DIOL DEHYDRATASE: PURIFICATION, STRUCTURAL CHARACTERIZATION, AND MECHANISM OF ACTION

Thesis by

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To Dorothy and my parents

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT TO CHAPTER 1

A new isolation procedure for propanediol dehydratase increases by a factor of about 16 the yield of enzyme obtainable from *Klebsiella pneumoniae*; the enzyme thus isolated has a specific activity of 95 $\pm$ 4 units/mg. The apoenzyme consists of four different subunits with molecular weights of 60 K, 51 K, 29 K, and 15 K daltons in the ratio of 2:1:2:2, respectively. In this new procedure, care was taken to prevent the partial proteolysis of the propanediol dehydratase which seems to occur in earlier procedures. The other novel aspect recognizes that the enzyme is associated with the cell membrane. Accordingly, after gentle sonication, the membrane fragments are separated from cytosol, and the enzyme is solubilized by extraction with buffers containing detergent. The amino acid compositions and N-terminal amino acid sequences for each of the subunits was also determined. From the amino acid compositions of the individual subunits, diol dehydratase appears to be a peripheral membrane protein.

ABSTRACT TO CHAPTER II

When diol dehydratase holoenzyme is inactivated by reaction with radioactive glycerol, one mole of glycerol appears to become tightly associated with each 250,000 daltons of the holoenzyme complex with a significant loss of tritium from C-2 being observed. However, denaturation of the inactivated complex releases the modified glycerol from the protein, indicating that the protein is not covalently modified by the inactivator. Similar experiments were carried out with radioactive isobutylene glycol, but due to the high level of nonspecific labeling, the results were not as definitive.

As described in Chapter I, former isolation procedures (Abeles, 1966; Poznanskaya *et al.*, 1979) yielded enzyme which had been proteolysed. For this reason inactivation studies employing various deuterated derivatives of glycerol and isobutylene glycol, as well as a new class of inactivators represented by hydroxyacetone and dihydroxyacetone, were carried out with native enzyme to compare results from similar studies with proteolysed enzyme (Bachovchin *et al.*, 1977; Moore, 1979). It was found that proteolysis had little effect on the constants associated with the glycerol inactivation, but an enormous effect on the constants describing the inactivation by isobutylene glycol.

The results of the radiolabeling studies and kinetic

experiments are consistent with the formation of a secondary alkylcobalamin upon inactivation of diol dehydratase by glycerol. Kinetic evidence also suggests that the inactivation of diol dehydratase by isobutylene glycol occurs after the abstraction of hydrogen from C-1, but before the substrate rearranges.

ABSTRACT TO CHAPTER III

A reinvestigation of trace label experiments with native diol dehydratase isolated by the method of McGee and Richards (1981) (see Chapter I) has shown that the probability of net intramolecular transfer for tritium is 0.33 ± 0.02 as opposed to the value of about 0.03 obtained earlier (Frey *et al.*, 1967a) with an enzyme preparation obtained by a different method. Our observed value of 0.33 is about 20 times larger than what one would predict on the basis of the mechanism for the migration of hydrogen given in the Introduction to this thesis. In contrast, tritium washout experiments, similar to those conducted by Essenberg *et al.* (1971), yielded a value of $k_{HH}/k_{HT} = 29 \pm 2$ which is approximately the value predicted by Moore *et al.* (1979). Also, tritium washout experiments were carried out in such a way that, in addition to the C-5' hydrogens of adenosylcobalamin containing tritium, any other multiple-hydrogen reservoirs should have contained tritium as well. These experiments yielded identical results to those in which only the C-5' hydrogens contained reactable tritium; therefore, it appears that only the C-5' hydrogens of C-5' deoxyadenosine participate directly in catalysis.

Also, the tritium isotope effect on the first hydrogen transfer was determined to be 6.1 ± 0.5 by measuring the isotopic enrichment in unreacted [1-^{3}H]-1,2-propanediol as a function of the extent of reaction. The results of our

study suggest that the generally accepted pathway for catalysis, which proceeds through the C-5' deoxyadenosine hydrogen reservoir, constitutes about 95% of the catalytic events with unlabeled substrate; however, there appears to be an alternate catalytic pathway whose contribution to catalysis can be enhanced by isotopic substitution at C-1 of substrate.

ABSTRACTS TO PROPOSITIONS

Proposition I

Nitroimidazoles are used in cancer radiotherapy to increase the sensitivity of tumors to radiation. A drawback to the use of these compounds are side effects which are symptomatically similar to thiamine deficiency. Studies are proposed to determine if these compounds are interferring with thiamine transport and to determine if these compounds need to be actively transported in order to be effective.

Proposition II

All adenosylcobalamin-dependent enzymes appear to have more than one subunit (Babior, 1975); however, the roles for the individual subunits are not understood. By preparing photoaffinity labels which are analogues of adenosine or cobalamin, one may be able to covalently link these labels to specific subunits of adenosylcobalamin-dependent enzymes and eventually determine individual amino acids involved in enzyme-cofactor interactions.

