value determined for gly-d_5 (3.9 mM) differs from that of glycerol (1.6 mM, Chapter I) obtained by the same method. The value of k_i obtained from the y-intercept is 0.085 min^{-1}, indicating an isotope effect of 15 on the inactivation rate constant. This represents reasonable agreement with the value of 14 obtained in Chapter I by comparison of the observed rate constants for gly-d_5 and glycerol at 0.04 M in the absence of 1,2-propanediol; the discrepancy can nevertheless be quantitatively explained by the different contributions made by oxygen inactivation to $k_{i,obs}$ due to the different K_G values.

Kinetics of Inactivation by (S)-, (RS)-, and (R)-1,1-Dideuterioglycerols. In the absence of substrate and in the presence of oxygen, diol dehydratase holoenzyme undergoes irreversible inactivation. The rate of this inactivation by oxygen is approximately 0.12 min^{-1} (W. W. Bachovchin, unpublished results). In the presence of glycerol under aerobic conditions, the inactivation rate is given by the following:

$$\frac{-d[E_a]}{dt} = k_o[E] + k_i[EG], \qquad (3)$$

where k_o and k_i are the rate constants for inactivation by oxygen and glycerol, respectively, and

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

the total amount of active enzyme present at a given time. With $K_G = [E][G]/[EG]$, we have by substitution and rearrangement

$$[E] = \frac{[E_a]}{1 + \frac{[G]}{K_G}}$$

$$[EG] = \frac{[E_a]}{1 + \frac{K_G}{[G]}} .$$

Substitution of these expressions into eq 3 and integration give

$$[E_a] = [E_a]_o e^{-k_{i,obs}(t)} \qquad (4)$$

where

$$k_{i,obs} = \frac{k_o}{1 + \frac{[G]}{K_G}} + \frac{k_i}{1 + \frac{K_G}{[G]}} .$$

The value of $k_{i,obs}$ was measured for (S)-1,1-dideuterioglycerol ((S)-gly-d_2) as a function of concentration of this inactivator (Fig. 1). The values of k_o, k_i, and K_G which provide the best fit of eq 4 to these data were determined by the method of Marquardt (1963) to be k_o= 0.14 min^{-1}, k_i= 0.82 min^{-1}, and K_G = 3.6 mM. $k_{i,obs}$ for (S)-gly-d_2 was also measured in the presence of 1 mM d-2,3-butanediol (K_I = 0.55 mM, Chapter I); in this case, the inactivation kinetics are described by (Chapter I)

Figure 1

Double reciprocal plot for inactivation of diol dehydratase by (S)-gly-d_2 in the presence of oxygen. The dotted line represents the best fit of eq 4 to these data as described in the text with k_o = 0.14 min^{-1}, k_i = 0.82 min^{-1}, and K_G = 3.6 mM. The uncertainties in $k_{i,obs}$ are approximately indicated by the size of the data points.

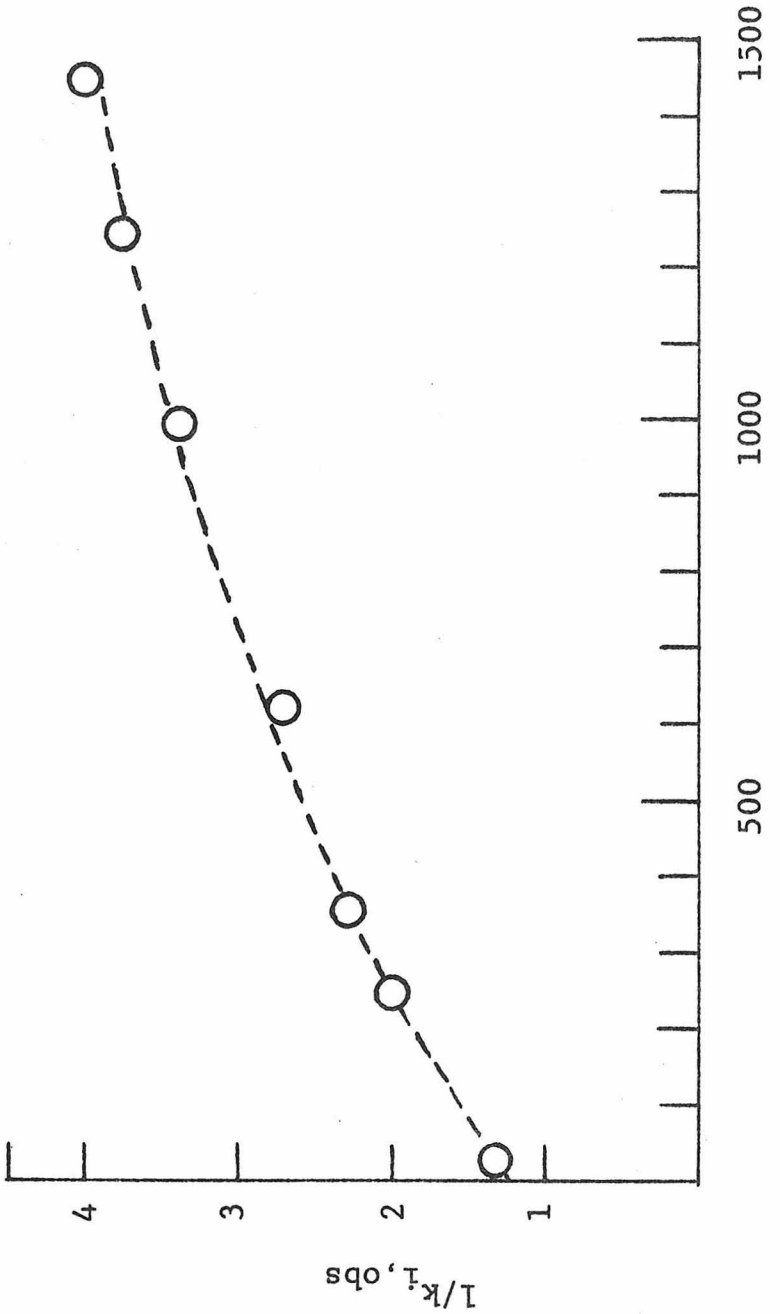


Figure 1

$$k_{i,obs} = \frac{k_i}{1 + \frac{K_G}{[G]}(1 + \frac{[I]}{K_I})}$$

From the slope and intercept of the double reciprocal plot of the resulting data we obtain $k_i = 0.8\ min^{-1}$ and $K_G = 3.8$ mM, in good agreement with the results described above.

The value of K_G for (RS)-1,1-dideuterioglycerol ((RS)-gly-d_2) was similarly determined in the presence of 1.74 mM dl-2,3-butanediol ($K_I = 0.107$ mM, Chapter I), and the results are shown in Fig. 2. The values of the slope and intercept gave $k_i = 0.69\ min^{-1}$ and $K_G = 2.1$ mM.

With (R)-1,1-dideuterioglycerol ((R)-gly-d_2), we find $k_i = 0.72\ min^{-1}$ and $K_G = 1.6$ mM, the same as the K_G of glycerol itself (Chapter I).

Kinetics of Glycerol Inactivation. Competition with (RS)-1,1-Dideuterio-1,2-propanediol. The rate of inactivation of holoenzyme in the presence of a fixed concentration of (RS)-1,1-dideuterio-1,2-propanediol (diol-d_2) was determined as a function of inactivator concentration for glycerol (gly), (RS)-gly-d_2, (R)-gly-d_2, (S)-gly-d_2, and gly-d_5. A fixed concentration of diol-d_2 of 1.08×10^{-4} M was chosen for two reasons: (1) This concentration is sufficiently large to validate the assumption that $\Delta[S] \sim 0$ over the time interval during which the inactivation rates were determined. (2) This concentration is sufficiently

Figure 2

Double reciprocal plot of $k_{i,obs}$ vs inactivator concentration for (RS)-gly-d_2 in the presence of 1.74 mM dl-2,3-butanediol.

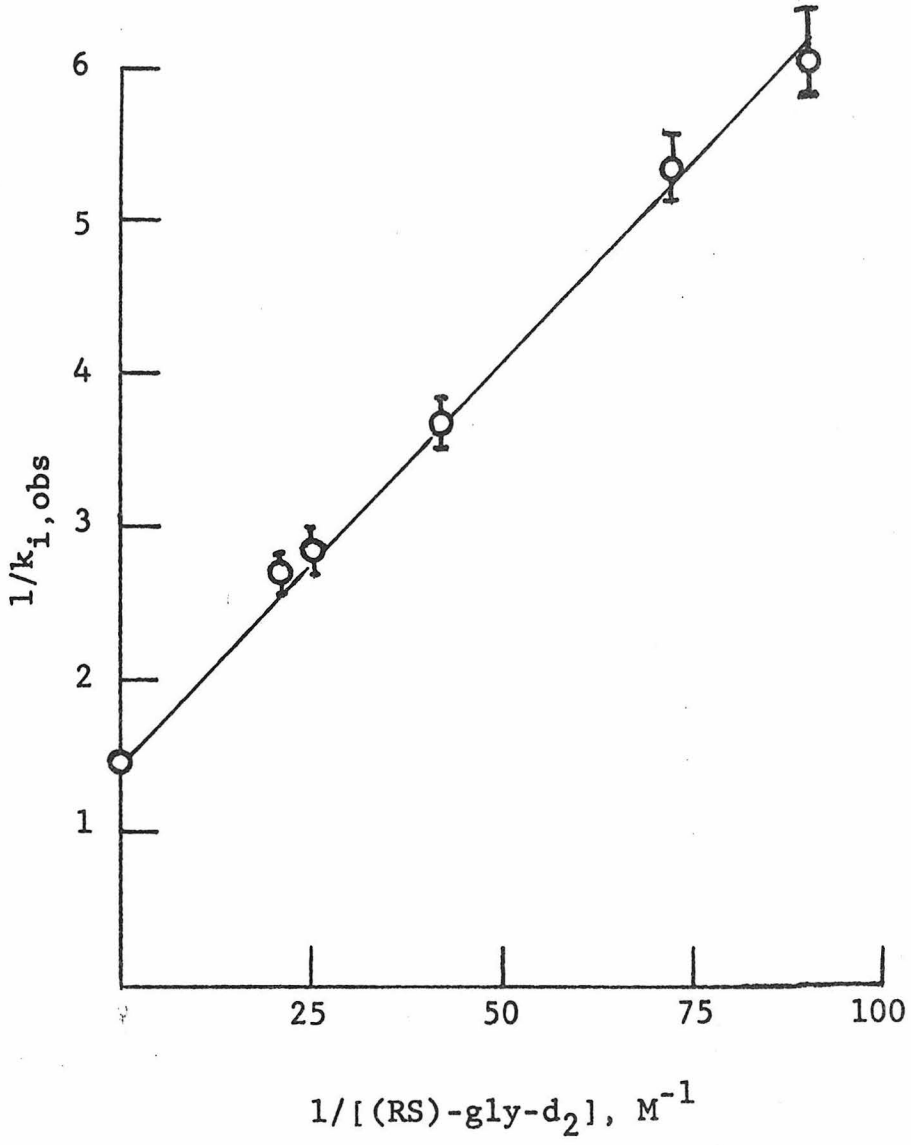


Figure 2

small to allow large inactivation rates to occur at reasonable concentrations of inactivators.

Figures 3 and 4 illustrate the results. With gly-d_5 as inactivator (Fig. 3), the double reciprocal plot is linear over the entire range of inactivator concentration. Moreover, in this case the y-intercept is nearly identical to that obtained for the substrate-free case. Thus, eq 5, derived in Chapter I to describe the inactivation kinetics of gly in the presence of undeuterated 1,2-propanediol (diol) should again apply.

$$k_{i,obs}(gly) = \frac{k_i(gly)}{1 + \frac{K_G(gly)}{[gly]}(1 + \frac{[diol]}{K_I(diol)})} \qquad (5)$$

The slope of the double reciprocal plot with gly-d_5 as inactivator should be given by

$$\text{slope} = \frac{K_G(gly\text{-}d_5)}{k_i(gly\text{-}d_5)}\,(1 + \frac{[diol\text{-}d_2]}{K_I(diol\text{-}d_2)}) \cdot \qquad (5a)$$

Since values of $K_G(gly\text{-}d_5)$ and $k_i(gly\text{-}d_5)$ are available, a value of $K_I(diol\text{-}d_2)$ of 3.0×10^{-5} M can be calculated. This is the same as the value of K_I for diol previously obtained in an analogous manner with glycerol as inactivator (Chapter I) and demonstrates that there is no deuterium isotope effect on K_I for the competitive inhibition by 1,2-propanediol of inactivation of holoenzyme by glycerol.

In contrast to data obtained for gly-d_5, double

Figure 3

Double reciprocal plot for inactivation by gly-d_5 in competition with 1.16×10^{-4} M diol-d_2. Slope = 0.22; intercept = 11.8.

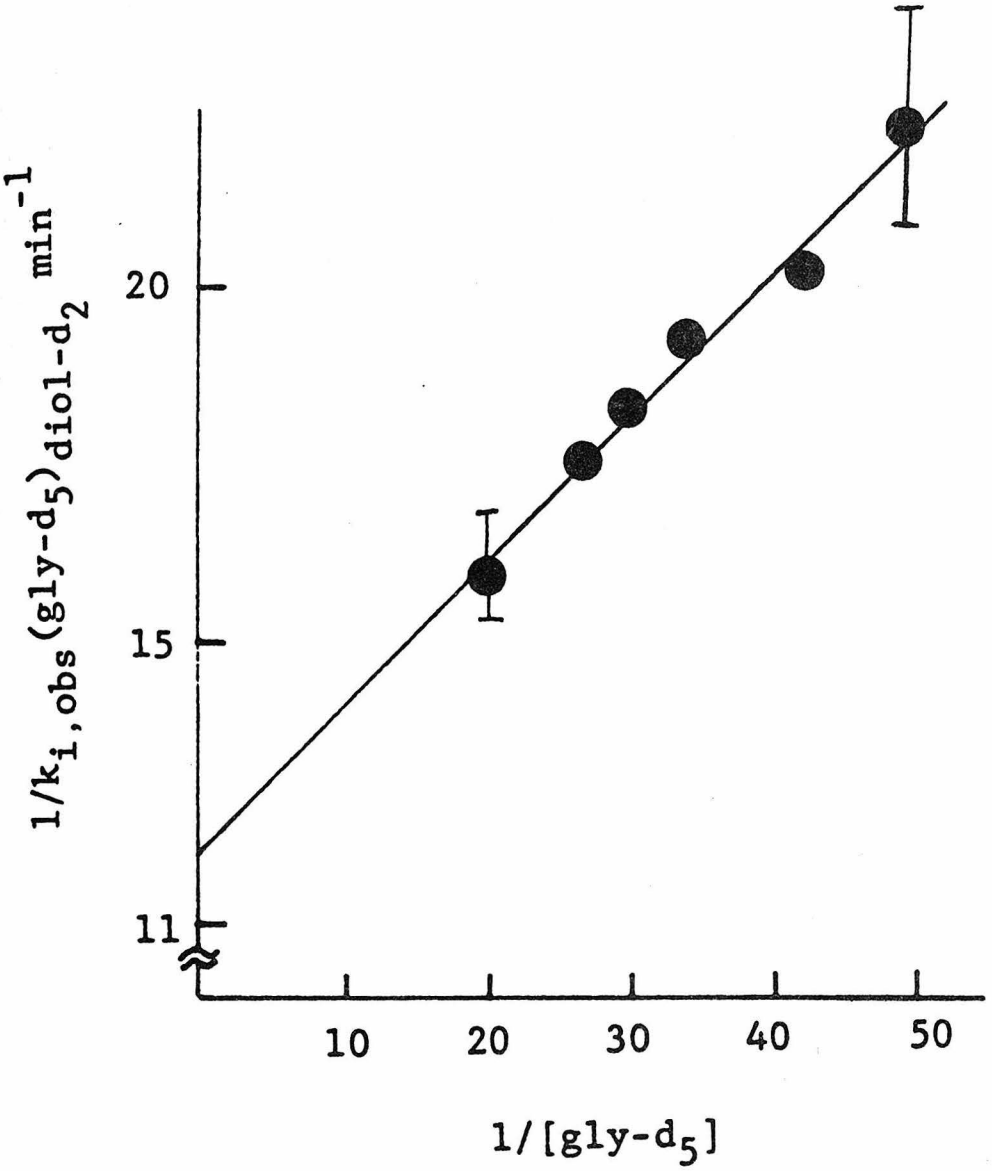

$1/k_{i,obs}(gly\text{-}d_5)_{diol\text{-}d_2}$ min^{-1}
20
15
11
10
20
30
40
50
$1/[gly\text{-}d_5]$

Figure 3

Figure 4

Double reciprocal plot of $k_{i,obs}$ in the presence of 1.08×10^{-4} M diol-d_2 as a function of inactivator concentration: (○), glycerol; (□) (R)-gly-d_2; (△), (RS)-gly-d_2; (●) (S)-gly-d_2. The values depicted at infinite inactivator concentration represent $k_{i,obs}$ measured experimentally at 40 mM inactivator concentration in the absence of 1,2-propanediol. Representative "error bars" are marked.

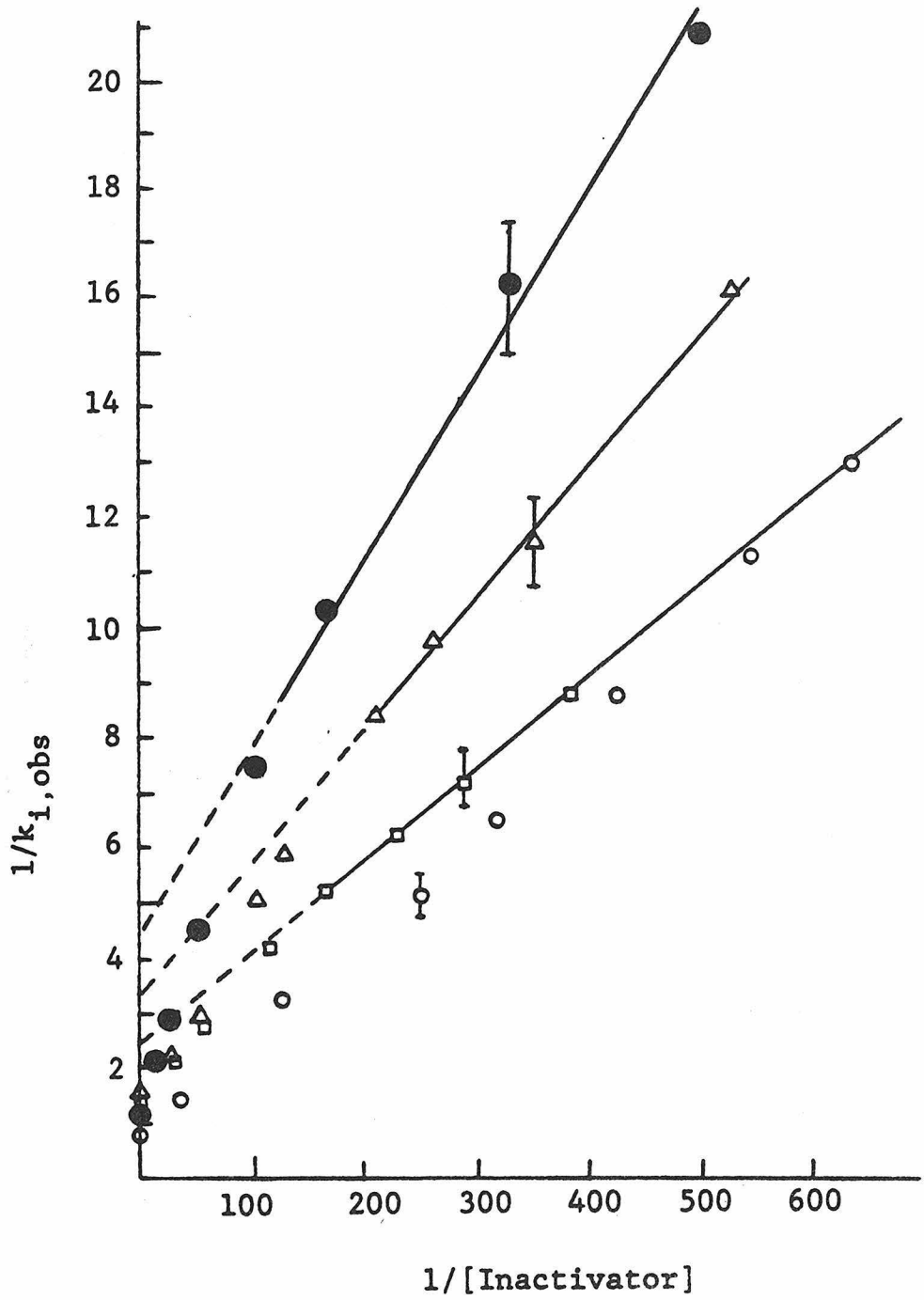

20
18
16
14
12
10
8
6
4
2
$1/k_{i,obs}$
100
200
300
400
500
600
1/[Inactivator]

Figure 4

reciprocal plots for (R)-, (S)-, and (RS)-gly-d_2 appear to become linear only at low inactivator concentrations (Fig. 4). Data for gly appear linear as plotted in Fig. 4, however, they actually exhibit behavior similar to that of (R)-, (S)-, and (RS)-gly-d_2: downward curvature at high inactivator concentration and linearity at low inactivator concentration (Fig. 5 better illustrates this behavior, which is discussed later in detail). In the linear regions, data for gly and (R)-gly-d_2 define the same line, whereas data for (S)- and (RS)-gly-d_2 give lines with different slopes and y-intercepts. The slopes of the linear regions should be given by the appropriate form of eq 5a. Assuming the y-intercept obtained by extrapolation of the linear region to infinite inactivator concentration to be the reciprocal of the respective k_i, one may use the value of K_I for diol-d_2 to calculate a value of K_G for each inactivator. In each case, this value is 1.6 mM, the same as that previously determined for gly independent of the presence of 1,2-propanediol (Chapter I). Thus, while values of K_G determined for (R)-, (RS)-, and (S)-gly-d_2 in the absence of 1,2-propanediol differ, these inactivators all exhibit the same apparent K_G in the linear regions of Fig. 4, where concentrations of each glycerol and of diol-d_2 are such that [EG]/[ES] < 1.

A plot of the observed isotope effect vs 1/[inactivator] (Fig. 5) more informatively illustrates the variation of

Figure 5

Plot of isotope effect on $k_{i,obs}$ as a function of the reciprocal of inactivator concentration in the presence of 1.08×10^{-4} M diol-d_2: (○) glycerol; (□) (R)-gly-d_2; (△) (RS)-gly-d_2; (●) (S)-gly-d_2. The isotope effects were calculated from the experimentally determined $k_{i,obs}$ as described in Results.

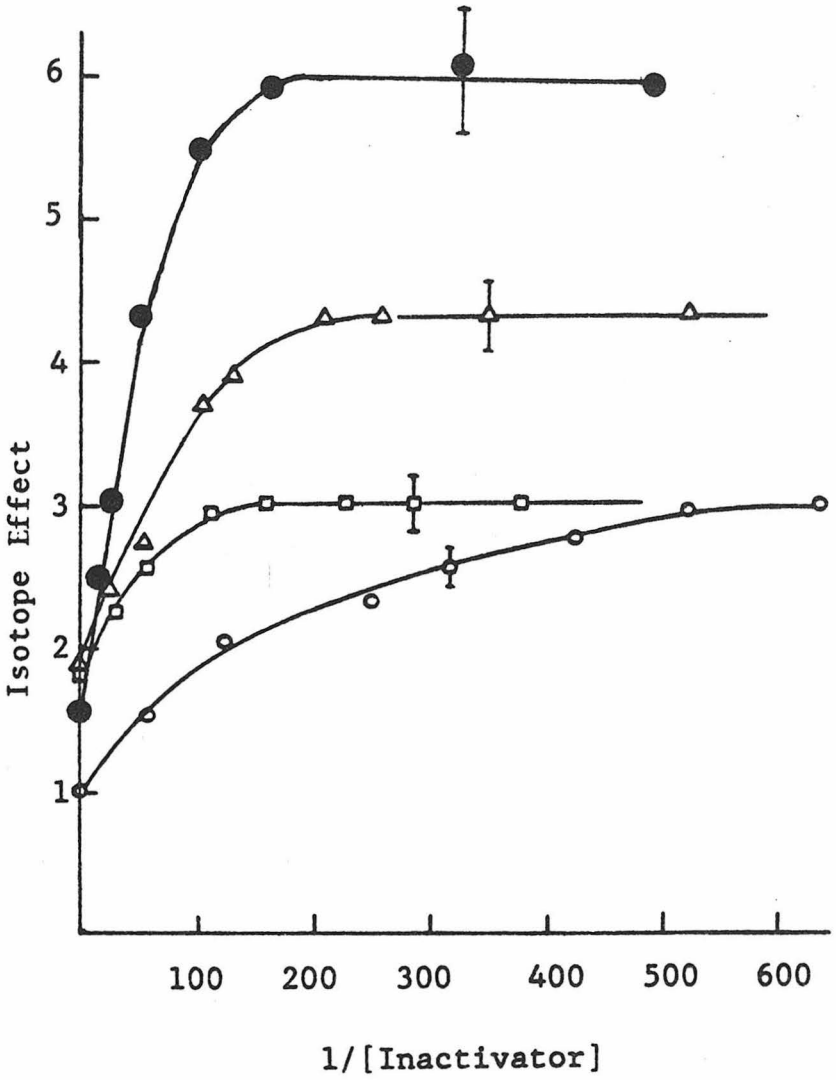


Figure 5

$k_{i,obs}$. The isotope effects were calculated by comparing $k_{i,obs}$ measured at each concentration of a given inactivator in the presence of diol-d_2 to the $k_{i,obs}$ obtained for the corresponding concentrations of undeuterated glycerol and propanediol. For example, the isotope effect on inactivation at a given concentration of (R)-gly-d_2 would be defined as:

$$\left(\frac{k_H}{k_D}\right)_i = \frac{k_{i,obs}(gly)_{diol}}{k_{i,obs}((R)\text{-gly-}d_2)_{diol\text{-}d_2}} \qquad (6)$$

$k_{i,obs}((R)\text{-gly-}d_2)_{diol\text{-}d_2}$ is the experimentally determined "observed" inactivation rate constant at the particular concentration of (R)-gly-d_2 in the presence of the fixed concentration of diol-d_2 and $k_{i,obs}(gly)_{diol}$ is the corresponding observed rate constant for gly in the presence of undeuterated diol. Use of eq 5 and the fact that [gly] = [(R)-gly-d_2] and [diol] = [diol-d_2] reduce eq 6 to

$$\left(\frac{k_H}{k_D}\right)_i = \frac{k_i(gly)}{k_i((R)\text{-gly-}d_2)} \quad .$$

The data in Fig. 5 show that for all four inactivators a limiting maximum isotope effect is approached at low inactivator concentration. For (S)-gly-d_2, this value is 6.0; for (RS)-gly-d_2, 4.3; for gly and (R)-gly-d_2, 3.0.

Kinetics of Glycerol Inactivation. Competition with (RS)-1,2-Propanediol. The rate of inactivation in the

presence of a fixed concentration of undeuterated propanediol was determined as a function of inactivator concentration for the following inactivators: (R)-, (RS)-, and (S)-gly-d_2, and gly-d_5. The analogous experiment with undeuterated glycerol has been described in Chapter I; the kinetics are described by eq 5 with k_i=1.3 min^{-1} and K_G = 1.6 mM. Figure 6 illustrates the results as a plot of the expressed isotope effect on inactivation vs the reciprocal of inactivator concentration. Isotope effects were calculated as described in the previous section. At low inactivator concentrations, the data of Fig. 6 indicate that a limiting minimum isotope effect is approached for (R)-, (RS)-, and (S)-gly-d_2; these values are 1.0 - 1.1 for (R)- and (RS)-gly-d_2, and 1.4 - 1.5 for (S)-gly-d_2.

At high inactivator concentrations (20 - 40 mM), the isotope effects for (RS)- and (S)-gly-d_2 (as calculated from eq 5 and 6) actually exceed those measured in the absence of 1,2-propanediol. However, if in calculating the isotope effect on $k_{i,obs}$ in this region the K_G values obtained in the absence of 1,2-propanediol are used, the resulting "isotope effects" are equal to those observed in the absence of substrate.

Thus, with (S)- and (RS)-gly-d_2 (and possibly gly-d_5) as inactivators in the presence of diol, the apparent K_G value observed at large inactivator concentrations is similar to that measured in the absence of substrate, and

Figure 6

Plot of the isotope effect on $k_{i,obs}$ as a function of the reciprocal of inactivator concentration in the presence of 1.4 mM diol: (○) gly-d_5; (●) (S)-gly-d_2; (△) (RS)-gly-d_2; (□) (R)-gly-d_2. Isotope effects were calculated as described under Results.

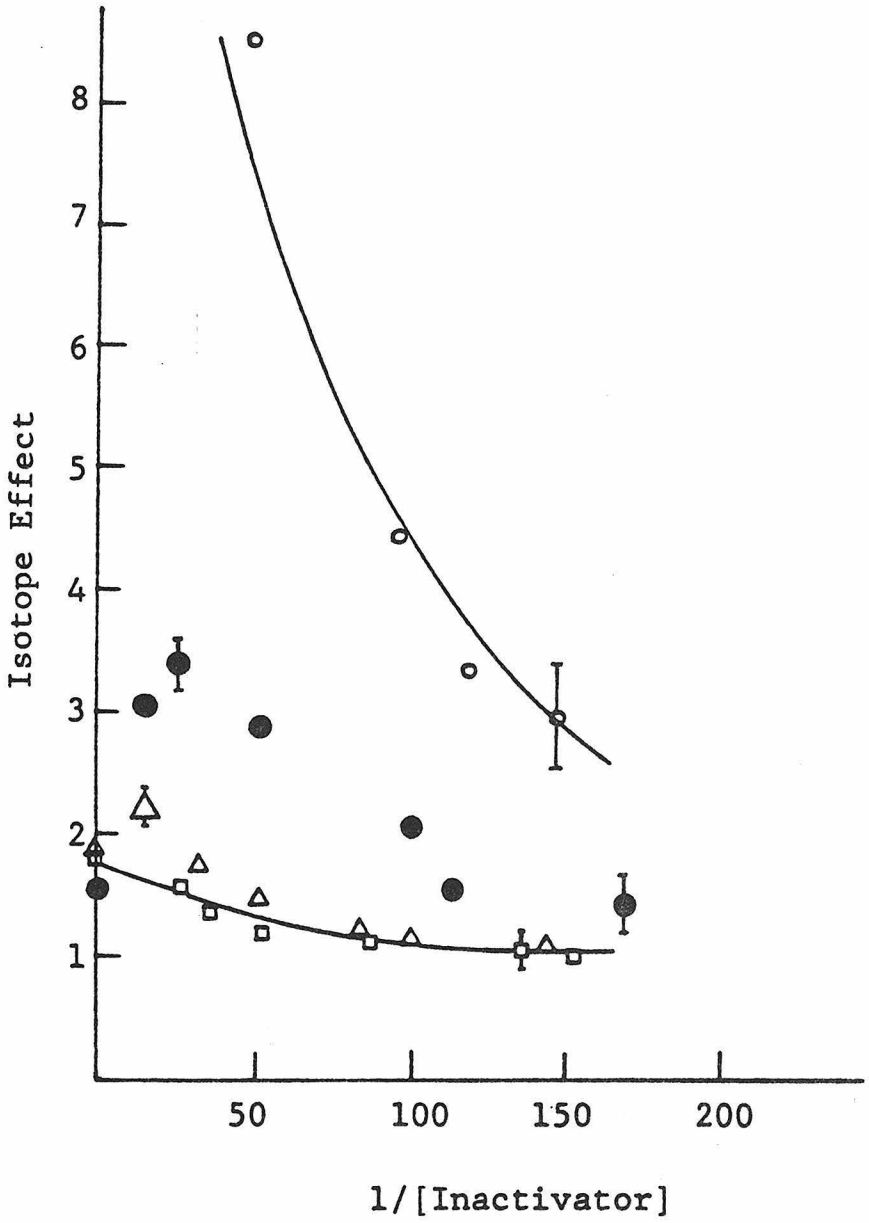


Figure 6

declines to a value identical to that of undeuterated glycerol at low inactivator concentration.

Isolation and Identification of 5'-Deoxyadenosine. When [1 -^{3}H]-glycerol inactivates diol dehydratase holoenzyme, tritium is transferred to the nucleoside moiety of AdoCbl. This nucleoside cochromatographs with authentic 5'-deoxyadenosine on silica gel and Whatman 3MM paper in a number of solvent systems. The amount of tritium in the 5'-deoxyadenosine isolated after each reaction is directly proportional to the amount of holoenzyme inactivated (Fig. 7); control experiments using [1 -^{14}C]-glycerol or heat-inactivated apoenzyme yielded no radioactivity associated with 5'-deoxyadenosine. A least squares fit of ^{3}H dpm in 5'-deoxyadenosine as a function of the amount of enzyme indicates that 5'-deoxyadenosine is enriched in tritium by a factor of approximately 2.1 over substrate, on a per mole basis. This calculation assumes an essentially quantitative liberation of 5'-deoxyadenosine from holoenzyme.

A control experiment in which the inactivated holoenzyme was not denatured prior to chromatography on BioGel P-2 showed that the nucleoside remains bound to the protein and does not exchange with unlabeled 5'-deoxyadenosine in solution.

Figure 7

Radioactivity associated with 5'-deoxyadenosine from diol dehydratase holoenzyme which has been inactivated by radiolabeled glycerol. (△) [1 -^{3}H]-glycerol, 0.49 mCi/mmole; (○) [1 -^{14}C]-glycerol, 3.7 mCi/mmole; (□) [1 - ^{3}H]-glycerol, 0.49 mCi/mmole and boiled apoenzyme.

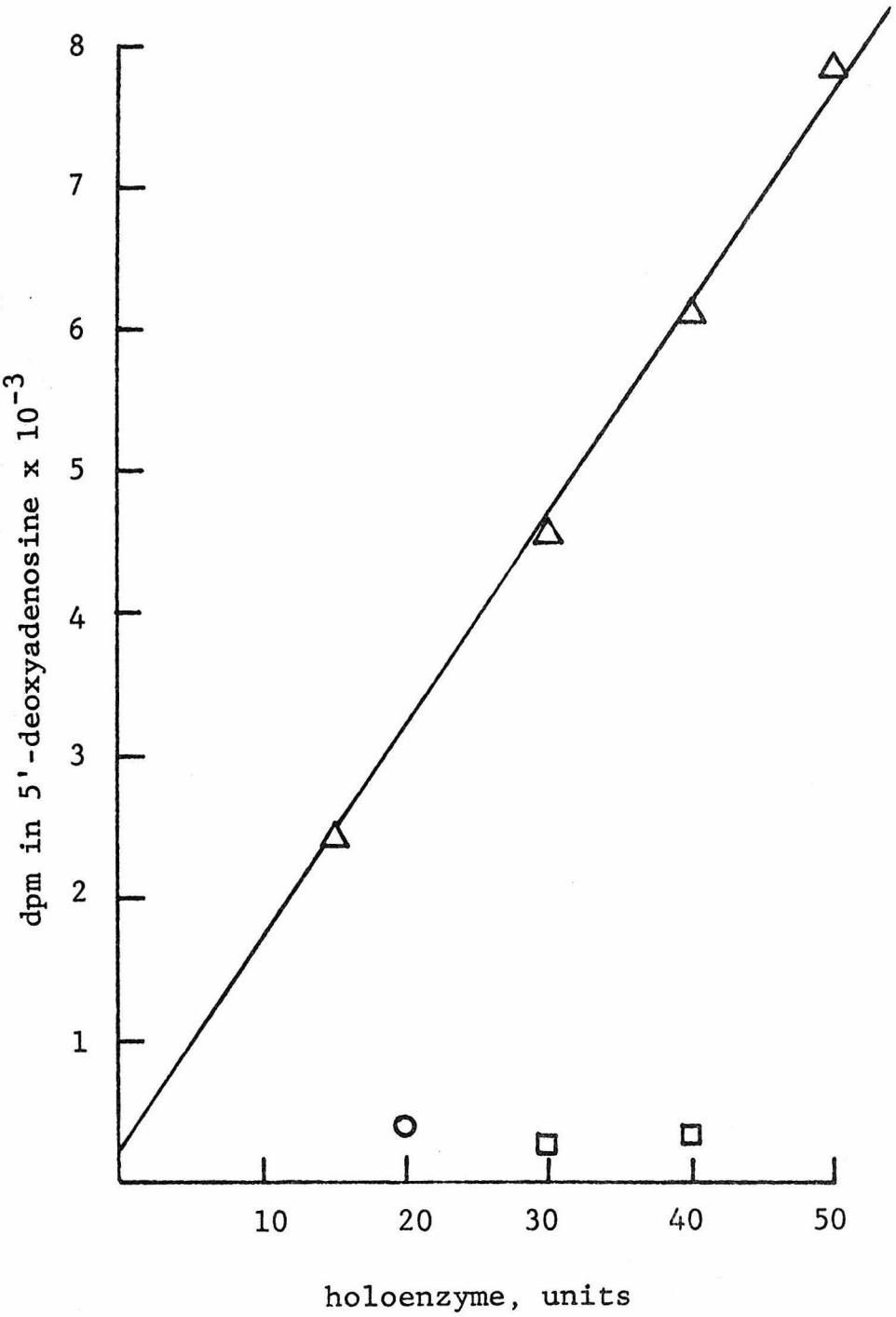

8
7
6
5
4
3
2
1
dpm in 5'-deoxyadenosine x 10^{-3}
10
20
30
40
50
holoenzyme, units

Figure 7

DISCUSSION

The kinetics of inactivation when both inactivator and inhibitor are "homogeneous" with respect to transferrable hydrogens (i.e., undeuterated glycerol with undeuterated propanediol, or gly-d_5 with diol-d_2) obey eq 5, with appropriate constant values for K_G, K_I, and k_i, for all concentrations of inactivator at the fixed "inhibitor" concentration. This is not true, however, for the isotopically mixed cases (i.e., undeuterated glycerol, (RS)-, (R)-, and (S)-gly-d_2 with diol-d_2; or gly-d_5 and these dideuterated glycerols with diol) as illustrated by the double reciprocal plots (for example, Fig. 4). Possible sources for this divergent behavior may be variation of K_G, K_I, and /or k_i with inactivator concentration.

A change of K_I for diol and diol-d_2 may be considered as an explanation for the shape of the double reciprocal plots in the isotopically mixed cases. This explanation, however, appears unlikely. The inhibitor constant K_I for 1,2-propanediol is unaffected by deuterium substitution, as is that of the substrate 3-fluoro-1,2-propanediol (Eagar et al., 1975). Moreover, the effects observed when undeuterated diol protects against inactivation by gly-d_5 are too large to be explained in terms of effects on K_I. Accordingly, an explanation for the observed behavior must be sought in effects on K_G and k_i.

Variation of K_G. In the absence of 1,2-propanediol,

the values of K_G, the Michaelis constant of the holoenzyme-glycerol complex, are 1.6 mM for gly and (R)-gly-d_2, 2.1 mM for (RS)-gly-d_2, and ~3.7 mM for both (S)-gly-d_2 and gly-d_5. These data suggest that substitution of deuterium for hydrogen at the pro-(S) hydroxymethyl group of glycerol (gly-d_5, (S)-gly-d_2, and (RS)-gly-d_2) adversely affects binding affinity, as measured in the manner described in Results, while the corresponding substitution at the pro-(R) hydroxymethyl group has no measurable effect. The K_G value exhibited by (RS)-gly-d_2 is the same as that which would be predicted for a 1:1 mixture of (R)- and (S)-gly-d_2. Eagar et al. (1975) have analyzed the kinetics of such a two-substrate mixture; the apparent K_m of the racemic substrate in terms of the respective constants for the (R)- and (S)-stereoisomers is

$$K_{RS} = \frac{2K_R K_S}{K_R + K_S} .$$

In the present case, the predicted K_G for (RS)-gly-d_2 is 2.2 mM, very close to the observed value of 2.1 mM. Thus, (R)- and (S)-gly-d_2 appear to compete for the same active site of diol dehydratase.

In this light, other results (Chapter I) suggest that substitution of deuterium at the pro-(S) hydroxymethyl group of glycerol adversely affects the binding of only the EG_S conformation. The isotope effect on catalysis (k_H/k_D), for (S)-gly-d_2 is 0.6; this substrate is converted to

product some 65 % faster than glycerol itself. As we know of no precedent for such a large inverse primary deuterium isotope effect, we attribute this result to weakened binding of the EG_S binding conformation of (S)-gly-d_2, with little or no concomitant effect on that of EG_R. Thus, the catalytic EG_R conformation competes more effectively for the active site with (S)-gly-d_2 as substrate; the actual microscopic turnover rate probably changes very little. This effect should also occur with gly-d_5; the observed $(k_H/k_D)_p = 8$ (Chapter I) is in reality a combination of two processes: reduction of k_p due to participation of heavy isotope, and enhancement of the catalytic rate due to more favorable competition of the EG_R conformation for the active site. Thus, the true deuterium isotope effect on catalysis is probably about 12. Similar reasoning suggests that the true deuterium isotope effect on inactivation $(k_H/k_D)_i$ may be somewhat smaller than the value of 15 observed with gly-d_5 as inactivator.

In the presence of diol or diol-d_2 as the inhibitor of inactivation, the apparent K_G values of (RS)- and (S)-gly-d_2 change, from the values measured above when [inactivator]/[diol] is relatively large, to the same value as gly and (R)-gly-d_2 (1.6 mM) when this ratio is relatively small (Fig. 4, 5, 6). However, this change is not observed when the inhibitor of inactivation is d- or dl-2,3-butanediol, purely competitive inhibitors which do not

undergo catalysis or inactivation. Thus, this effect is associated in some fashion with the ability of diol and diol-d_2 to undergo catalysis. The nature of this phenomenon was further probed by measuring $k_{i,obs}$ of (S)-gly-d_2 as a function of inactivator concentration in the presence of 1,2-propanediol of an isotopic composition (2.25 % D at C-1) chosen to result in a hydrogen isotopic composition at C-5' of AdoCbl (Moore et al., 1979; Chapter III) which is approximately the same as that generated by (S)-gly-d_2 itself. The results indicate that in this case, the apparent K_G of (S)-gly-d_2 is 3.7 mM, whereas in the presence of diol or diol-d_2, it is observed to be 1.6 mM.

These results suggest that K_G is probably not a simple dissociation constant; rather, it is more analogous to a Michaelis constant ("K_m"). Consider the simple mechanism

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E + P \ .$$

For this reaction, $K_m = (k_2 + k_3)/k_1$, and we see that the catalytic rate constant contributes to determining the value of K_m. In the present case, K_G is clearly influenced by reaction steps subsequent to binding in which hydrogen attached to C-5' participates. However, an explicit description of this phenomenon in kinetic terms has not yet emerged.