Proposition III

The development of substances which would covalently bond to dentine will lead to simpler dental procedures (with less etching and grinding being necessary) and inhibit the

formation of subsequent caries in the area of the prosthetic replacement. The inclusion of copolymerizable compounds, which contain the photosensitive diazirine group, in acrylic mixtures used to rebuild portions of teeth, could provide the additional adhesive strength desired.

Proposition IV

Diol Dehydratase can utilize a variety of monovalent cations (besides K^+) as a cofactor ion; however, the observed maximal velocity varies with the ionic radius (Toraya *et al.*, 1971). Studies are proposed to determine if various cofactor ions could affect differently the contribution of the alternate kinetic pathway (discussed in Chapter III) to catalysis.

Proposition V

From α-secondary deuterium isotope effects, one can determine the extent to which the bond-making and bond-breaking processes have developed in the transition state of Cope rearrangements (Gajewski and Conrad, 1979). By studying appropriately deuterated bis(methylene-cycloalkanes), one could compare the effects of radial-stabilizing substituents on the "boat" and "chair" transition states of the Cope rearrangement

INTRODUCTION TO THESIS

Cobalamin, commonly referred to as B_{12}, and its derivatives make up a class of compounds of both clinical and chemical interest. The most common clinical manifestations of cobalamin deficiencies are pernicious (megaloblastic) anemia and neurological abnormalities associated with the formation of lesions on, and demyelination of, the spinal cord. Minot and Murphy (1926) successfully abated symptoms in patients with pernicious anemia by giving them 120 to 140 grams of liver per day. Shortly thereafter, Cohn and coworkers (1928), in conjunction with Eli Lilly and Company, commercially produced a yellow powder known as Liver Extract 343 (Lilly) which, according to Castle (1966), conserved most of the hematopoietic activity of 300 grams of beef liver in a daily oral dose of 12.75 grams. Although many different groups were trying to further fractionate the liver, progress was slow. Laland and Klem (1936) in Norway reported a partially purified substance which, upon intravenous injection, was clinically active against pernicious anemia with a daily dose being only 0.7 milligrams. The material was obtained by extraction of minced liver with 50% acetone, followed by phenol extraction from water and adsorbtion on charcoal. Unfortunately, further efforts in that laboratory were hampered due to the German invasion. The red, crystalline substance known as vitamin B_{12} (cyanocobalamin) was finally purified in 1948

at the Merck (Rickes *et al.*, 1948) and Glaxo Laboratories (Smith and Parker, 1948). Berk and coworkers (1948) reported that a daily oral dose of five micrograms of vitamin B_{12} was effective against pernicious anemia; however, as little as one microgram daily has been shown to be sufficient (Rosenberg, 1978).

While the purification of the anti-pernicious anemia factor ended an era of intensive effort, it also opened the doors to fascinating areas of chemical and biochemical research. The structure of vitamin B_{12} was determined by X-ray crystallography (Hodgkin *et al.*, 1955) (Figure 1a). The molecule contains a cobalt atom (Co^{3+}, d_6 low spin) coordinated to four nitrogen atoms of a tetrapyrrole macrocycle known as corrin. Corrin lacks the completely conjugated π-electron system and strictly planar configuration of porphyrin; however, there is sufficient similarity between the two macrocycles that the same numbering system is used for both (Figure 1b). Since corrin has a methine (instead of a methylene) bridge between rings A and D, the number 20 is omitted when numbering its atoms (Hogenkamp, 1975),

While cyanocobalamin is the most common commercial form of B_{12}, it is not found in microorganisms, plants, or animal tissues. The only naturally occurring forms of cobalamin in mammalian tissue are hydroxycobalamin, methylcobalamin and adenosylcobalamin (Figure 1a).

Methylcobalamin and adenosylcobalamin are unusual for both biological compounds contain a carbon-cobalt bond and they are the only known coenzymic forms of cobalamin in mammalian systems. Some synthetic analogues of adenosylcobalamin have shown coenzymic activity (Babior, 1975). For example, ribonucleotide reductase can utilize a variety of synthetic Coβ-adenosylcobamides having Coα ligands other than 5,6-dimethylbenzimidazole as cofactors (Blakely, 1965; Vitols *et al.*, 1967; Morely *et al.*, 1968; Blakely, 1966); adenosyl-13-epicobalamin (a diastereomer of adenosylcobalamin) showed about 14% of the coenzymic activity of adenosylcobalamin with diol dehydratase from *Aerobacter aerogenes* (Toraya *et al.*, 1975).