Variation of k_i. One can construct a mechanism whereby k_i may vary by drawing upon the currently accepted catalytic mechanism and the supporting experimental data. Catalysis by diol dehydratase holoenzyme and other AdoCbl-dependent enzymes involves two hydrogen-transfer steps: (1) from C-1 of substrate to C-5' of AdoCbl and (2) from C-5' of AdoCbl to C-2 of product (Abeles, 1971). Evidence for the formation of 5'-deoxyadenosine as an intermediate and evidence for the equivalence of the C-5' hydrogens, although not conclusive, are quite extensive (Babior, 1975b; Frey et al., 1967; Miller & Richards, 1969; Eagar et al., 1972). Thus, both intramolecular and intermolecular transfers of hydrogen are possible (Abeles & Zagalak, 1966). Moreover, the isotope effect on the second hydrogen-transfer step (cofactor → product) considerably exceeds that for the first hydrogen-transfer step; thus, deuterium (or tritium) accumulates at the 5'-carbon of cofactor during catalysis. Indeed, enrichment in tritium of enzyme-bound AdoCbl by as much as 20-fold over that in substrate has been observed during catalysis (Essenberg et al., 1971). Thus, a mechanism exists for the "inhibitor" of inactivation to contribute a hydrogen (or deuterium) to the inactivation pathway. Furthermore, the isotopic composition of the 5' carbon of cofactor, and thus the isotope effect expressed on hydrogen transfer from cofactor, should vary with inactivator concentration. For the "homogeneous" cases, no

such variation should occur, since only hydrogen or only deuterium is involved in the reaction. Thus, the divergent behavior observed between the homogeneous and mixed cases can be explained in terms of the variation of k_i and requires only that a rate-contributing or rate-limiting step for inactivation occur at some point along the pathway after the initial abstraction of hydrogen from C-1 of glycerol. This step could be hydrogen abstraction either from C-5' of the resulting 5'-deoxyadenosine intermediate or from C-1 of glycerol after equilibration between this hydrogen and those attached to C-5' of AdoCbl. However, hydrogen exchange between cofactor and unreacted substrate could not be demonstrated in the diol dehydratase (Frey et al., 1967) and ethanolamine deaminase (Carty et al., 1971) reactions. In the case of diol dehydratase, ethylene glycol, a substrate which also inactivates holoenzyme was used. Thus, equilibration of hydrogen between AdoCbl and reversibly bound substrate is unlikely. If such exchange occurs after an irreversible step such as reaction 1 above, no distinction can be drawn between these two suggested mechanisms; both require formation of an intermediate containing three equivalent hydrogens.

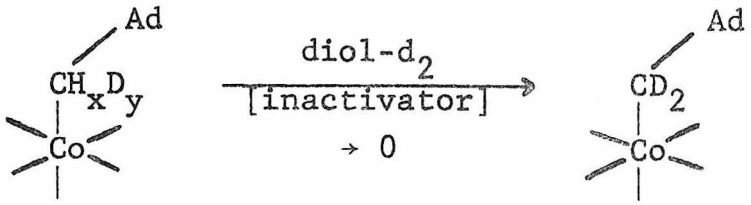
We shall therefore argue that not only does transfer of hydrogen from cofactor contribute to the observed inactivation rate constant, thereby implicating the presence of an intermediate such as 5'-deoxyadenosine in the inacti-

vation pathway, but that transfer of hydrogen from this intermediate is the rate-determining step in the inactivation process. Virtually all our kinetic data accord with this hypothesis; in particular, the limiting value of the isotope effects observed at low inactivator concentrations (Fig. 5 and 6) can with perhaps one exception be quantitatively explained by this hypothesis.

We shall consider first the "mixed" cases in which diol-d_2 functions as the inhibitor of inactivation. At very high concentrations of inactivator, diol-d_2 should be effectively excluded from the holoenzyme, and the rate of inactivation should, and does, extrapolate to that observed for inactivation in the absence of diol inhibitor (Fig. 4 and 5; Table II lists the rates of inactivation and the corresponding isotope effects observed for each inactivator in the absence of propanediol). As the concentration of inactivator decreases, an increasing proportion of holoenzyme interacts with diol-d_2, and enzyme-bound AdoCbl becomes increasingly rich in deuterium. Thus, the inactivation rate constant reflects an increasing isotope effect (Fig. 5).

Figure 5 also illustrates that, as inactivator concentration decreases, the observed isotope effect reaches a limiting maximum value for each inactivator, a behavior consistent with our hypothesis. Considering the much larger isotope effect on the second hydrogen-transfer ste

(cofactor → product) one might expect that, as the concentration of inactivator relative to inhibitor is decreased, a point should be reached where essentially all transferrable hydrogens at C-5' of enzyme-bound AdoCbl have been replaced with deuterium.



(x and y represent the isotopic composition that would characterize the cofactor.) Thus, when the cofactor is saturated with deuterium, no further increase in the isotope effect with decreasing inactivator concentration would be possible, and a maximum value for the isotope effect for each particular inactivator would be reached as demonstrated experimentally in Fig. 5.

The inactivator concentration at which this maximum, limiting value is expressed differs for each inactivator. With (R)-gly-d_2, this "saturation" effect occurs at a relatively high concentration of (R)-gly-d_2; with (RS)-gly-d_2 and (S)-gly-d_2 somewhat lower concentrations are required; with undeuterated glycerol, this saturation effect is observed only at very low concentrations of inactivator. This order likewise accords with the proposed hypothesis. We have previously shown that glycerol not only inactivates holoenzyme, it can also serve as a

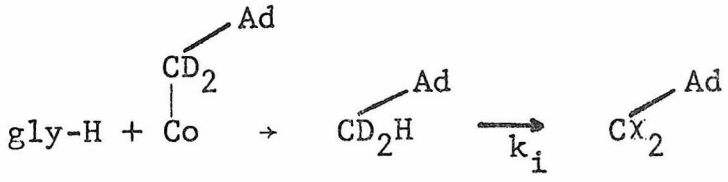
Table II: Comparison of the Limiting Values of the Inactivation Rate Constants and Resulting Isotope Effects

	Substrate free		1,2-Propanediol-d_2		1,2-Propanediol	
	$k_i(min^{-1})$	$(k_H/k_D)_i$	$k_i(min^{-1})$,[a] $1.08X10^{-4}M$ diol-d_2	k_H/k_D	k_i,[a] $1.4X10^{-3}M$ diol	$(k_H/k_D)_i$
Glycerol	1.3	1.0	0.43	3.0	1.30	1.0
(R)-Glycerol-d_2	0.72	1.8	0.43	3.0	1.30	1.0
(RS)-Glycerol-d_2	0.68	1.9	0.30	4.3	<1.30	>1.0
(S)-Glycerol-d_2	0.82	1.6	0.22	6.0	0.9	1.4
Glycerol-d_5	0.085	15	0.087	15	∿0.9	∿1.4

[a]The k_i values listed in the competition with substrate experiments are the constant k_i's fitting eq 5 at low inactivator concentrations.

substrate (involving predominantly the pro-(R) end of the glycerol molecule) (Bachovchin et al., 1977). (R)-gly-d_2 contains only deuterium on the pro-(R) carbon; thus, catalysis involving this glycerol as substrate should more readily enrich enzyme-bound AdoCbl with deuterium than would catalysis involving (RS)-gly-d_2, which contains both deuterium and hydrogen in equal amounts on the pro-(R) carbon. Undeuterated glycerol and (S)-gly-d_2 acting as substrates should actually retard the saturation of enzyme-bound AdoCbl with deuterium. Thus, the observed order follows from the hypothesis and the ability of glycerol to act both as substrate and inactivator.

The maximum limiting values of the isotope effects are 3.0 both for undeuterated glycerol and for (R)-gly-d_2, while for (RS)-gly-d_2 the experimental value is 4.3 (Fig. 5). These values can be quantitatively explained by the hyptohesis. At very low concentrations, glycerol in the presence of diol-d_2 finds enzyme-bound AdoCbl saturated with deuterium.

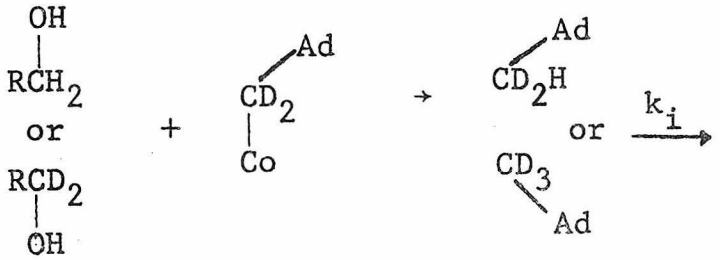


where χ = D or H

Since glycerol contains only hydrogen, the resulting 5'-deoxyadenosine must be $-CD_2H$. Using 15 as the value for the primary isotope effect in transfer away from 5'-deoxyadenosine and assuming that the three hydrogens have a statistically equal chance to be removed in this rate-determining step, one calculates that the isotope effect should be:

$$(k_H/k_D)_i = [(2/3)(1/15) + (1/3)(1)]^{-1} = 2.7$$

This agrees reasonably well with the experimental value of 3.0. With (R)-gly-d_2, again only hydrogen can be transferred to AdoCbl during inactivation, since inactivation occurs predominantly from the pro-(S) end of glycerol (Bachovchin et al., 1977); thus, the calculated value is the same as that for undeuterated glycerol which agrees with the experimental value. However, with (RS)-gly-d_2 either deuterium or hydrogen can be transferred. so that two isotopically substituted types of 5'-deoxyadenosine are possible:



Assuming an equal contribution of both types of 5'-deoxyadenosine to the rate and the statistical equivalence of

the three hydrogens at C-5', one calculates that the isotope effect in this case should be

$$(k_H/k_D)_i = \left[\frac{0.5}{[(2/3)(1/15) + (1/3)(1)]^{-1}} + \frac{0.5}{15}\right]^{-1} = 4.5$$

which agrees well with the experimental value.

In contrast, the isotope effects calculated for a mechanism involving an intermediate with two equivalent hydrogens would be 1.9 for gly and (R)-gly-d_2 and 3.3 for (RS)-gly-d_2. Thus, the experimental data favor a mechanism involving the intermediacy of a species with three equivalent hydrogens.

With (S)-gly-d_2 as inactivator, the above treatment predicts a limiting maximum isotope effect of $\sim$ 14. However, a value of 6 is observed (Fig. 5). A possible explanation for this result is that the (S)-gly-d_2 as synthesized (Chapter I) is partly ($\sim$ 20%) racemic, and contains some of the R isomer. As deuterium is the only source of stereoisomerism after removal of the isopropylidene protecting group in step six of the eight-step synthesis, there is no sufficiently sensitive method for detecting such a small degree of racemization during either of the last two steps. However, in control experiments, all kinetic data for inactivation and product formation obtained with (RS)-gly-d_2 were reproduced by an equimolar mixture of (R)- and (S)-gly-d_2; accordingly we believe this explanation to be unlikely. Alternatively, as

discussed in the previous section, the true isotope effect on inactivation may be less than the value of 15 observed with gly-d_5 as inactivator. If for example the true isotope effect is 11-12, the observed limiting maximum isotope effect of 6 observed for (S)-gly-d_2 in the presence of diol-d_2 could be explained by a small fraction ($\leq$ 15 %) of inactivation occurring via the pro-(R) conformation. In fact, this possibility would also account for the limiting maximum isotope effect observed with (R)-gly-d_2, which as noted is somewhat higher than predicted on the basis of the proposed mechanism (no change in the value observed for (RS)-gly-d_2 would be expected). We recognize that this explanation is at root conjectural, and cannot really be verified. However, of these limiting maximum isotope effects, that observed with (S)-gly-d_2 is by far the most sensitive to small deviations from the behavior predicted by the (admittedly simplified) hypothesized inactivation mechanism.

Further support for the proposed mechanism comes from evaluation of the Michaelis constants for inactivator, K_G, and inhibitor, K_I, as measured at low inactivator concentrations in the linear regions of Fig. 4. With k_i constant, eq 5 should apply, and in fact double-reciprocal plots of these data points are linear with slopes and y-intercepts indicating the appropriate new values of k_i

while indicating no effect on either K_G or K_I for each inactivator (Fig. 4). Thus the proposed hypothesis accounts for the features of the experimental results presented in Fig. 4 and 5.

We now consider the corresponding "mixed" cases in which undeuterated propanediol is the inhibitor of inactivation. In these cases, the deuterium content of enzyme-bound AdoCbl should decrease with decreasing concentration of inactivator and the observed isotope effect should also decrease.

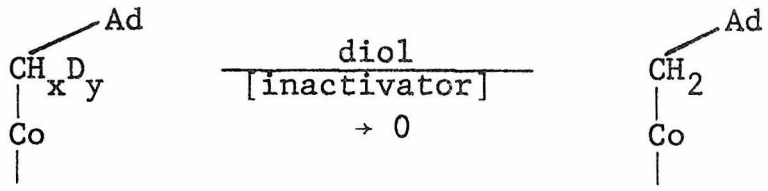


Figure 6 illustrates the agreement between this expectation and the experimental results. This effect is more clearly illustrated with gly-d_5 as inactivator; at high concentrations of gly-d_5, the isotope effect is quite large, but with decreasing concentrations of gly-d_5 the observed isotope effect decreases dramatically.

Again, as one lowers the concentration of inactivator relative to diol inhibitor, enzyme-bound AdoCbl should become saturated with the isotope present in the diol, which in this case is hydrogen rather than deuterium. Thus, one anticipates a limiting minimum value for the isotope effect. The results in Fig. 6 suggest that such a limit is in fact

approached for each inactivator. However, in practice, reliable experimental data in this region of "constant k_i" are difficult to obtain; the value of [S] (1.4 mM) chosen to ensure $\Delta[S] \sim 0$ was so large that the observed inactivation rates are quite small.

Nevertheless, limiting minimum values for the isotope effects expected for each inactivator can be calculated on the basis of the above hypothesis; these values agree rather well with those approached experimentally (Fig. 6). For example, (R)-gly-d_2 with only hydrogen at the pro-(S) carbon can generate only an Ad-CH_3 intermediate; the calculated isotope effect for inactivation is thus 1.0. The calculated value for (RS)-gly-d_2 is only slightly larger (1.2). With both these inactivators, the experimental value appears to approach 1-1.1 as the limiting minimum value. (S)-Gly-d_2 and gly-d_5, however, should only contribute deuterium; the intermediate in the limiting case is Ad-CH_2D, and the isotope effect calculated for this intermediate is 1.4. The data for (S)-gly-d_2 do indeed seem to attain approximately this value at low inactivator concentrations. As this isotope effect is considerably smaller than the value of 15 observed at large gly-d_5 concentrations, the data obtained for gly-d_5 present the theory with a challenging test. In fact, these experimental data do indicate such a large change, and seem to approach a minimum value somewhat greater than 1 (Fig. 6).

SUMMARY AND CONCLUSIONS

The results of these kinetic investigations may be summarized as follows. First, while substitution of deuterium for hydrogen at C-1 of 1,2-propanediol has no effect on the K_I of this substrate, the same substitution at the pro-(S) (but not the pro-(R)) hydroxymethyl group of glycerol causes an increase in the value of K_G. In the presence of another substrate which undergoes catalysis, this value of K_G is observed to vary as a function of inactivator concentration. The characteristics of this variation suggest that K_G is not a simple dissociation constant, and that reaction steps subsequent to formation of the holoenzyme-glycerol complex, involving the intermediate hydrogen reserovir at C-5' of AdoCbl, contribute to determining its value.

Second, the results of this work indicate the occurrence of an intermediate such as 5'-deoxyadenosine along the inactivation pathway and, furthermore, that transfer of hydrogen from such an intermediate containing three equivalent hydrogens is the rate-determining step in the inactivation process. This hypothesis can account quantitatively for the limiting values of the isotope effects on k_i observed at low inactivator concentrations in the presence of fixed concentrations of diol and diol-d_2, which function as competitive inhibitors of inactivation.

The hypothesis also accounts for the variation of k_i with the concentration of inactivator in the cases which are isotopically "mixed" and for the invariance of k_i in the isotopically "homogeneous" cases. Accordingly, we conclude that the mechanism of inactivation, like that of catalysis, involves as the first step abstraction of hydrogen from C-1 of glycerol and transfer of this hydrogen to the 5'-carbon of cofactor. The three hydrogens thereby attached to C-5' of deoxyadenosine become equivalent and removal of one of them is involved in the rate-determining step for inactivation. Whether this transfer from 5'-deoxyadenosine involves readdition of hydrogen to the glycerol skeleton or transfer to some other group or species associated with the enzyme or coenzyme remains unclear, as does the nature of the inactivated holoenzyme complex. The identification of the nucleoside in the inactivated complex as 5'-deoxyadenosine suggests that the 5'-deoxyadenosyl moiety which obtains after the second hydrogen-transfer step is capable of acquiring a third hydrogen, possibly either from substrate or a nearby amino acid residue, since intact AdoCbl cannot be recovered following glycerol inactivation (Bachovchin et al., 1977).

A 5'-deoxyadenosine moiety containing three equivalent hydrogens has long been considered as an intermediate in catalysis. The significance of these results for the mechanism of catalysis is that they establish kinetically

the feasibility of such an intermediate in the mechanism of glycerol inactivation, a mechanism which is very similar to and may in fact be identical to the mechanism of catalysis. The major unique feature of inactivation could be that enzyme-bound "product" formed in the normal way from glycerol bound in an "EG_S" fashion effects irreversible inactivation of the holoenzyme complex.

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CHAPTER III

HYDROGEN TRANSFER IN CATALYSIS BY DIOL DEHYDRATASE

INTRODUCTION

Diol dehydratase is an adenosylcobalamin-dependent enzyme which catalyzes the conversion of 1,2-propanediol to propionaldehyde, of ethylene glycol to acetaldehyde (Lee & Abeles, 1963), and of glycerol to β-hydroxypropionaldehyde (Toraya et al., 1976; Bachovchin et al., 1977; Chapters I, II).

The purpose of the work described in this chapter is to investigate the nature of the intermediate(s) on the catalytic pathway between the 1,2-diol substrate and the 1,1-diol intermediate which is subsequently dehydrated to form the aldehyde. Experimental values of k_{cat} were determined for several mixtures of 1,2-propanediol and 1,1-dideuterio-1,2-propanediol. These data were then analyzed in terms of an equation derived to describe the kinetics which would result for several different mechanisms. This method facilitates discrimination among the suggested mechanisms, and also allows calculation of isotope effects on k_{cat} caused by substitution of deuterium for hydrogen in a single step of the reaction pathway. Furthermore, the results of this analysis were used to predict a value for the tritium isotope effect in the diol dehydratase reaction, and this predicted isotope effect was measured experimentally. Analogous experiments were also carried out with mixtures of glycerol and perdeuterioglycerol as substrate.

The experiments and analysis of this work are somewhat

similar to those previously applied to methylmalonyl CoA mutase (Miller & Richards, 1969) and to glutamate mutase (Eagar et al., 1972). Moreover, these methods should prove applicable to the study of intermediates in other enzymatic processes.

EXPERIMENTAL

Propanediol dehydratase was obtained from Klebsiella pneumoniae by a procedure adapted from Lee & Abeles (1963) as described in Chapter I. Fraction E-8 with a specific activity of 35 - 58 was used for all determinations.

Adenosylcobalamin (AdoCbl) was purchased from Sigma Chemical Co.

Assays were performed as previously described (Bachovchin et al., 1977; 1978; Chapter I). Solutions generally contained: yeast alcohol dehydrogenase (ADH, Sigma), 10 units; NADH, 0.2 mM; apodiol dehydratase, 0.15 unit; bovine serum albumin, 0.1 mg; potassium phosphate buffer, pH 8, 30 mM; AdoCbl, 0.02 mM; and the desired mixture of deuterated and undeuterated substrate, 20 mM. Total volume was 2.3 ml and all reactions were carried out at 37° C. The reaction was initiated by addition of AdoCbl in a 0.03 ml aliquot. The rate for each substrate mixture was determined from at least three kinetic experiments.

Values of k_{cat} for glycerol-perdeuterioglycerol mixtures were obtained as described in Chapter I from

experimentally determined values of $k_{i,obs}$, the inactivation rate constant, and P_∞, the amount of product β-hydroxypropionaldehyde formed before complete inactivation. The glycerol concentration employed was at least 50 mM, approximately 14 - fold greater than the K_G of perdeuterioglycerol. At most 7 % of the substrate was converted to product during any determination.

Tritium Isotope Effect. The tritium isotope effect was determined by measuring the increase of the $^3H/^{14}C$ ratio of residual 1,2-propanediol during catalysis by diol dehydratase. Reaction mixtures contained: apodiol dehydratase, 2.3 units; bovine serum albumin, 0.09 mg; potassium phosphate buffer, pH 8, 50 mM; AdoCbl, 0.03 mM; and 1,2-propanediol, 20 mM, containing 0.13 μCi ^3H and 0.04 μCi ^{14}C. Volume 0.45 ml, 37° C. The reaction was initiated by addition of AdoCbl in a 0.01 ml aliquot and quenched by addition of 0.05 ml 2 N HCl. Three 0.02 ml aliquots were removed for aldehyde assay by colorimetric determination of propionaldehyde 2,4-dinitrophenylhydrazone (Eagar et al., 1975) to determine the extent of reaction. A 0.15 aliquot of 7 M 1,2-propanediol was added to the sample as carrier, and 0.5 ml (10 μmole) of methanolic 2,4-dinitrophenylhydrazine (DNP) added. The solution was allowed to stand for 2 - 3 hr and then centrifuged. The supernatant was extracted with three 0.36 ml portions of chloroform. The aqueous layer was added to 20 ml chloroform and dried

with stirring over excess sodium sulfate. This solution was filtered and evaporated under vacuum. The yellow, oily residue was taken up in 1 ml water, decolorized with charcoal, and centrifuged. An 0.9 ml aliquot was added to PCS:xylene (2:1) and assayed for radioactivity by double isotope counting methods. Acid was added to one sample prior to AdoCbl as a control.

Substrate. (RS)-1,2-Propanediol and (RS)-1,1-dideuterio-1,2-propanediol (diol-d_2) were obtained as described in Chapter I.

(RS)-1,1,2-Trideuterio-1,2-propanediol (diol-d_3) was prepared by reduction of ethyl pyruvate with lithium aluminum deuteride (Stohler, 99 % D).

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

(RS)-[1 -^{14}C]-1,2-Propanediol was purchased from Dhom.

(RS)-[1 -^{3}H]-1,2-Propanediol was synthesized by reduction of dl-ethyl lactate with $LiAl^3H_4$ (New England Nuclear). Radiochemical purity was at least 98 % as determined by thin-layer chromatography on silica gel with 5:1 chloroform:methanol as eluting solvent.

Perdeuterioglycerol was purchased from Merck, Sharp,

and Dohme, Ltd.

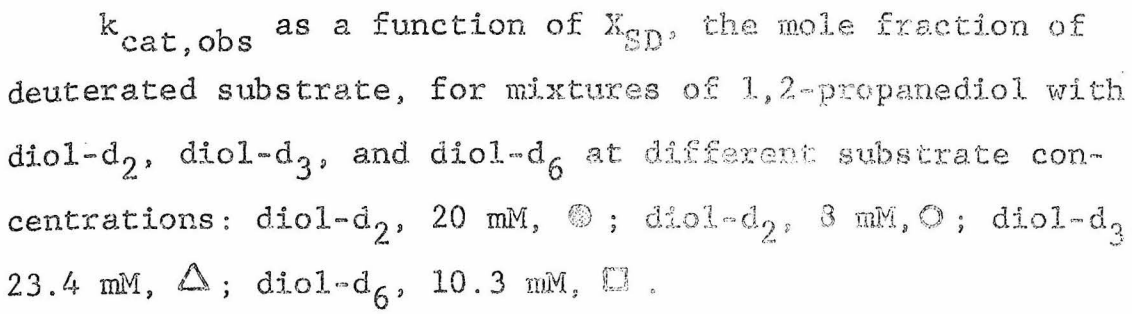
Computing. Computer programs based on the algorithms described by Marquardt (1963) were used to fit the experimental rate data to an equation derived to describe the kinetics of AdoCbl-dependent reactions.

RESULTS

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

Figure 2 shows the temperature dependence of k_{cat} for 1,2-propanediol and diol-d_2. Values of the activation energy E_a and entropy $\Delta S^{\dagger}$ (37° C) obtained from these data are 9.2 ± 1 kcal/mole and -17.8 ± 2 e.u. for 1,2-propanediol

Figure 1



$k_{cat,obs}$ as a function of X_{SD}, the mole fraction of deuterated substrate, for mixtures of 1,2-propanediol with diol-d_2, diol-d_3, and diol-d_6 at different substrate concentrations: diol-d_2, 20 mM, ◉ ; diol-d_2, 3 mM, ○ ; diol-d_3, 23.4 mM, △ ; diol-d_6, 10.3 mM, □ .

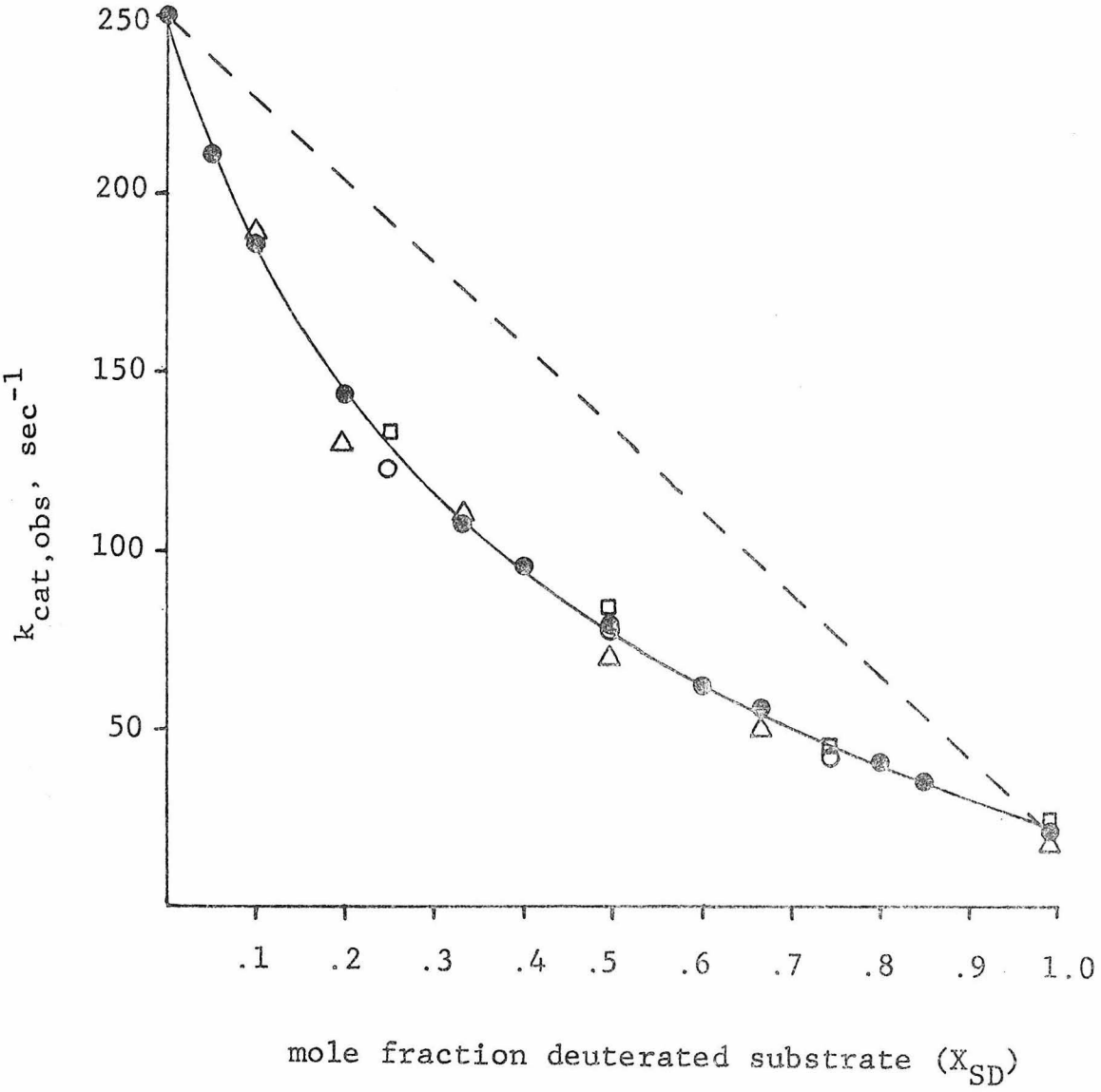

250
200
150
100
50
$k_{cat,obs}$, sec^{-1}
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
mole fraction deuterated substrate (X_{SD})

Figure 1

Figure 2

Plot of $\ln(k_{cat,obs})$ as a function of 1/T for 1,2-propanediol (O, right), and diol-d_2 (◍, left).

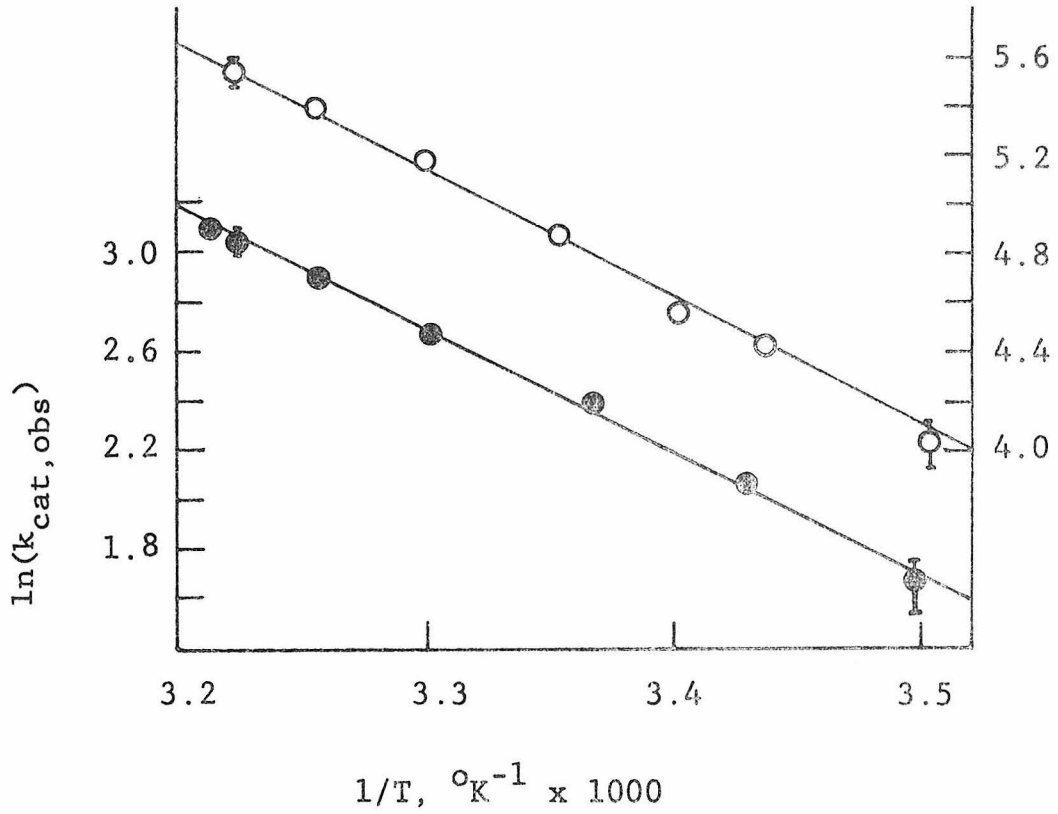


Figure 2

and 9.8 ± 1 kcal/mole and -20.8 ± 2 e.u. for diol-d_2 (Moore, 1962).

Derivation of Rate Expression: A quantitative interpretation of the data of Fig. 1 requires an equation which relates the observed catalytic rate to the isotopic composition of substrate. Such an equation may be derived by consideration of the generally accepted machanism of catalysis, which involves a minimum of two steps: (i) transfer of hydrogen (or deuterium) from C-1 of substrate to an intermediate reservoir of hydrogen, and (ii) transfer of hydrogen (or deuterium) from this reservoir to C-2 of product. Figure 3 outlines a simple hypothetical mechanism in which hydrogen abstracted from substrate becomes one of two equivalent hydrogens in an intermediate reservoir. Substrate may contain either deuterium (mole fraction X_{SD}) or hydrogen (mole fraction $X_{SH} = 1 - X_{SD}$), and the reservoir may likewise have either deuterium (mole fraction X_D) or hydrogen (mole fraction X_H). As illustrated in Fig. 3, conversion of one molecule of substrate to product may occur by any one of six microscopic pathways (a-f), each of which is characterized by a particular overall rate constant (k_{HH}, k_{HD}, k_{DH}, or k_{DD}). The contribution of each pathway to the overall observed rate constant ($k_{cat,obs}$) is the rate constant appropriate for that particular path multiplied by the probability that such a path will be followed. Accordingly, the catalytic rate observed for a

Figure 3

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

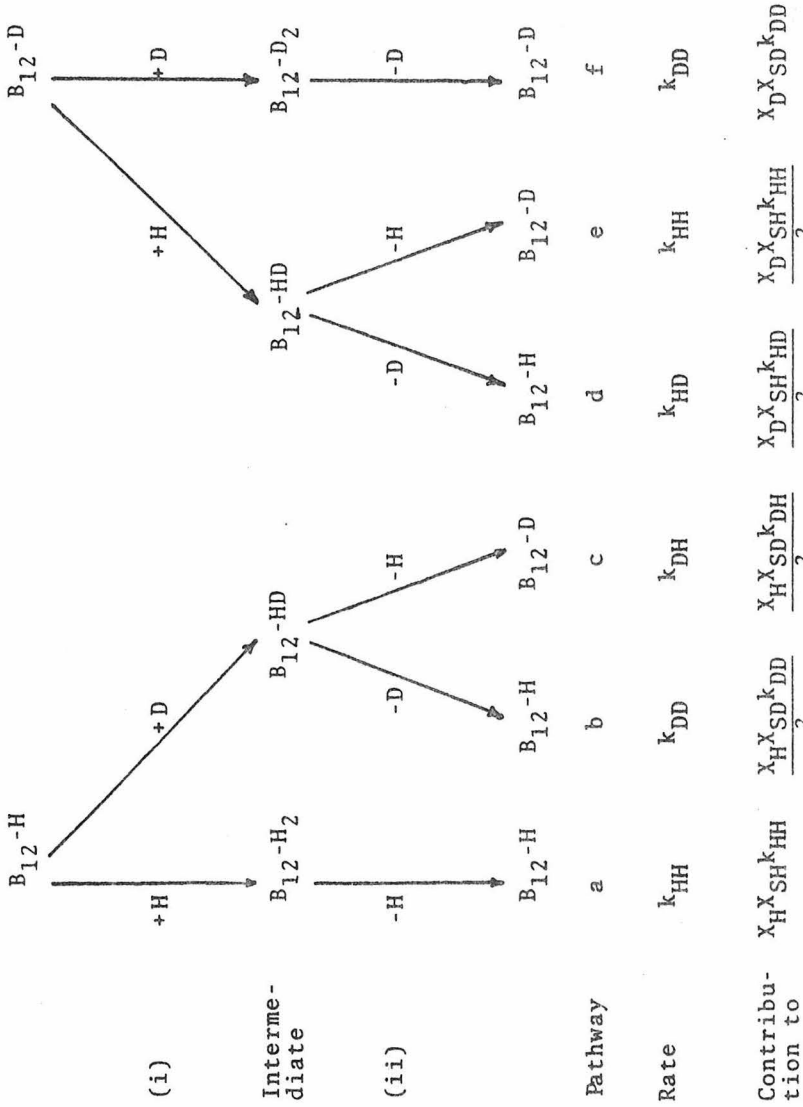

B_{12}-H
B_{12}-D
+H
+D
B_{12}-H_2
B_{12}-HD
B_{12}-D_2
(i)
Intermediate
(ii)
-H
-D
B_{12}-H
B_{12}-D
Pathway
a
b
c
d
e
f
Rate
k_{HH}
k_{DD}
k_{DH}
k_{HD}
k_{HH}
k_{DD}
Contribution to k_{cat}
$X_H X_{SH} k_{HH}$
$\frac{X_H X_{SD} k_{DD}}{2}$
$\frac{X_H X_{SD} k_{DH}}{2}$
$\frac{X_D X_{SH} k_{HD}}{2}$
$\frac{X_D X_{SH} k_{HH}}{2}$
$X_D X_{SD} k_{DD}$

Figure 3

given isotopic composition of substrate will be the sum of terms at the bottom of Fig. 3.

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

This expression can be simplified by deriving X_D (and X_H) in terms of X_{SD}, assuming that the isotopic composition of the coenzyme has reached a steady state. (This is a reasonable expectation, as each molecule of holoenzyme turns over thousands of molecules of substrate even during the very early stages of the reaction.) In the mechanism of Fig. 3, only pathways c and d affect the isotopic composition of hydrogen attached to the coenzyme. At steady state, their contributions to $k_{cat,obs}$ become equal, so that:

$$\frac{1}{2} X_H X_{SD} k_{DH} = \frac{1}{2} X_D X_{SH} k_{HD}$$

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

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

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

Equation 1 may generalized to describe a mechanism in which the intermediate hydrogen reservoir contains n

hydrogens and in which that hydrogen transferred to the reservoir from substrate has a statiscal probability f/n of being returned to product in step (ii) (f = 1 for a mechanism with n equivalent hydrogens and f = 0 for a "merry-go-round" mechanism in which hydrogen can never be returned to the same substrate molecule from which it was abstracted (Miller & Richards, 1969; Eagar et al., 1972)).

$$k_{cat,obs} = \sum_{i=1}^{m} \binom{n-1}{i-1} X_H^{[n-i]} X_D^{[i-1]} \left\{ X_{SH} \left[\left(\frac{f}{n} + B\right) k_{HH} + C k_{HD} \right] + X_{SD} \left[B k_{DH} + \left(\frac{f}{n} + C\right) k_{DD} \right] \right\} \qquad (3)$$

where

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

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

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

Equation 3 includes several assumptions. Hydrogen in the intermediate reservoir may not exchange with product. In our experiments both the high substrate concentrations employed (which should saturate holoenzyme with propanediol) and the immediate reduction by ADH/NADH of propionaldehyde released from the enzyme preclude significant recombination between enzyme and product. In addition, hydrogen in the

reservoir may not exchange with unreacted substrate to such an extent that the isotopic compositions of either are appreciably affected. The absence of appreciable exchange of hydrogen between cofactor and unreacted substrate has previously been demonstrated (Frey et al., 1967a; Carty et al., 1971). Furthermore, the finding that AdoCbl becomes considerably enriched in tritium relative to substrate during catalysis (Essenberg et al., 1971) indicates that the hydrogen of substrate does not equilibrate with that of AdoCbl. Equation 3 further requires that all (n-1) hydrogens initially in the reservoir become chemically equivalent in the intermediate. At least two results support this requirement. Both hydrogens attached to C-5' of AdoCbl can participate in the reaction (Frey et al., 1967b) and ^{1}H NMR studies of ethanolamine deaminase (Hull et al., 1975) indicate that the methyl group of enzyme-bound 5'-deoxyadenosine. the putative form of the coenzyme during the intermediate stages of the enzymatic reaction, rotates at a rate exceeding 10^7 sec^{-1}, which is many orders of magnitude larger than the rate of hydrogen transfer from the intermediate to product. Lastly, eq 3 neglects any secondary isotope effect; for example, abstraction of hydrogen should occur with equal facility from intermediates CH_3Ad, CH_2DAd, and CHD_2Ad. Although no definitive support exists for this assumption, such secondary isotope effects should be small relative to the primary isotope effects included

in eq 3 (Richards, 1970). Moreover, the kinetics of inactivation of diol dehydratase by glycerol of varying isotopic composition can be explained quantitatively without postulating such isotope effects (Chapter II).

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

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

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

Table I

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

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

k_{DH}, and k_{DD}).

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

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

Glycerol as Substrate. Figure 4 shows the variation of k_{cat} with substrate isotopic composition for glycerol, a substrate - inactivator of diol dehydratase. While these data are qualitatively similar to those of 1,2-propanediol, the upward curvature of the data is much less marked than that of Fig. 1. The corresponding k_{cat} value for (RS)-1,1-dideuterioglycerol is also shown in Fig. 4; it is the

Table II

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

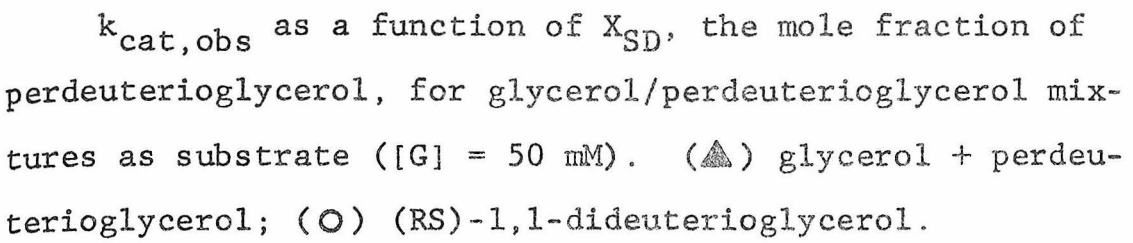
Optimized rate constants (sec^{-1}) and f for mechanisms with an intermediate containing n hydrogens.

Table III

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

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

Figure 4


$k_{cat,obs}$ as a function of X_{SD}, the mole fraction of perdeuterioglycerol, for glycerol/perdeuterioglycerol mixtures as substrate ([G] = 50 mM). (▲) glycerol + perdeuterioglycerol; (O) (RS)-1,1-dideuterioglycerol.

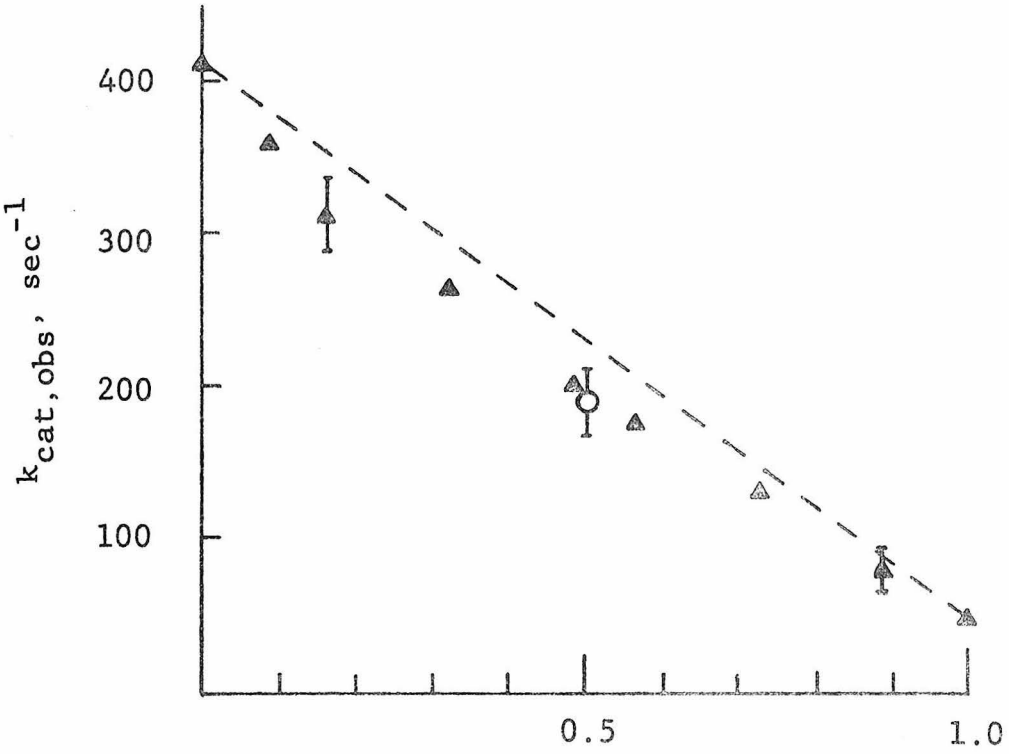

400
300
200
100
$k_{cat,obs}$, sec^{-1}
0.5
1.0
mole fraction perdeuterioglycerol
in substrate (X_{SD})

Figure 4

same as that expected for an equimolar mixture of glycerol and perdeuterioglycerol. As the values of K_G, the enzyme-glycerol Michaelis constant, are different for glycerol and perdeuterioglycerol, these data could not be analyzed meaningfully in the manner described above for the data of Fig. 1. However, a qualitative analysis was carried out as described in the next section.

Tritium Isotope Effect. The results of the tritium isotope effect determination are shown in Fig. 5, a plot of the relative $^3H/^{14}C$ (dpm) ratio of residual 1,2-propanediol as a function of the fraction of substrate converted to product, x. As only one of the two C-1 hydrogens of 1,2-propanediol can be abstracted during catalysis (Zagalak et al., 1966), only 50 % of the tritium in [1 -^{3}H]-1,2-propanediol participates in the reaction. The specific activity of a tritiated substrate as a function of x is (Melander, 1960)

$$\frac{S_T/S_H}{S_{To}/S_{Ho}} = (1 - x)^{[\frac{k_T}{k_H} - 1]},$$

where S_T and S_H are the amounts of reactive tritium and hydrogen. However, when a fraction m of the total tritium T in substrate does not participate in the reaction,

$$S_{To} = To(1 - m)$$

$$S_T = T - m(1 - x)To,$$

Figure 5

Relative $^{3}H/^{14}C$ ratio (dpm) of residual 1,2-propanediol as a function of the amount of substrate consumed, x. The dashed and dotted lines are the calculated curves for k_H/k_T = 3.5 and 5.5, respectively.

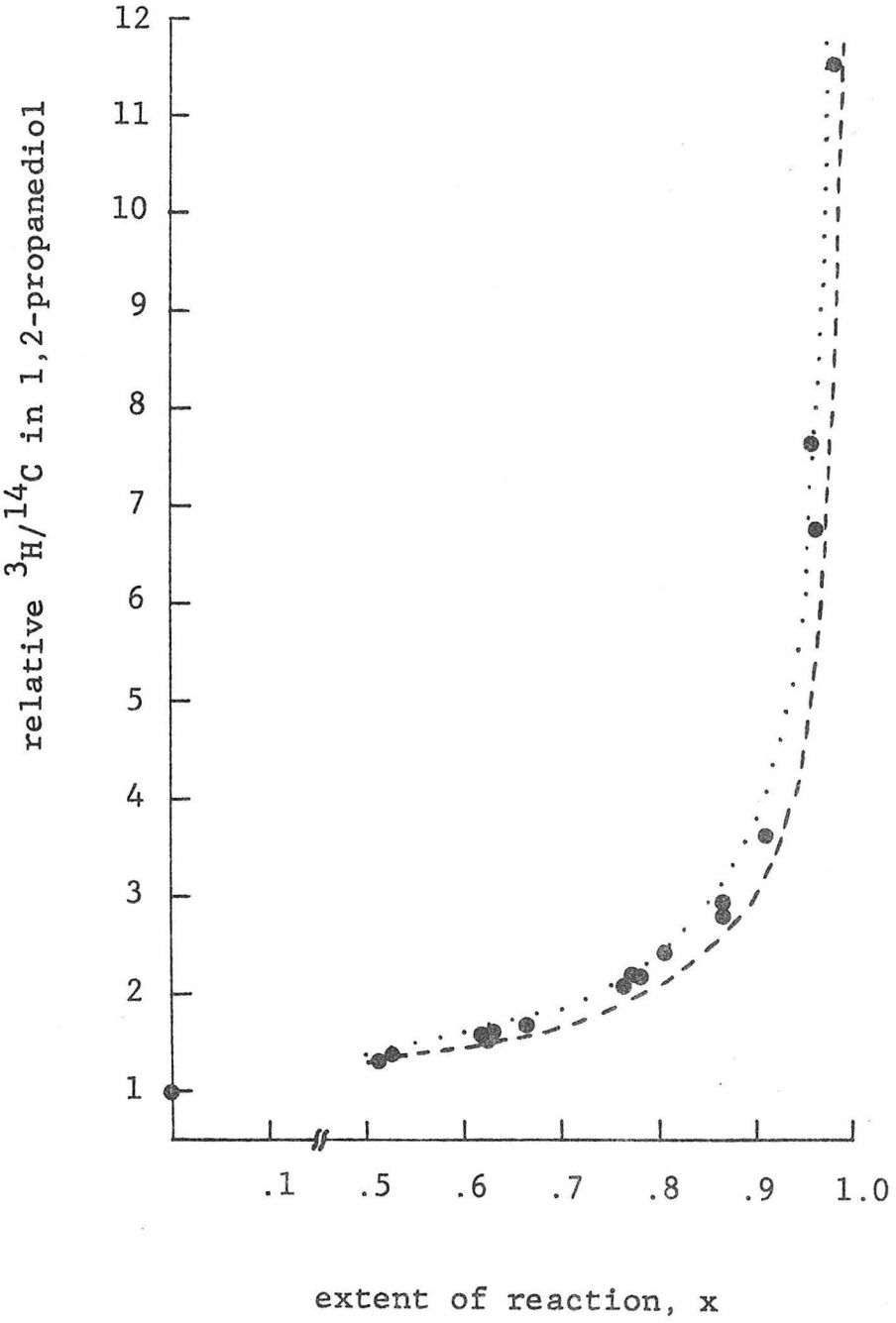

relative $^{3}H/^{14}C$ in 1,2-propanediol
12
11
10
9
8
7
6
5
4
3
2
1
.1
.5
.6
.7
.8
.9
1.0
extent of reaction, x

Figure 5

and the above relation becomes

$$\frac{T/S_H}{To/S_{Ho}} = (1 - m)(1 - x)^{[\frac{k_T}{k_H} - 1]} + m .$$

In the present case, m = 0.5 for [1 -^{3}H]-1,2-propanediol. The dashed and dotted curves for Fig. 5 are theoretical curves for (k_H/k_T) = 3.5 and 5.5, respectively. As the experimental data lie between these curves, we conclude that for diol dehydratase, $(k_H/k_T) = 4.5 \pm 1$.

DISCUSSION

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

A second possibility involves a reaction pathway with concerted transfer of two or more hydrogens. A relation between the isotopic composition of substrate and reaction velocity has been derived (Kresge, 1964; Gold, 1969) for such reactions. In the present case, in which no exchange

of substrate hydrogens with water occurs (Abeles & Lee, 1962), such an explanation would require that $(k_{cat})^{1/n}$, where n is the number of hydrogens simultaneously in flight, should be a linear function of X_{SD}. Such behavior has in fact been reported in studies of the mechanism of action of serine proteases (Hunkapiller et al., 1976) and amidohydrolases (Schowen, 1977). Our results do not show such a dependence. Moreover, concerted transfer of two or more hydrogens, from C-1 of substrate to C-5' and from C-5' to C-2 of product, appears unlikely in view of the finding that hydrogen abstracted from C-1 of substrate has a significant probability of being returned to its parent substrate molecule (Frey et al., 1967a).

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

Models for Hydrogen Transfer. Equation 3 allows

evaluation of pathways with intermediates containing n hydrogens in which hydrogen derived from substrate has a probability f/n of participating in the second transfer step (Fig. 3, reaction (ii)). The best fit of the experimental rate data in Fig. 1 to this equation was determined by systematic variation of the rate constants k_{HH}, k_{DH}, k_{HD}, and k_{DD} by the method of least squares estimation of nonlinear parameters for 2 to 5 equivalent and 2 to 5 hydrogen merry-go-round mechanisms (Table I). In Table II, these rate constants, as well as the parameter f were allowed to vary. The rate constants k_{HH} and k_{DD} are turnover rates when both transfer steps involve only hydrogen or only deuterium, respectively; k_{HD} and k_{DH} are turnover rates in which deuterium is transferred only in the second, or only in the first step, respectively.

As shown in Table I, row 1, a close fit for a mechanism involving two equivalent hydrogens requires the physically meaningless condition that k_{HD} and k_{DH} assume negative values. On this basis we may eliminate this proposed mechanism. Similar evidence also argues against the merry-go-round mechanisms in which $k_{DH} \sim 280\ sec^{-1}$, compared to $k_{HH} \sim 250\ sec^{-1}$ ($k_{HH}/k_{DH} \sim 0.89$). Such an inverse primary isotope effect has no precedent in studies of AdoCbl-dependent enzymes and little precedence in enzymology in general (Jencks, 1969; Richards, 1970). Thus, the mechanisms most adequately describing experimental data obtained with

1,2-propanediol are those involving an intermediate with three or more equivalent hydrogens (Table I, rows 2 - 4). The analysis in Table II, in which f is allowed to vary as well as the rate constants, indicates that, even when n differs from 3, hypothetical mechanisms in which the ratio $n/f \sim 3$ still provide the best fit to the experimental data. (In these cases, however, hydrogen derived from substrate is not considered statistically equivalent to those initially present at C-5'.) The improvement in this second analysis of the agreement between calculated and experimental data is, however, slight, in view of uncertainties inherent in the rate measurements. Thus, these data and analyses provide evidence for a mechanism involving an intermediate containing three equivalent hydrogens, one of which was abstracted from substrate. The possibility of more than three equivalent hydrogens cannot however be rigorously eliminated on the basis of our data alone.

Rate-Determining Step. The value of k_{DD} (20.7 sec^{-1}, $k_{HH}/k_{DD} = 12$) indicates that the transfer of hydrogen is an important rate-contributing step of the reaction. Which step, (i) or (ii) is slower can be ascertained from the values of k_{DH} and k_{HD} in Table I. In the three equivalent hydrogen mechanism, substitution of deuterium for hydrogen in step (i) causes the turnover rate to decrease from 250 sec^{-1} (k_{HH}) to 112 sec^{-1} (k_{DH}) for an isotope effect of $k_{HH}/k_{DH} = 2.2$. The same substitution in reaction (ii)

causes a much larger decrease, to k_{HD} = 23 sec^{-1} for k_{HH}/k_{HD} = 10.8, an isotope effect similar to that observed when deuterium participates in both reactions (k_{HH}/k_{DD} = 12). (We emphasize that the rate constants k_{HD} and k_{DH} represent turnover rates when deuterium replaces hydrogen in only one of steps (i) or (ii); they are not true "microscopic" rate constants for a particular step of the reaction.) These data do nevertheless clearly identify reaction (ii), the transfer of hydrogen from cofactor intermediate to product, as the slowest step in the conversion of 1,2-propanediol to propionaldehyde catalyzed by diol dehydratase. This accords with conclusions drawn for ethanolamine deaminase (Weisblat & Babior, 1971) and for inactivation of diol dehydratase by glycerol (Chapter II), but differs from a previous conclusion (Essenberg et al., 1971).

The possibility that the rate-limiting step is changed by participation of heavy isotope was tested by determining the energies of activation E_a for catalysis with diol and diol-d_2 as substrates. Presumably, a change in the rate-determining step effected by the presence of deuterium would involve a concomitant change in E_a (Weisblat & Babior, 1971). Of course, this assumption would not be correct if the two reaction steps had similar activation energies, or if substitution of deuterium for hydrogen caused an activation energy change much larger than expected from a change in the zero-point energy of a carbon-hydrogen

bond. However, in the former case, neither step could be considered "rate-determining." In the latter case, quantum mechanical tunneling (Lewis & Robinson, 1968) could conceivably cause such a large energy change, but even in this event, failure to observe a change in E_a would be quite fortuitous.

The observed E_a values (Results) were virtually identical for the two substrates, and we conclude that hydrogen transfer from AdoCbl to C-2 of product is the predominantly rate-determining step of the reaction.

Glycerol as Substrate. The data of Fig. 4 show much less marked nonlinear character than those of 1,2-propanediol (Fig. 1). However, the variation of K_G of glycerol with isotopic composition (Chapter II) precludes an analysis of these data such as that carried out for 1,2-propanediol (Table I). Values for K_G are 1.6 mM for glycerol (and (R)-1,1-dideuterioglycerol) and about 3.8 mM for perdeuterioglycerol (and (S)-1,1-dideuterioglycerol). The "apparent" K_G for a mixture of two stereoisomeric substrates R and S in relative amounts x_R and x_S is given by

$$K_G = \frac{K_R K_S}{x_R K_S + x_S K_R} ,$$

with $x_R + x_S = 1$. Thus, in the neighborhood of $x_S = 1$ ($X_{SD} = 1$, Fig. 4) and $x_R = 1$ ($X_{SD} = 0$, Fig. 4), K_G changes relatively slowly, and the variation of $k_{cat,obs}$ with X_{SD}

may be compared with that of 1,2-propanediol. For example, at $X_{SD} = 0.1$, the $k_{cat,obs}$ of glycerol has decreased from 412 sec^{-1} to 360 sec^{-1}, a ∿13 % decrease. In contrast, $k_{cat,obs}$ of 1,2-propanediol at $X_{SD} = 0.1$ is 183 sec^{-1}, a 27 % decrease from the all-hydrogen rate. Similarly, at $X_{SD} = 0.9$ the rate of conversion of glycerol to β-hydroxypropionaldehyde is 80 sec^{-1}, a 60 % increase over the rate observed with perdeuterioglycerol alone, while at $X_{SD} = 0.9$ the 1,2-propanediol $k_{cat,obs}$ has increased only 43 %, from 21 sec^{-1} at $X_{SD} = 1.0$ to about 30 sec^{-1}. While analysis of the 1,2-propanediol rate data indicates that $(k_{DH}/k_{HD}) \sim 5$ for a three-equivalent hydrogen mechanism (Table I, row 2), a manual fit to eq 3 of data obtained for glycerol near $X_{SD} = 0$ and $X_{SD} = 1$ indicates that in this case $(k_{DH}/k_{HD}) \sim 1.5 \pm 0.3$. Accordingly, we conclude that with glycerol as substrate neither the first nor the second hydrogen transfer step is strictly rate-determining in catalysis. This conclusion is supported by the observation (Chapter II) that 5'-deoxyadenosine isolated from [1 -^{3}H]-glycerol-inactivated diol dehydratase is enriched only about two-fold in tritium relative to substrate, while for AdoCbl isolated from holoenzyme and tritiated 1,2-propanediol, up to a 20-fold enrichment has been observed (Essenberg et al., 1971). The enrichment of the intermediate hydrogen reservoir in heavy isotope is proportional to the ratio k_{DH}/k_{HD}, as discussed later.

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

$$E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} ES \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES' \overset{k_3}{\rightarrow} E + P$$

k_1 is the rate constant for hydrogen transfer from substrate to cofactor, while k_3 is the net rate constant for the remainder of the pathway, which includes hydrogen transfer from cofactor to product. When holoenzyme is saturated with substrate, k_{cat} is given by (Cleland, 1975)

$$k_{cat} = \frac{V_{max}}{E_o} = \frac{k_1 k_3}{k_1 + k_2 + k_3} \quad .$$

Each of the rate constants k_1, k_2, and k_3 may manifest a primary deuterium isotope effect. This expression may be used to derive expressions for the observed isotope effects k_{HH}/k_{HD}, k_{HH}/k_{DH}, and k_{HH}/k_{DD} in terms of the microscopic constants. For the first of these,

$$k_{HH}/k_{HD} = \frac{k_1^H k_3^H (k_1^H + k_2^H + k_3^H)}{k_1^H k_3^D / (k_1^H + k_2^H + k_3^D)} = \frac{k_3^H (k_1^H + k_2^H + k_3^H)}{k_3^D / (k_1^H + k_2^H + k_3^D)}$$

Because the values of k_1, k_2, and k_3 are unknown, k_{HH}/k_{HD} cannot be evaluated explicitly. However, limiting values of k_{HH}/k_{HD} may be obtained for the cases $k_3 >> (k_1 + k_2)$, for which $k_{HH}/k_{HD} = 1$ and $k_3 << (k_1 + k_2)$, for which $k_{HH}/k_{HD} = k_3^H/k_3^D$. Similar consideration of k_{HH}/k_{DH} and k_{HH}/k_{DD} (neglecting equilibrium isotope effects) gives the results in Table IV.

In view of the values $k_{HH}/k_{HD} = 10.8$, $k_{HH}/k_{DD} = 12$, and $k_{HH}/k_{DH} = 2.2$ obtained experimentally, we conclude that the diol dehydratase reaction with 1,2-propanediol as substrate corresponds more closely to the left column of Table IV. In contrast, the glutamate mutase reaction (Eagar et al., 1972), with isotope effects of k_{HH}/k_{DD} ("k_H/k_D") = 7.5, $k_{HH}/k_{HD}\left(\frac{k_H/k_D}{\Delta_1}\right) = 7.5/7.0 = 1.1$, and $k_{HH}/k_{DH}\left(\frac{k_H/k_D}{\Delta_2}\right) = 7.5/1.5 = 5.0$, corresponds to the right side of Table IV, with k_1 most likely rate-limiting. Methylmalonyl CoA mutase (Miller & Richards, 1969), which exhibits values of 3.5, 1.1, and 1.2 respectively (and glycerol with diol dehydratase) constitutes an intermediate case in which both hydrogen abstraction from substrate and hydrogen return to form product influence significantly the overall reaction rate.

Relation to Previous Studies. The existence of an intermediate in which the cofactor exists as

TABLE IV

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

	$k_3 << (k_1 + k_2)$	$k_3 >> (k_1 + k_2)$
k_{HH}/k_{HD}	k_3^H / k_3^D *	1
k_{HH}/k_{DH}	1†	k_1^H / k_1^D
k_{HH}/k_{DD}	k_3^H / k_3^D	k_1^H / k_1^D

*† If the hydrogens in the intermediate are equivalent with respect to k_2 , the * and † labeled quantities will be somewhat reduced and increased, respectively.

5'-deoxyadenosine with three equivalent hydrogens has been inferred from studies of ethanolamine deaminase (Babior, 1969; Weisblat & Babior, 1971) and diol dehydratase (Frey et al., 1967a; Essenberg et al., 1971), using substrates and AdoCbl in which mobile hydrogens are enriched with tritium. The results and analysis described here accord with the qualitative conclusions reached earlier. More importantly, these deuterium isotope effects observed during rearrangement of 1,2-propanediol catalyzed by diol dehydratase unambigously require the intervention of a "reservoir" containing at least three equivalent hydrogens. This same point has been established for two other AdoCbl-dependent enzymes, methylmalonyl CoA mutase (Miller & Richards, 1969) and glutamate mutase (Eagar et al., 1972).

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

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

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

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

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

which upon integration becomes:

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

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

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

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

where B and C have been defined previously. On substitution of n = 3 and f = 1, and neglect of terms containing

X_T^2, $X_{ST}X_T$, or $X_{ST}X_T^2$, this expression simplifies to:

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

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

$$\frac{\text{Specific Activity, } x}{\text{Specific Activity, } x = 0} = (1-x)^{[(2k_{TH}+k_{TT})/3k_{HH} - 1]} \tag{9a}$$

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

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

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

When X_T is given by eq 10, the amount of tritium entering

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

$$\frac{\text{Product specific activity, } x}{\text{Substrate specific activity, } x=0} = \frac{1-(1-x)^{[(2k_{TH}+k_{TT})/3k_{HH}]}}{x} \tag{11}$$

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

In view of these conclusions, an earlier report (Essenberg et al., 1971) merits scrutiny. Based on an experiment of the type described by eq 8, a tritium isotope effect of $250\cdot(2/3)$ was calculated for hydrogen

transfer from AdoCbl to product at 10°C. This value is unprecedentedly large and is, further, not consistent with the deuterium isotope effects observed in this work. In another experiment, Essenberg et al. measured the specific activities of AdoCbl, propionaldehyde and residual substrate as a function of time with [1-^{3}H]-1,2-propanediol as substrate. Their first observation, that AdoCbl becomes enriched in tritium relative to unreacted substrate by a factor of 19, accords with the conclusions reached in the present work. From the present results obtained with deuterium, approximate values of k_{TH} and k_{HT} can be calculated (Swain et al., 1958):

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

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

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

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

When applied to the diol dehydratase reaction, our data on the deuterium isotope effects, together with the above treatment (eq 12), predict a tritium isotope effect

of

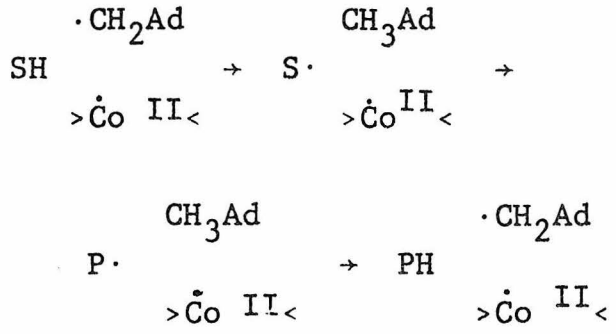
$$k_H/k_T = \frac{3k_{HH}}{2k_{TH} + k_{TT}} = 4.5,$$

compared to a deuterium isotope effect of 12. The data in Fig. 4 confirm this prediction, and are consistent with a value of $k_{TH} \simeq 81$ sec^{-1} ($k_{HH}/k_{TH} = 3.1$). As k_{TT} is much smaller than $2k_{TH}$, the value of k_H/k_T is fairly insensitive to the value of this rate constant. However, the value of k_{TT} predicted from the observed value of k_{DD} is 7 sec^{-1} ($k_{HH}/k_{TT} = 36$), and the observed k_H/k_T is not inconsistent with this prediction.

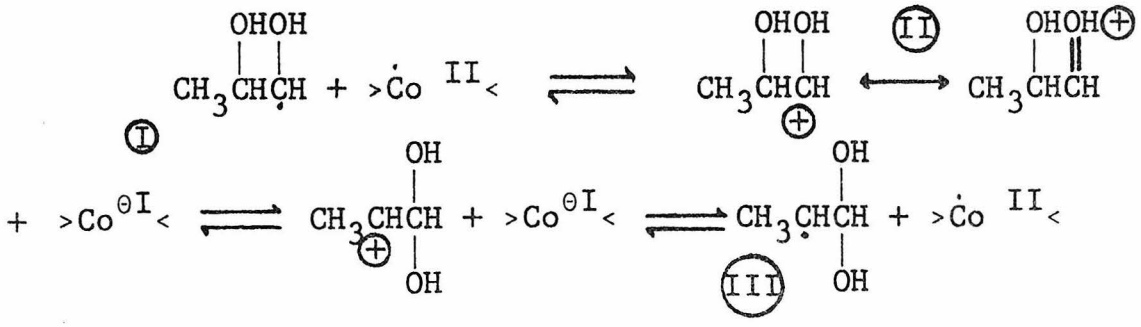
Thus, the overall tritium isotope effect, as well as the data of Essenberg et al. (1971) concerning the enrichment of AdoCbl in tritium during catalysis with [1-^{3}H]-1,2-propanediol as substrate, are consistent with the deuterium isotope effects observed in this work.

The deuterium isotope effects observed in this study are within the range of those reported for free radical reactions involving cleavage of a carbon-hydrogen bond. For example, Wilen & Eliel (1958) reported isotope effects ranging from 3 to 10 for abstraction of hydrogen from α-deuterated toluene and p-xylene by various peroxides. Howard et al. (1968) observed isotope effects of 8 to 20 for the oxidation of deuteriocumenes and perdeuteriotetralin by cumene hydroperoxide and t-butylhydroperoxide. Thus, our data are compatible with a mechanism involving

hydrogen transfer between free radicals (Babior, 1970; Finlay et al., 1972; Finlay et al., 1973).



However, with the exception of the work of Walling & Cioffari (1972) and Julia (1971), and of calculations carried out by Golding & Radom (1976), there is little precedent for a 1,2-rearrangement of a radical species. In contrast, 1,2-rearrangement of a carbonium ion, such as might be generated by oxidation of the 1,2-propanediol-1-yl radical by cob(II)alamin, has ample precedent. In this event, the following mechanism could obtain as suggested earlier (Eagar et al., 1972; Halpern, 1974);



Free radical I is more stable than its precursor, $\cdot CH_2Ad$. Similarly, the rearranged radical III is less stable than

$\cdot CH_2Ad$, and formation of the latter, by transfer of a C-5' hydrogen to C-2 of III, would be energetically favored. The relative stability of the oxocabonium ion II could help to facilitate oxidation of the radical I by Cbl^{II}, while the eventual elimination of H_2O could provide a driving force for rearrangement of II and reduction of the resulting carbonium ion by Cbl^{I}.

CONCLUSIONS

By analysis of the variation of k_{cat} with mole fraction deuterated substrate in the AdoCbl-dependent conversion of 1,2-propanediol to propionaldehyde, we have inferred the obligatory intervention of an intermediate hydrogen reservoir in which the hydrogen derived from substrate becomes equivalent with at least two other hydrogen atoms; one of these three equivalent hydrogen atoms is returned to form product in the principal rate-determining step of the reaction. With glycerol as substrate, neither hydrogen transfer reaction appears to be a major rate-contributing step. The magnitude of the primary deuterium isotope effects observed in these two hydrogen transfer steps are consistent with these steps' involving cleavage of a carbon-hydrogen bond. These results should, therefore, be immune to the criticism (Schrauzer, 1971) of experiments using tritium as a trace label. The role of the 5'-methyl group of 5'-deoxyadenosine as the reservoir of the three

equivalent hydrogens has ample precedence (for example: Babior et al., 1974; Bachovchin et al., 1978).

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APPENDIX

GROWTH OF KLEBSIELLA PNEUMONIAE AND ISOLATION OF DIOL DEHYDRATASE

During the past three years, modifications in the isolation procedure have significantly improved the yield and purity of diol dehydratase preparations in this laboratory. Yields have increased by up to a factor of four, and purity by up to 30 % over previous work. This appendix summarizes the current procedure used and makes tentative suggestions for further improvements.

General References

Lee, H. A., Jr., & Abeles, R. H. (1963) J. Biol. Chem. 238, 2367. Original reference.

Poznanskaya, A. A., Tanizawa, K., Toraya, T., Soda, K., & Fukui, S. (1977) Bioorg. Khim. 3, 111. Recently published work.

Some of the work described below has been carried out in collaboration with Dennis E. McGee.

The Culture. Klebsiella pneumoniae (ATCC 8724) is obtained from the American Type Culture Collection as a freeze-dried culture.

Nutrient Agar Stabs – for long term storage.

0.9 g	Difco "Bacto" nutrient broth powder
0.5 g	NaCl
0.75 g	Difco "Bacto" agar powder
	to 100 ml with distilled water

Dissolve ingredients with short (2 - 3 min) heating in an autoclave (slow exhaust). Swirl to mix thoroughly,

and dispense 1 ml aliquots into screwcap culture tubes. Autoclave these tubes for 20 min, allow to cool in an upright position.

Nutrient Agar Slopes:

5.26 g	Difco "Bacto" nutrient broth powder
1.76 g	NaCl
4.42 g	Difco "Bacto" agar powder
	to 350 ml with distilled water

Dissolve ingredients with brief autoclaving, swirl to mix thoroughly, and dispense 6 - 7 ml into each culture tube. Cap the tubes and autoclave 20 min. Allow the tubes to cool at an angle and store upright at 4° C until use.

Nutrient Agar Plates:

2.53 g	Difco "Bacto" nutrient broth powder
1.9 g	Difco "Bacto" agar powder
	to 150 ml with distilled water

Autoclave ingredients for 20 min, swirl, and dispense 20 - 25 ml into sterile petri dishes in a uv-hood under draft-free conditions. Allow plates to cool and store upside down at 4° C until needed.

The Klebsiella pneumoniae culture is maintained on agar slopes. Every other week a single colony is isolated from an agar plate which has been grown overnight at 37 ° C and placed on a new slope. One slope is used as a master culture to avoid contamination of the bacteria. All manipulations are made with a sterile platinum wire loop in a uv-hood in a draft-free room.

The Inoculation Broth:

12 g	Yeast extract	Autolyzed brewer's yeast fraction, 50X concentrated liquid. Amber laboratories, Juneau, Wisc. 53039 414-386-2691.
90 ml	glycerol	
31.2 g	$K_2HPO_4 \cdot 3H_2O$	
5.6 g	KH_2PO_4	
7.05 g	$(NH_4)_2SO_4$	
1.2 g	$MgSO_4 \cdot 7H_2O$	
	to 6 l with distilled water	

Predissolve all salts. Add each ingredient in the order listed while diluting to volume with distilled water. Dispense aliquots of approximately 200 ml into 250 ml screw-cap erlenmeyer flasks. These flasks are autoclaved for 30 min, allowed to cool, and the caps are tightened until use.

Cells from a slope which has been grown 12 hr at 35 - 37° C are suspended in 1 ml of sterile distilled water and transferred to the sterile inoculation broth in a uv-hood in a draft-free room. The broths are incubated at 35 - 37° C with loosened caps for 12 hr.

The Growth Medium:

50 ml	1,2-propanediol
100 ml	glycerol (glycerin)
3 - 5 g	yeast extract
105 g	$K_2HPO_4 \cdot 3H_2O$
20 g	KH_2PO_4
24 g	$(NH_4)_2SO_4$
4 g	$MgSO_4 \cdot 7H_2O$
	to 18 l with distilled water

Predissolve all salts. Add each ingredient in the order listed while diluting to volume with distilled water

in a five-gallon carboy. The carboy is closed with a rubber stopper with a glass vent tube loosely plugged with non-absorbent cotton or glass wool. The opening is covered with a double layer of aluminum foil and the medium autoclaved for 90 min. Cool and bring to 35 - 37° C in a hot room or large bath.

A fully grown inoculation broth is rapidly added to the center of an 18 l medium at 35 - 37° C without stirring. The cells are allowed to grow for 24 hr (or until a fair amount of froth appears), and are harvested by centrifugation over a period of 3 - 4 hr for an eight bottle batch, starting with the most frothy carboys.

These large bottles should serve as the first point of variation if either growth or enzyme activity are unsatisfactory. Approximately 60 - 85 g wet cells should be obtained from an eight bottle harvest. Often better activity correlates with poorer growth. The first variable is the yeast extract, which can be varied from 1.5 - 10 g. If low activity persists, the glycerol/propanediol ratio may be changed. The cells may be somewhat sensitive to the water source.

The Harvest. A Beckman J21C centrifuge and JCF-Z rotor are used, at 12 KRPM and 4° C, with flow rates of 0.5 - 0.9 l/min. The effluent should be examined at intervals for residual bacteria.

The Cells. All procedures from this point on are done at 0-5°C, and only glass-distilled deionized water is used. After harvesting, the cell paste is weighed and suspended (a blender may be used) in 0.01 M Tris base (5 ml/1g wet cells). The suspension is stirred for 15-30 minutes and centrifuged in a GSA rotor for one hour at 10 KRPM. The cells are suspended (may use a blender) in a minimum amount of water (maximum 2 ml/1g wet cells) and lyophilized.

The Sonicate. The lyophilized cells are sonicated by the following procedure as soon as possible after the harvest. The lyophilized cells may be stored in a deep freeze (-80°C) if immediate sonication is not possible.

The dry cells are added to water (10 ml/1g dry cells) and homogenized in a blender. Dithiothreitol is added to a concentration of 15-20 mM, and the pH adjusted to 8.8-9.0 with 40% KOH with efficient stirring. After 30 minutes, the cells are sonicated with a Bronson sonicator (setting of "60") in an ice-methanol bath. The resulting enzyme activity is seemingly a function of both total sonication time and the duration of each burst. Therefore, the cells are sonicated for a total time of 5 min/1g dry cells in bursts which are as long as possible while still keeping the temperature below 15°C. Bursts of up to 30 minutes may be attained by stirring the ice-methanol bath. The bath cools most efficiently when it is mostly liquid.

After sonication, a cold suspension of Darco G60 charcoal, H_2O, and distilled 1,2-propanediol (30g:115ml: 8 ml/30g dry cells) is added and efficiently stirred at 4°C for 15-20 min. The suspension is centrifuged 90 min at 11 KRPM in a GSA rotor. The supernatant is decanted, an aliquot removed for activity assay (0.05-0.1 ml, diluted to 10 ml in Buffer E, below), and lyophilized for use in the enzyme preparation. The dry sonicate is stored in a deep freeze, and should contain at least 200 units/g before continuing to the end of the purification.

The Assay. General reference:

Bachovchin, W. W., Eagar, R. G., Jr., Moore, K. W., & Richards. J. H. (1977) Biochemistry 16, 1082.

The assay solution should contain 60 mM 1,2-propanediol; 40-60 mM potassium phosphate buffer, pH 8.0; 10 unit/ml yeast alcohol dehydrogenase (ADH, Sigma); 0.2-0.25 mM nicotinamide adenine dinucleotide, reduced form (NADH, Sigma); and 20-40 µg/ml bovine serum albumin. A 2.0 ml aliquot of this solution is placed in a cuvet with a magnetic stirrer, and 0.2 ml of an enzyme-containing solution added to this. The cuvet is incubated with stirring at 37°C for 25 min., and 25 µl of 2.5 mg/ml adenosylcobalamin (AdoCbl, coenzyme B_{12}; Sigma) solution is added to start the reaction. The absorbance at 340 nm is monitored with time to determine enzyme activity.

Activity may also be assayed by the method of Eagar,

R. G., Jr., Bachovchin, W. W., & Richards, J. H. (1975) Biochemistry 14, 5523, via colorimetric determination of propionaldehyde-2,4-dinitrophenylhydrazone.

Protein concentration is determined by the method of Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) J. Biol. Chem. 193, 265.

Enzyme Purification. These are not rigorous procedures, and the purification should be regarded as more of an art than a science. For those buffers containing dithiothreitol (DTT), best results are obtained by adding this reagent immediately before the buffer is to be used.

Buffer A: 0.05 M $(NH_4)_2SO_4$ (26.4 g)
0.01 M $K_2HPO_4 \cdot 3H_2O$ (40 ml, 1 M solution)
2% 1,2-propanediol (80 ml)
0.01% NaN_3 (0.4 g)
to pH 8.0 (40% KOH)
to 4.0 l with H_2O
2 mM DTT (0.3 g/l)

Buffer B: 0.5 M $(NH_4)_2SO_4$ (66.05 g)
0.01 M $K_2HPO_4 \cdot 3H_2O$ (10 ml, 1 M solution)
2% 1,2-propanediol (20 ml)
to pH 8.0 (40% KOH)
to 1.0 l with H_2O

Buffer D: 0.005 M $K_2HPO_4 \cdot 3H_2O$ (5 ml, 1 M solution)
0.1 M 1,2-propanediol (7.6 ml)
0.01% NaN_3 (0.1 g)
to 1.0 l with H_2O

Buffer E: 0.01 M $K_2HPO_4 \cdot 3H_2O$ (60 ml, 1 M solution)
2% 1,2-propanediol (120 ml)
0.01% NaN_3 (0.6 g)
to 6.0 l with H_2O
1 mM DTT (optional, 0.15 g/l)

Buffer F: 0.524 g/ml $K_2HPO_4 \cdot 3H_2O$ in H_2O

Buffer G: 10% glycerol, reagent (200 ml)
5% 1,2-propanediol (100 ml)
0.01 M potassium phosphate, pH 8.0 (20 ml, 1 M, pH 8 solution)
0.01% NaN_3 (0.2g)
to 2.0 l with H_2O
0.02% DTT (0.2g/l)

Assay buffer: 0.2 M potassium phosphate, pH 8.0

Assay buffer + 2 mg/ml bovine serum albumin.

The fate of Buffer C is unknown. All 1,2-propanediol should be distilled and not yellowish. The scale is for 21 g sonicate. All assay points and dilution factors are noted. All glassware should be very clean and at 25° or below (not hot!).

Crush 21 g of sonicate to a powder in a mortar and pestle and dissolve in a minimum amount of Buffer A (180-220 ml), crushing lumps with a small spoon (not silver) if desired. Maintain pH at or above 8.0 by addition of 40% KOH while stirring. Stir for 30 min in an ice bath. E-1: 0.2 ml/10 ml Buffer E.

Centrifuge 2 hr at 10 KRPM in a GSA rotor. Decant supernatant and record volume. Suspend pellet in a minimum amount of Buffer A and stir in the cold. Repeat two or three times with the supernatant, or until there is no pellet after a centrifugation. Add each pellet to the pellet (P) fraction, with more Buffer A as necessary to maintain a reasonably thin suspension. E-1s: 0.2 ml/10 ml; P-1: 0.05 ml/10 ml Buffer E.

At this point, P-1 may contain a substantial amount of enzyme activity, and the A_{280}/A_{260} ratio should be between 0.7 and 1.0. If P-1 contains 2500 units or more, it is probably worthwhile to work with it further, as discussed later.

During the final spin of E-1s, prepare protamine sulfate solution by dissolving 3.0 g protamine sulfate (Calbiochem) in 147 ml Buffer A at room temperature, with pH adjusted to 8.0 with 40% KOH.

Add 80 ml protamine sulfate solution dropwise with stirring to E-1s in an ice bath, keeping the pH above 8.0 with 40% KOH. Stir for 20 min, centrifuge 75 min at 11 KRPM in a GSA rotor. Discard the pellet. During the spin, grind $(NH_4)_2SO_4$ (3 g/10 ml E-1s). Supernatant is E-2. E-2: 0.2 ml/10 ml Buffer E. The A_{280}/A_{260} ratio should be between 0.7 and 1.0. If not, add 10-20 ml more protamine sulfate and treat as before.

Add crushed $(NH_4)_2SO_4$ (3 g/10 ml E-2) to E-2 slowly

while stirring in an ice bath and maintaining the pH above 8.0 with 40% KOH. After completing the addition, stir for 20 min and centrifuge 100 min at 11 KRPM in a GSA rotor. Supernatant is E-3s: 0.2 ml/10 ml. If E-3s contains substantial activity, add more crushed $(NH_4)_2SO_4$ (up to 90-95% saturation) and centrifuge again.

The pellets from the ammonium sulfate precipitation are suspended in 55-65 ml Buffer A and allowed to stir in the cold, overnight if desired. The suspension is E-3: 0.05 ml/10 ml. Dialyze P-1 overnight in the rocking dialyzer against Buffer E.

Centrifuge E-3 for 2 hr at 15.5 KRPM in an SS-34 rotor. Supernatant is E-4: 0.1 ml/10 ml. If E-4 contains all or a large fraction of the enzyme activity, set aside and proceed as described later. If not, suspend E-4's pellet in Buffer B, stir for 20 min.

The P-1 dialysate is P-6: 0.05 ml/10 ml.

Centrifuge the Buffer B suspension for 90 min at 15.5 KRPM in an SS-34 rotor. Supernatant is E-5a: 0.2 ml/10 ml. Suspend the pellet in 25 ml Buffer B, stir for $\geq$ 20 minutes.

Centrifuge this suspension 70 min at 15.5 KRPM in an SS-34 rotor. Supernatant is E-5b: 0.2 ml/10 ml.

Suspend the pellet in a minimum amount of Buffer E and stir for several hours to solubilize the protein. Dialyze overnight against Buffer E in a rocking dialyzer.

The resulting dialysate is E-6: 0.1 ml/10 ml.

Centrifuge E-6 and/or P-6 for 2-2.5 hr at 40 KRPM in a Ti60 rotor. Remove the supernatant carefully (E-6s; P-6s: 0.1 ml/10 ml) with a pasteur pipet, stopping when the dense, yellow suspension is reached. The pellets may be resuspended in Buffer E, solubilized, and centrifuged again to extract more diol dehydratase. P-6s may contain a large fraction of the activity present in P-6.

Transfer P-6s and E-6s to corex centrifuge tubes. Chill the tubes in ice, and add Buffer F (0.03 ml/1 ml 6s supernatant) in 0.1 ml portions, while stirring with a glass rod. Allow solutions to stand for 30 min - 1 hr in the ice bath. Centrifuge at 10 KRPM for 10 min in an SS-34 rotor. Decant supernatants, and suspend the pellets in 0.5-3 ml Buffer D, according to their apparent size. Cover and allow to solubilize for 1-2 days before assaying.

P-8a; E-8a: 0.02 ml + 0.4 ml Buffer E. Assay 0.02 ml for enzyme activity, and 0.4 ml for protein.

The 8a pellets may be combined, diluted with Buffer D to about 200 unit/ml, and dialyzed against Buffer D or E to solubilize the protein.

The supernatants from these pellets are P-7 and E-7. If they contain substantial activity (> 25-30 unit/ml), treat as for E-4 described below.

The enzyme-containing solution (E-4, E-7, or both) is treated with ammonium sulfate as described previously,

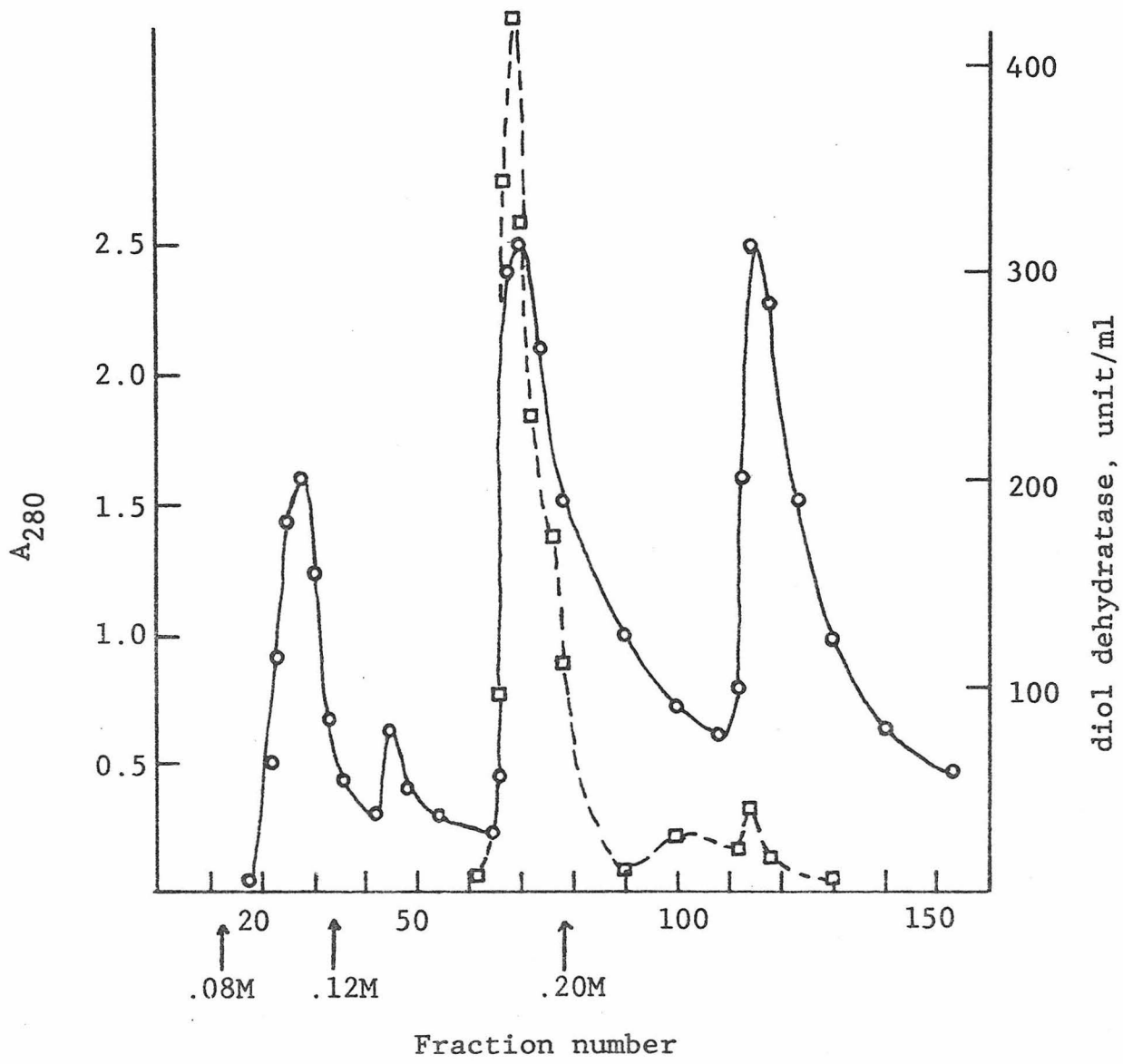
and centrifuged. An effort should be made to precipitate as much enzyme activity as possible. Suspend the pellet in a minimum amount of Buffer E and dialyze against two or three 500 ml portions of Buffer E (the rocking dialyzer may be used). This solution is then dialyzed against two 500 ml portions of Buffer G and centrifuged for one hour at 10 KRPM in an SS-34 rotor. The supernatant is passed onto a 2.6 x 8 cm column of Cellex D, equilibrated with Buffer G. The column is washed with ∿ 10 ml Buffer G. Diol dehydratase is then eluted in 1.5-2 ml fractions with a stepwise gradient of 0.08 M, 0.12 M, and 0.2 M KCl in Buffer G.

A typical elution profile is shown in Fig. 1. Enzyme activity is associated with two protein bands. Most of the activity elutes at 0.12 M KCl, and is pooled as desired. We have obtained enzyme concentrations near 600 unit/ml with a specific activity of nearly 80 units/mg (see Poznanskaya et al. (1977) cited in the beginning of this appendix) in this band: E-8b: 0.015 ml + 0.4 ml Buffer E.

However, enzyme activity also elutes at 0.2 M KCl, although the quantity is much smaller. When enzyme from E-8a or P-8a is added to the E-4/E-7 solution prior to Cellex-D chromatography, the activity of enzyme eluting at 0.2 M KCl is enhanced. No other property could be found which distinguishes these two forms of diol dehydratase. Their reactions with 1,2-propanediol and glycerol were identical in terms of the kinetic parameters k_p, k_i, K_S,

Figure 1

Elution profile of diol dehydratase during Cellex-D chromatography in Buffer G. (-o-) A_{280}; (--□--) diol dehydratase activity, unit/ml. Fraction volume 2 ml.


A_{280}
2.5
2.0
1.5
1.0
0.5
400
300
200
100
diol dehydratase, unit/ml
20
50
100
150
.08M
.12M
.20M
Fraction number

and K_G. Furthermore, the kinetic isotope effects for catalysis and inactivation were the same for each fraction. Their molecular weights as measured by chromatography on Sepharose 6B were identical, as was the four band pattern obtained by SDS-gel electrophoresis (Poznanskaya et al., 1977).

Preliminary results indicate that the relative proportions of these two fractions (and of the amounts of enzyme appearing in E-8a/P-8a and in the E-4 supernatant) are related to the total time and the duration of each burst of sonication. For a given total sonication time, an increase of the "burst time" seems to correlate with decreased activity in the 8a fractions and the 0.2 M KCl fraction, and with increased enzyme activity in the E-4 supernatant and 0.12 M KCl fraction. In addition, longer bursts of sonication seem to give a higher overall yield, even when the total sonication time does not change. The reason for this behavior remains obscure.

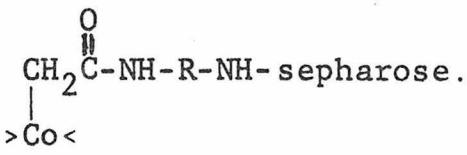
Suggested Further Studies. Alternative means of rupturing the bacteria should probably be tried, such as pulverizing with glass beads (Poznanskaya et al., 1977). The sonication procedure is susceptible to problems involving oxidation. Although the introduction of 15-20 mM DTT is a good prophylactic measure, some active enzyme is probably still destroyed by oxidation during sonication.

The nature of the two forms of diol dehydratase observed during Cellex D chromatography could be further probed by a systematic, thorough study of the effects of sonication time and time of burst on the enzyme activity profile.

Finally, attempts could be made at constructing an affinity column, using a cobalamin derivative coupled to a solid support. One such attempt could be the following:

$$\text{CN-Cbl} + \text{ICH}_2\text{COOH} \xrightarrow[\text{NaBH}_4]{\text{Co}^{2+}} \text{carboxymethylcobalamin}$$

$$\text{carboxymethylcobalamin} + \text{aminoalkylsepharose} \xrightarrow{\text{carbodiimide}}$$



The advantage of this method would be that the enzyme-cobalamin complex could be liberated by photolysis, and the apoenzyme resolved of OH-Cbl by the method described in Chapter I.

The last four years have seen considerable improvements in the purification of diol dehydratase. With luck and effort, further improvements could be made, perhaps to the point where amounts of enzyme sufficient to do even NMR studies could be isolated.

PROPOSITIONS

PROPOSITION I

The current body of knowledge concerning adenosylcobalamin (AdoCbl)-dependent rearrangements has been reviewed in this thesis (Introduction) and in the literature (Babior, 1975a,b; Abeles & Dolphin, 1976). In view of the results described in this thesis, the following investigations are proposed for further studies of the mechanism of AdoCbl-dependent rearrangements.

Kinetic Isotope Effects. Rate Determining Step. In the reactions catalyzed by ethanolamine deaminase (Weisblat & Babior, 1971) and diol dehydratase (Moore et al., 1979; Chapter III), evidence has been obtained implicating the second hydrogen transfer step, from C-5' of 5'-deoxyadenosine to C-2 of product aldehyde, as the major rate-contributing step in catalysis. This conclusion differs from that reached by Essenberg et al. (1971), although the reasons for their conclusions are somewhat unclear. In view of this disagreement, and as a complement to the study described in Chapter III, catalysis with [1 -^{14}C]-1,2-propanediol may be studied. If hydrogen abstraction from C-1 of substrate makes a significant contribution to the rate of catalysis, then a $^{12}C/^{14}C$ isotope effect should be expressed, resulting in an increase of the ^{14}C-specific activity of unreacted substrate as a function of the amount of substrate consumed. $^{12}C/^{14}C$ isotope effects

of 1.06 - 1.17 have been observed (Schmitt & Daniels, 1953; Fry, 1970), and are about twice the magnitude of $^{12}C/^{13}C$ isotope effects (Stern & Vogel, 1971). In the range of 80 - 98 % depletion of substrate, ^{14}C enrichment of the residual substrate in the range 10 - 51 % relative to the initial specific activity should be observed for an isotope effect of 1.06 - 1.12 (Melander, 1960).

Double-isotope counting methods should be useful in this regard. An appropriate standard, [3 -^{3}H]-1,2-propanediol, could be synthesized from [^{3}H]-CH_3I by the procedures outlined by Kollonitsch (1966a,b). Use of this standard, which should be converted to propionaldehyde without an isotope effect, would obviate the need for quantitative recovery of unreacted 1,2-propanediol from the reaction mixture.

While a $^{12}C/^{14}C$ isotope effect of 1.06 - 1.17 would indicate that the first hydrogen transfer step is significantly rate-contributing, the observation of no or a very small (<1.03) isotope effect would support conclusions based on previous work with isotopes of hydrogen (Chapter III).

Tritium Isotope Effect. Diol dehydratase and ethanolamine deaminase exhibit large isotope effects ($k_H/k_T \sim 160$) for transfer of tritium from [5'-^{3}H]-AdoCbl to product derived from unlabeled substrate (Essenberg et al., 1971; Weisblat & Babior, 1971). In contrast, studies employing

deuterium substitution, and measurement of the overall tritium isotope effect (Chapter III) indicate k_H/k_T ∿30-40 for transfer of tritium from AdoCbl to product. A tritium isotope effect of this magnitude (30 - 40) is also indicated by measurements of the specific activity of AdoCbl and unreacted substrate during catalysis with [1 -^{3}H]-1,2-propanediol. The discrepancy between these two values of the tritium isotope effect is quite significant in relation to the catalytic mechanism. If k_H/k_T is in the range 30 - 40, then all known data on primary hydrogen isotope effects with diol dehydratase can be reconciled in terms of the "generally" accepted catalytic mechanism (Fig. 3, Introduction). On the other hand, if k_H/k_T = 160, then this mechanism represents an oversimplification and must be considerably modified to account for the large tritium isotope effect.

Thus, a careful reexamination of this isotope effect is warranted. In the case of propanediol dehydratase (Essenberg et al., 1971), a determination of the number of active sites in a diol dehydratase preparation involved measurement of the amount of tritium transferred in 2 min at 10^o C from [5'-^{3}H]-AdoCbl to propionaldehyde derived from from unlabeled 1,2-propanediol as a function of the amount of [5'-^{3}H]-AdoCbl added to the reaction mixture. At low concentrations of [5'-^{3}H]-AdoCbl, the amount of tritium released to product was dependent upon the [5'-^{3}H]-AdoCbl

concentration, whereas at high concentrations it was not. Thus, the available sites could be saturated with [5'-^{3}H]-AdoCbl, leading to the important conclusion that AdoCbl does not exchange between solution and enzyme at an appreciable rate at 10° C. This result, along with the observed specific activity of the most highly purified enzyme preparation available at that time (60 unit/mg), led to an estimate of a molecular weight of 2.5×10^5 daltons. This agrees well with the molecular weight obtained from gel chromatography (Essenberg et al., 1971; Moore & Richards, unpublished results) and subunit composition (Poznanskaya et al., 1977). However, recent results (Poznanskaya et al., 1977; Moore, McGee, & Richards, unpublished results) suggest that the actual specific activity of pure diol dehydratase lies in the range 75 - 100 unit/mg. Thus, in order to have obtained a correct molecular weight estimate from the results of the tritium washout experiment described above, Essenberg et al. (1971) may have measured 25 - 60 % more "active sites" (as defined by tritium release from [5'-^{3}H]-AdoCbl) than were actually present. If this were in fact the case, a significant amount of exchange between free and enzyme-bound AdoCbl would have occurred in 2 min at 10° C. In this event, an even greater degree of exchange would be expected at 37° C where control experiments were carried out for the isotope effect in tritium washout. Such exchange would lead to an artifactually

high estimate of the amount of tritium released to product at infinite time at 37° C, and by comparison, an apparently slow release of tritium as a function of time in the determination of the isotope for tritium washout into product propionaldehyde at 10° C.

Several control experiments can be carried out to resolve complications in the determination of this tritium isotope effect. Exchange between free and enzyme-bound AdoCbl can be tested in two ways. First, incorporation into propionaldehyde of tritium derived from [5'-^{3}H]-AdoCbl can be measured by adding labeled AdoCbl to a solution containing unlabeled holoenzyme and 1,2-propanediol. The appearance of tritium in propionaldehyde would suggest exchange between free and enzyme-bound AdoCbl. Second, free AdoCbl may be isolated from a reaction mixture containing holoenzyme, excess AdoCbl, and [1 -^{3}H]-1,2-propanediol. The observation of tritium associated with free AdoCbl would also suggest exchange.

The tritium isotope effect can be measured by allowing enzyme and [5'-^{3}H]-AdoCbl to react with [1 -^{14}C]-1,2-propanediol. The expected half-life for tritium transfer from [5'-^{3}H]-AdoCbl to product is less than 1 sec; this experiment is best carried out with apoenzyme in excess over [5'-^{3}H]-AdoCbl and with amounts of [1 -^{14}C]-1,2-propanediol equivalent to 20 - 150 turnovers per holoenzyme active site. The reaction can be quenched with acid,

neutralized, carrier aldehyde added, and propionaldehyde isolated as the semicarbazone for $^{3}H/^{14}C$ assay. The ^{3}H content of water from which AdoCbl and aldehyde have been removed should be determined to ascertain the degree of exchange of tritium from aldehyde into water.

One possible interpretation of the apparently anomalous large tritium isotope effect in the transfer of tritium from AdoCbl to product is the presence of a second reservoir of mobile hydrogen (in addition to C-5' of AdoCbl). Indeed Babior (1979) has presented some preliminary evidence for such a second reservoir in the ethanolamine deaminase reaction. He found that interaction of [1 -^{3}H]-ethanolamine with holoenzyme in the presence of NADH and ADH (to reduce product aldehyde to ethanol as soon as formed) followed by (i) treatment with trichloroacetic acid to quench the reaction and denature the protein and (ii) removing ethanol by azeotropic distillation, led to tritium being recovered in water as well as in substrate, ethanol, and recovered AdoCbl. If unlabeled ethanolamine was added as a chase to holoenzyme previously exposed to [1 -^{3}H]-ethanolamine, little or no tritium was found in the water. These results provide a basis for inferring that there exists a reservoir of hydrogen attached to such a site(s) on the protein that they are exchangeable with solvent water after denaturation of the protein; these hydrogens cannot exchange with water during the normal course of the reaction, as

under these conditions no tritium from tritiated substrate or tritiated AdoCbl appears in solvent (Brownstein & Abeles, 1961; Babior, 1969). Such a reservoir could explain the anomalously large tritium isotope effects. The majority (∿80 %) of catalytic events would involve the reservoir as intermediate hydrogen carrier, while the 5'-carbon of AdoCbl would participate the remainder (∿20 %) of the time. Thus the factor of five discrepancy between the predicted (30 - 40, Chapter III) and observed (160) tritium isotope effects would be of a statistical nature.

Experiments are proposed to examine the possibility that such a second reservoir for migrating hydrogen exists in the case of diol dehydratase. A key to the proper execution of these experiments is to separate any product or unreacted substrate from holoenzyme without oxygen inactivation of the holoenzyme and without exchange with solvent of any tritium acquired by the putative second "exchangeable" reservoir. To accomplish this purpose, one can carry out a reaction with [1 -^{3}H,^{14}C]-1,2-propanediol as substrate. The resulting solution can then be passed down a BioGel P-60 column in a buffer containing a saturating concentration (>1 mM) of dl-2,3-butanediol, which functions as a purely competitive inhibitor (Chapter I; Moore & Richards, 1979). Labeled 1,2-propanediol and aldehyde will be retarded by the column while the holoenzyme-dl-2,3-butanediol complex will elute in the void volume. The

resulting enzyme-inhibitor complex would be denatured with heat or trichloroacetic acid and lyophilized. To determine the amount of exchangeable hydrogen associated with protein, the distillate can be assayed for ^{3}H (as well as ^{14}C to ensure that no diol or aldehyde is present). A possible way for aldehyde still to be present in the protein fraction is by formation of a Schiff's base with free amino groups of lysine residues.

The possibility of such aldehyde-protein derivatives is suggested by the observation that [^{14}C]-β-hydroxypropionaldehyde and [^{14}C]-acetaldehyde (Moore & Richards, unpublished results) extensively label diol dehydratase during the enzymatic reaction. Treatment of this protein with trichloroacetic acid would then hydrolyze these Schiff's base functionalities, releasing aldehyde to solution, where it could by enolization release its hydrogen to water. Were this the source of any tritium observed in the distillate, ^{14}C should be present also.

Another approach is to reduce the enzyme-dl-2,3-butanediol complex with KBH_4 to convert any imine groups to alkyl amines, which would be stable to hydrolysis by trichloroacetic acid. (The NADH/ADH reducing system does not accomplish this conversion.) If in the first case, the distillate contains ^{3}H and ^{14}C and in the second case does not, one would know the source of radioactivity was aldehyde-enzyme Schiff's base derivatives. If in the second case ^{3}H is still present in the distillate (and

especially so if no ^{14}C was present in the distillate in the first case) one could conclude that some exchangeable site on the enzyme has acquired tritium.

An additional useful experiment would then be measurement of the rate of transfer of this "exchangeable" tritium to product aldehyde by procedures analogous to those described previously for measuring the washout of tritium from [5'-^{3}H]-AdoCbl by catalysis with unlabeled substrate. If such an alternate hydrogen reservoir does serve in a majority of turnovers to store migrating hydrogen, one should observe a nearly normal kinetic isotope effect for transfer of tritium from this reservoir to product.

The possibility that label appears in solvent via Schiff's base formation between protein and aldehyde can also be tested by carrying out the diol dehydratase reaction in the presence of some other protein (albumin, myoglobin, etc.), isolating this protein by gel chromatography, and subjecting it to analysis for exchangeable tritium as outlined above.

Protein. We have obtained evidence that inactivation by oxygen and glycerol involves alteration of the protein as well as of AdoCbl (Bachovchin et al., 1977; Chapter I), and from these results infer that one or more amino acid residues in the active site are essential for catalytic activity.

To determine which residues are at or near the active

site, studies to identify those that are altered during inactivation by glycerol or oxygen are proposed. Holoenzyme which has been inactivated in one of these ways can be digested proteolytically or cleaved with cyanogen bromide, and the peptide mixture subjected to chromatography in one dimension and electrophoresis in a second. The resulting peptide maps can be compared to those obtained from apoenzyme or from holoenzyme which has been exposed to 1,2-propanediol and then treated just as the inactivated enzyme before being cleaved into fragments. A similar procedure could be applied to the separated subunits (Toraya et al., 1973; Poznanskaya et al., 1977) of inactivated holoenzyme in an attempt to localize any altered amino acid residues to a particular subunit. Ultimately, isolation, followed by sequence analysis of the altered peptides should identify those residues involved in inactivation, and therefore presumably near the cataytic site and likely to play a significant role in catalysis. While glycerol-inactivated holoenzyme presents some special problems in this regard, due to extensive labeling of protein by β-hydroxypropionaldehyde, we have reduced the degree of this nonspecific labeling by a factor of ten or more by such strategies as trapping the aldehyde product by KBH_4 added to the reaction mixture. It is certain that the inactivation of holoenzyme by glycerol does not result from interaction of the protein with product

β-hydroxypropionaldehyde (or its dehydration product acrolein) because of the stereospecificity of inactivation relative to catalysis. Furthermore, free β-hydroxypropionaldehyde added to the reaction mixture does not inactivate holoenzyme.

Another method for identifying residues at or near the active site is provided by the photolability of alkyl cobalamins (Hogenkamp, 1975). Accordingly [^{14}C]-methyl-, ethyl-, and carboxymethyl- (etc.) cobalamins may serve as useful active-site directed photoaffinity labels. Such derivatives can be readily prepared by alkylation of cob(I)-alamin with the appropriate [^{14}C]-alkyliodide (Dolphin, 1971). Binding of these modified forms of AdoCbl, which are competitive inhibitors (Lee & Abeles, 1963), to apoenzyme followed by photolysis of the carbon-cobalt bond should generate reactive primary alkyl radicals which may then alkylate nearby amino acid residues, or modify them in a way which would be detectable by the techniques outlined above. Appropriate control experiments involving competitive inhibition of this photoaffinity labeling by binding of photochemically inert OH-Cbl or by unlabeled alkylcobalamin should also be carried out.

NMR Studies. Recent advances in the purification of diol dehydratase (Appendix) make possible studies of this enzyme by nuclear magnetic resonance. Currently, concentrations of active sites on the order of 30 - 40 μM

are attainable; possibly, concentrations of up to 0.1 mM may be achieved in the near future. Useful information could be gained by studies involving the following nuclei: ^{19}F, ^{31}P, and $^{205}Tl^{+}$.

(i) ^{19}F. 3,3,3-Trifluoro-1,2-propanediol binds to diol dehydratase, and undergoes both catalysis and inactivation, although at greatly reduced rates compared to 1,2-propanediol and glycerol, respectively. The three ^{19}F nuclei at C-3 of this substrate analog constitute a very sensitive probe for NMR studies of interactions of substrate with enzyme, both in the presence and absence of analogs of AdoCbl. As the reaction of this substrate with holoenzyme is rapid on the time scale of the NMR experiment, studies of the holoenzyme-3,3,3-trifluoropropanediol complex itself will probably not be feasible (except for the inactivated complex). However, binding studies such as those reported by Hull et al. (1975) with ethanolamine deaminase should be informative about the interaction of enzyme with substrate when carried out in the presence of OH-Cbl, CN-Cbl, Me-Cbl, or other catalytically inactive cobalamins.

(ii) ^{31}P. Recently, a preliminary ^{31}P NMR study of cobalamin has been carried out (Satterlee, 1979). The ^{31}P resonance of the phosphate group in the nucleotide side chain (Introduction, Fig. 1) is sensitive to the ionization state of cobalt, to pH changes, and to whether or not the benzimidazolyl nitrogen is coordinated to cobalt.

Moreover, the ^{31}P resonance is both shifted and broadened when the cobalt atom is in the Co^{2+} oxidation state and carries an unpaired electron. Thus, ^{31}P NMR should be a valuable tool for investigating the binding of cobalamins to diol dehydratase. In particular, the ionization state of cobalt in the inactive holoenzyme complexes formed by glycerol and thioglycerol, which exhibit Cbl^{III} and Cbl^{II} uv-visible spectra, respectively, could be further probed by ^{31}P NMR. The importance of the phosphate group to binding of Cbl to enzyme could also be studied, since the chemical shift of ^{31}P is sensitive to the involvement of phosphate oxygen in hydrogen bonds (Moon & Richards, 1973; Goetze & Richards, 1978). However, the relatively low sensitivity of ^{31}P ($\sim$360 times that of naturally abundant ^{13}C) will require accumulation of numerous free-induction decays, so study of rapid reactions such as catalysis will not be feasible.

(iii) $^{205}Tl^{+}$. Diol dehydratase requires a monovalent cation for activity; this cation seems to play a role in binding of AdoCbl to apoenzyme (Toraya et al., 1971; Toraya & Fukui, 1972). Maximum enzyme activity is observed with cations having ionic radii of about 1.4 Å (K^{+}, NH_4^{+}, Rb^{+}, Tl^{+}) (Toraya et al., 1971). Tl^{+} is known to mimic potassium in a variety of biological processes (Britten & Blank, 1968; Manners et al., 1970), and has two spin ½ isotopes, $^{203,205}Tl$. ^{205}Tl has a sensitivity about twice

that of ^{31}P (which results in a time saving of a factor of four in obtaining a spectrum), is 70.5 % naturally abundant, and has a chemical shift range of over 5000 ppm (Schneider & Buckingham, 1962). The use of ^{205}Tl as an NMR probe of diol dehydratase thus seems attractive. Binding of $^{205}Tl^+$ to protein could be studied by this method, as could binding of Cbl derivatives to the protein-$^{205}Tl^+$ complex. Conceivably, the $^{205}Tl^+$ resonance chemical shift may also be sensitive to inactivation of holoenzyme by oxygen and by glycerol and thioglycerol as described above for ^{31}P; these results could be compared to data obtained with the competitive inhibitor dl-2,3-butanediol, which does not cause inactivation or cleavage of the carbon-cobalt bond of enzyme-bound AdoCbl (Chapter I).

With $^{205}Tl^+$ in this system, chemical shift measurements will be considerably more feasible than measurements of relaxation times. For example, Chan & Reeves (1974) and Bangerter (1977) have determined that $^{205}Tl^+$ relaxation times are extremely sensitive to the presence of small amounts of dissolved oxygen.

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PROPOSITION II

Cancer fatalities are rarely caused by a single, or primary tumor; it is the proliferation of multiple tumors throughout the body which most often causes death. This ability of tumor cells to colonize distant parts of the body remote from the main tumor mass is called metastasis, and is the most deadly aspect of cancer. At present little is understood about the various processes involved in metastasis, such as tumor cell detachment, infiltration of and transport through the circulation and lymphatic systems, and invasion and colonization of the secondary sites. These mechanisms are the subject of recent reviews (Roos & Dingemans, 1979; Nicholson, 1979).

Introduction into the organism of microbial stimulating agents, such as Bacillus Calmette-Guerin (BCG) and Corynebacterium parvum (C. parvum) or C. granulosum, under suitable conditions appears to inhibit formation of metastases and even causes regression of the primary tumor mass. The effect is observed with either living or killed BCG or C. parvum preparations (Baldwin et al., 1974). In most cases, these observations have been confined to animal cancer models, but use in immunotherapy of human malignant melanoma has been reported (Gutterman et al., 1973).

Injection of a BCG suspension into a primary tumor

results in a decreased level of metastasis (Zban et al., 1972; Baldwin & Pimm, 1973a; Sparks et al., 1974; Smith et al., 1975; Lee, 1977), and in some cases in regression of the primary tumor (Bomford & Olivotto, 1974). Apparently, contact of tumor cells and BCG is required for the effect to be observed; intravenous administration of BCG seems effective only against the appearance of tumors in the lung, where BCG accumulates (Baldwin & Pimm, 1973b, 1974). BCG appears to enhance an immune response against tumor-specific antigens, and is relatively more effective against tumors which are antigenic (Baldwin & Pimm, 1973a). However, macrophages have also been implicated in this effect (Hanna et al., 1972).

In contrast, anti-metastatic activity by C. parvum does not seem to require cell-cell contact; both intravenous and intraperitoneal administration are effective (Jones & Castro, 1977; Sadler & Castro, 1976a,b; Bomford & Olivotto, 1974). Evidence has been obtained implicating a role for circulating T-cells in this effect (Sadler & Castro, 1976b; Milas et al., 1975), although an intact thymus may not be necessary. Conflicting results have been obtained on the role of macrophages (Jones & Castro, 1977; Bomford & Olivotto, 1974).

The mechanisms responsible for these effects are poorly understood. BCG and C. parvum may potentiate an immune response to tumor antigens, or cause an enhancement of

"nonspecific resistance" (Bomford & Olivotto, 1974) to the cancer cells. Conceivably, these microbial agents may challenge the host's immune system with antigens which somehow mimic those on cancer cells, but are significantly more antigenic, thus enhancing an immune response against the tumor cells. Some experiments are proposed which should help to resolve these questions.

First, the "antigen mimicry" hypothesis may be tested by immunizing tumor-free organisms with BCG or C. parvum and then testing the resulting antisera for cross-reaction against the appropriate cancer cells. The immunized animals can then be tested for the ability of these microorganisms to suppress metastasis of implanted tumors. Detection of immunologically cross-reactive antigens present in both bacteria and cancer cells would suggest a possible mode of action for the observed suppression of metastasis. In the event of a positive result, one might predict that the immunized animals would display an overall decreased frequency of metastasis, which would probably not be further depressed by introduction of BCG or C. parvum. A negative result, that no "antigen mimicry" is observed, would suggest that this direct stimulation of the immune system is not a factor in the observed antimetastatic behavior.

The possibility that administration of these microorganisms effects a stimulation of the immune system, thereby rendering it more competent to deal with cancer

cells, can be tested in the following way. Fetal mice or neonatal rats which are not yet immunologically mature may be tolerized to BCG or C. parvum by the techniques described by Billingham et al. (1953; 1955a,b). Fetal mice are best exposed to the antigen at the 16th or 17th day of fetal life (Billingham et al., 1955b); while neonatal rats seem to yield better results than neonatal mice (Woodruff & Simpson, 1955). The tolerant organisms may then be employed to determine the effect of BCG and C. parvum on metastasis of tumors subsequently introduced. If the mechanism of metastasis suppression in any way involves the recognition by the immune system of BCG and C. parvum antigens as "foreign," then these microorganisms should have no effect upon proliferation of tumors in the tolerant host animals; such an effect should be observed in control animals which were injected with antigen-free solutions during the fetal or neonatal period.

The observation that BCG and C. parvum could still inhibit tumor metastasis in tolerant organisms would seemingly rule out any role in this process for those components of the immune system involved in the recognition of "foreign" antigens.

Several host-tumor systems are available for the proposed studies in which the antimetastatic activity of BCG and C. parvum has been demonstrated: (C57Bl/Rij x CBA/Rij) F1 mice and osteosarcoma C22LR (van Putten et al., 1975);

CBA-T6T6 mice and T3 fibrosarcoma (Bomford & Olivotto, 1974); Sewall-Wright guinea pigs and a transplantable hepatocellular carcinoma (Smith et al., 1975) are a few examples. The effect appears to be general; and any host-tumor model in which it can be demonstrated should be adequate for the proposed study.

The proposed experiments should clarify the mechanism of the antimetastatic effects of BCG and C. parvum; in any case, the current confusing picture should be improved.

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PROPOSITION III

The complement system of nine serum proteins (C1-C9) is an important component of the immune system of vertebrate organisms. These proteins participate in several reactions involved in defense of the organism, such as inflammation and cytotoxicity.

The complement proteins are produced as nonfunctional precursors which under suitable conditions may become activated and thus enabled to participate in complement-mediated reactions. The final portion of the complement reaction sequence may be preceded by either the classical activation pathway (Muller-Eberhard, 1969), which involves interaction of the antibody-antigen complex with C1 (specifically, $C1_q$), or the alternate (or "properdin") pathway, whose origins are less well characterized (Gotze and Muller-Eberhard, 1971; Muller-Eberhard & Gotze, 1972; Arrogave, 1972). Two recent reviews summarize current knowledge of the activation process (Porter, 1979; Reid & Porter, 1975).

The classical activation pathway is initiated by interaction of the complement protein $C1_q$ with immunoglobulins IgM, and IgG subclasses IgG1, IgG2, and IgG3 (Augener et al., 1971). Although a $C1_q$ molecule can bind up to six Ig molecules, as few as two are required to initiate the classical activation pathway (Borsos & Rapp, 1965). Whether an antigen-induced conformational change

in the Ig heavy chain region (Schur & Christian, 1965; Press, 1975), or simple aggregation of antibody molecules (Hyslop et al., 1970, Augener, 1971), or both, are required for Cl_q binding is currently unresolved. Nonetheless, evidence has been accumulated implicating the existence of specific sites of interaction, in the CH2 domain of IgG (Kehoe & Fougerean, 1969; Utsumi, 1969; Ellerson et al., 1972) and Fc_μ of IgM (Plaut et al., 1972).

In human IgG1 and rabbit IgG, amino acid residues between 271 and 291 of the CH2 region appear to be involved in complement fixation. Allen & Isliker (1974a,b) showed that modification of rabbit IgG and its Fc fragment with the tryptophan-directed reagent 2-hydroxy-5-nitrobenzyl bromide decreased complement-fixing activity, despite the observation that this modification did not detectably alter the Cl_q binding capacity, and suggested that tryptophan at position 277 is important in complement fixation. Johnson & Thames (1976) modified IgG1 with reagents directed against tryptophan (2-hydroxy-5-nitrobenzyl bromide), tyrosine (tetranitromethane), and arginine (2,3-butanedione); the complement-fixing abilities of these modified proteins were reduced by up to 77%.

These workers also synthesized peptides with amino acid sequences similar to those of the Ig heavy chain region suspected to be involved in complement fixation, and determined the extent to which these peptides could

inhibit complement fixation by IgG1. A particularly effective inhibitor was

GlyTrpTyrGluArgGly, (I)

which may be compared to the CH2 region sequence

AsnTrpTyrValAspGly (II)
276 281

(Edelman, 1970).

Interpretation of chemical modification studies such as those described above is complicated by uncertainty of the effect of a given modification. Alteration of amino acids may have a direct effect on the interaction under study, or an indirect effect: a change in the conformation of the site of interaction could be caused by modification of an amino acid elsewhere in the protein.

In order to resolve these ambiguities, and to investigate the nature of the antibody-$C1_q$ interaction, an investigation of the inhibitory behavior of peptides such as (I) which have been chemically modified is proposed. Of particular utility should be peptides containing 3-nitrotyrosine produced by nitration with tetranitromethane. This procedure is selective, simple to execute, and results in a chromophore which can be conveniently exploited for concentration assay purposes (Means & Feeney, 1971). Furthermore, the resulting nitrated tyrosine has a pK of 7.0-8.0 for deprotonation of the phenolic hydroxyl group.

Thus, a study of inhibition by such a modified peptide of complement fixation over the pH range 6-8.5 should be informative about the role played by the tyrosyl residue in binding of Cl_q. Moreover, a similar study of tetranitromethane-treated IgG1 may also be valuable. The experiments described by Johnson & Thames (1976) were carried out at pH 7.4, and one or more of the seven modified tyrosine residues may have been ionized at physiological pH (manipulation of conditions of the nitration reaction could conceivably decrease the number of altered amino acids).

Methods for assay of complement activity are well known (Kabat & Mayer, 1961; Lachmann & Hobart, 1978), as are those for peptide synthesis (Finn & Hofmann, 1976; Erickson & Merrifield, 1976). Peptides could perhaps also be obtained from a proteolytic or chemical digest of the Fc portion of IgG1. Control experiments should also be carried out to determine the extent of complement fixation via the alternate pathway by assaying for the consumption of C2 or by repeating the assay in the absence of Ca^{2+}, which is required by the classical pathway.

The pH-dependent behavior of complement fixation by IgG1 and its inhibition by the peptide inhibitors which contain nitrated tyrosine could illuminate the nature of the interaction, between Cl_q and the heavy chain constant region of immunoglobulins, which initiates the classical pathway of complement fixation.

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PROPOSITION IV

The immune system is one of the most complex biological systems, the molecular, genetic, and cellular aspects of which have been studied intensively. The immunoglobulin molecule is composed of two light and two heavy polypeptide chains, each of which may be divided into an NH_2-terminal variable (V) and one (light chain) or three (heavy chain) constant regions (C). Three unlinked families of genes code for immunoglobulins; one codes for the V and C regions of heavy chains, while two (λ and κ) code for those of the light chains (Hood et al., 1975). The V and C regions are encoded by separate genes which become joined to form a single gene (Dreyer & Bennett, 1965).

Until recently, immunogeneticists were limited to serological and protein sequence studies in their investigations of antibody genes. With the introduction of recombinant DNA technology, the tools are now available for direct examination of the organization of antibody genes in the DNA of both antibody-producing and undifferentiated cells.

Studies of immunoglobulin light chain genes with probes derived from purified light chain mRNA generally confirm the V-C joining hypothesis (Leder et al., 1977; Rabbits et al., 1975; Hozumi & Tonegawa, 1976; Seidman & Leder, 1978). Hozumi & Tonegawa (1976) showed that the

mouse V_κ and C_κ genes lie on different DNA restriction fragments in early emhryonic tissues, while in the mature (differentiated) antibody-producing myeloma from which the mRNA was obtained, these genes lie on the same restriction fragment (in both chromosomes). A particularly intriguing result was that even in a differentiated myeloma cell, the V_λ and C_λ genes remain separated by a 1250 base pair sequence; this sequence is absent from the mRNA (Brack & Tonegawa, 1977). Such intervening sequences, termed "introns" (Gilbert, 1978), have also been observed in other gene systems, such as κ-light chains (Rabbitts & Forster, 1978), β-globin (Jeffreys & Flavell, 1977), ovalbumin (Breathnach et al., 1977), and yeast tRNA (Goodman et al., 1977; Valenzuella et al., 1978). Recent evidence (Rabbitts, 1978) indicates that these introns disappear during the processing of the initial RNA transcript of the mouse light chain genes.

Because of difficulties in preparation of the desired mRNA, the organization of immunoglobulin heavy chain genes has only recently been studied (Honjo & Kataoka, 1978; Early et al., 1979; Sakano et al., 1979). The latter two studies, of IgA and IgG1 heavy chains, respectively, indicate that the CH1, CH2, CH3, and hinge regions of the heavy chain constant region are each encoded by uninterrupted DNA sequences which are separated by introns of 100-

350 base pairs. In the IgA case (Early et al., 1979) the V and C genes are separated by 6.8 kilobases in embryonic DNA, and rearrangement apparently does occur upon differentiation.

The function of the introns remains unclear, although current evidence favors a role in splicing of the primary nuclear RNA transcript. In a large majority of introns characterized so far, the intron-exon junction may be chosen so that the intron begins with G-T and ends with A-G (Bernard et al., 1978; Breathnach et al., 1978; Reddy et al., 1978; Van den Berg et al., 1978; Sakano et al., 1979). This observation suggests that splicing sites may be quite similar in vastly different species.

For the purposes of both further characterization of immunoglobulin heavy chain genes, and examination of the structure of introns within a gene family, studies of the structures of the μ(IgM), δ(IgD), and ε(IgE) heavy chain constant region genes of the mouse are proposed. In addition to valuable information on the arrangement of these genes in the mouse genome, a comparative study of the intron sequences may provide insights into their function, in evolutionary and structural terms. For example, the observation that intron sequences between CH regions are highly conserved, while the $CH_{\mu,\gamma,\alpha,\delta,\varepsilon}$ coding regions are considerably more diverse, might suggest that quite different selective pressures operate on the coding

and intervening sequences. On the other hand, if the intron sequences in the CH gene family differ considerably, one might speculate that perhaps, with the exception of the intron-exon junction region, sequence plays a secondary role to that of size, and mutational drift could be the source of the sequence differences.

The appropriate mRNA is required as the source of a probe for the CH genes. A convenient source of this mRNA would be a myeloma cell line secreting antibody of the desired class. IgA, IgG, and IgM-secreting mouse myeloma cell lines are readily available. An IgD-producing rat myeloma tumor could also be used; its heavy chain mRNA should cross-hybridize with mouse C_δ genes. The technique of cell hybridization to produce myeloma-like cell lines secreting monoclonal antibody of known antigen specificity (Kohler & Milstein, 1975) can also be used; IgM and IgG-secreting mouse "hybridoma" cell lines specific for the dinitrophenyl group have been produced in this laboratory (R. R. Hardy, personal communication). Recently a monoclonal IgE-producing cell line with specificity for ovalbumin has been reported (Bottcher et al., 1978). These induced and hybrid myeloma cell lines should provide a ready source of heavy-chain mRNA for the proposed studies.

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PROPOSITION V

The explosive growth of the chemical industry in recent decades has played a major role in technological and agricultural development. However, the accompanying release into the environment of large amounts of synthetic, nonbiological compounds is of major concern, particularly in regard to their potential carcinogenicity and activity as mutagens (McCann et al., 1975; McCann & Ames, 1976). Recently, the Department of Health, Education and Welfare has advanced the theory that as much as 40% of all cancer may soon be caused by industrial or environmental exposure to carcinogens. Of particular potential danger are those substances which are not degraded by bacteria and fungi, or are lipophilic and thus become concentrated in the fat depots of organisms of the food chain (for example, dioxin, DDT, and halogenated aromatics).

The techniques of bacterial mutagenesis, especially recent developments (Bachovchin & Roberts, 1977) can be employed to "create" new strains of microogranisms whose mutant enzymes are capable of converting a target compound to less toxic or biodegradable forms. Such organisms could then be used directly to detoxify these compounds.

Techniques are available which could be used to generate mutant bacteria with a desired characteristic. In general, this procedure would involve treatment of the wild-type organism (the soil bacteria Pseudomonas might be a

good selection) with a mutagen such as nitrosoguanidine, followed by selection of a clone of cells, using for example the technique of replica plating, of the desired phenotype (Bachovchin & Roberts, 1977). This method was used to produce a histidine auxotroph of Myxobacter 495 for a study of the mechanism of serine protease action (Bachovchin & Roberts, 1978). In the proposed study, the selection criterion could be for the ability to survive in the presence of, or even utilize as a carbon source, the target compound. Naturally occurring strains of Pseudomonas which can metabolize halobenzoic acids and halophenols have been reported (Dorn et al., 1974; Knackmuss & Hellwig, 1978), as have conjugate strains which can utilize halobenzoic acids as a carbon source, converting them to metabolites, CO_2, and halide ion (Reineke & Knackmuss, 1979).

Possible adaptive mutations which would result in the desired characteristics include gene duplication leading to hyperproduction of an enzyme (Rigby et al., 1974) alteration of structural genes to give new enzymes with different specificities (Betz et al., 1974; Senior et al., 1976), or changes in regulatory processes (Wood, 1966; LeBlanc & Mortlock, 1971).

Once a mutant strain capable of metabolizing or suitably altering the target compound is isolated, the fate of the compound may be studied using radioisotope

tracer methodology with either the whole organism or cell-free extracts. The results of this investigation could suggest which enzyme(s) had been altered by mutagenesis, and would also identify products derived from the labeled target compound.

The "synthetic" organisms produced by these methods could be used for on-site treatment of wastes containing the target compound. Alternatively, but only after thorough characterization of the organism, areas known to be contaminated with the target compound could be "seeded" with the mutant bacterium, which would presumably compete effectively against native microorganisms only so long as its "substrate", the target compound, is present in sufficient quantities.

This proposed study would likely involve a great deal of labor, and the screening of prodigious numbers of colonies for the desired characteristics. However, the potential rewards are sufficiently great to justify the effort involved.

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