Since mammals do not biosynthesize cobalamin, they must extract it from the food they ingest via an intricate absorbtion and transport system (Ellenbogen, 1975). Ingested cobalamin becomes bound to a protein called intrinsic factor (Bunge *et al.*, 1956). The intrinsic factor-cobalamin complex then transverses the small bowel to the distal ileum where it becomes attached to the microvilli of the epithelial cells (Strauss and Wilson, 1960). Next, by an energy-requiring process, the vitamin is released from the intrinsic factor into the intestinal cell. Eventually the cobalamin enters the bloodstream (Doscherholmen and Hagen, 1957) where the majority of it is bound to a plasma β-globulin called transcobalamin II. The remainder of the

cobalamin is bound to an α-globulin called transcobalamin I which appears to be concerned with the storage of the vitamin in the circulation (Hall and Finkler, 1965; Horn, 1967). The transcobalamin II-cobalamin complex is moved to the cytosol of tissue cells via an endocytotic process where the transcobalamin II is then degraded by proteases, thereby liberating free cobalamin into the cytosol (Rosenberg, 1978). Cobalamin is then converted to methylcobalamin in the cytosol, or it enters the mitochondria and is converted to adenosylcobalamin (Pletsch and Coffey, 1972; Pierce *et al.*, 1975; Rosenberg *et al.*, 1975).

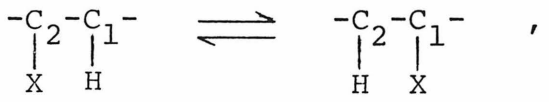
Although there is some evidence that methylcobalamin-dependent enzymes have a role in the biosynthesis of methane and acetate in certain bacteria (Poston and Stadtman, 1975), the most studied function of methylcobalamin is to serve as a cofactor for methyltransferase found in bacteria, fungi, higher plants, and animals. Methyltransferase catalyzes the reaction of N^5-methyltetrahydrofolate with homocysteine to yield tetrahydrofolate and methionine. The discovery of this reaction provided a key in understanding the relationship between folate and cobalamin metabolism.

An early clinical observation (Spies *et al.*, 1945) demonstrated that daily doses of 29 milligrams of folic acid, given to patients with pernicious anemia, caused a remission of symptons. A plausible explanation for this, and other

apparent relationships between cobalamin and folate metabolism, was independently put forth by Herbert and Zalusky (1962), Noronha and Silverman (1962), and Larrabee and coworkers (1963), and has been called the "folate trap hypothesis". The hypothesis is based on the conversion of tetrahydrofolate to N^5-methyltetrahydrofolate, followed by the regeneration of tetrahydrofolate from N^5-tetrahydrofolate as a result of the methyltransferase reaction. If methionine biosynthesis is the only major use for N^5-methyltetrahydrofolate, a cobalamin deficiency will interfere with such a folate cycle and cause an accumulation of N^5-methyltetrahydrofolate and depletion of other folate derivatives. Severe depletion would affect other pathways requiring folate derivatives, such as the biosynthesis of purines and pyrimidines or the conversion of formiminoglutamate to glutamate. Since there is some evidence that the direct or indirect involvement of cobalamin-dependent enzymes in the biosynthesis of deoxyribonucleotides and metabolism of fatty acids (Beck, 1975; Kishimoto *et al.*, 1973; Cardinale *et al.*, 1975) relates to the symptoms associated with pernicious anemia, one can understand how the administration of folic acid can ameliorate the symptoms in certain cases.

In man, the only known adenosylcobalamin-dependent enzyme catalyzes the reversible conversion of (2R)-methylmalonyl-coenzyme A to succinyl-coenzyme A. However,

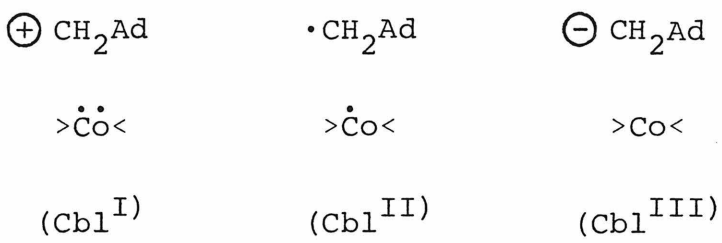
bacterial adenosylcobalamin-dependent enzymes catalyze a variety of interesting and widely studied reactions (Figure 2): ten rearrangements and a reduction. While the rearrangements are quite diverse, they are generalized by the following scheme:



a vicinal interchange of hydrogen and some group "X". The variations of group "X", which may be alkyl, hydroxyl, amino, or an acyl group, are responsible for the diversity of the rearrangements. Moreover, this diversity makes adenosylcobalamin-dependent enzymes unique, for this single family of enzymes is capable of breaking carbon-carbon (glutamate mutase, methylmalonyl coenzyme A mutase, and α-methyleneglutarate mutase), carbon-nitrogen (ethanolamine deaminase, L-β-lysine mutase, d-α-lysine mutase, d-ornithine mutase, and leucine 2,3-amino mutase), carbon-oxygen (diol dehydratase and glycerol dehydratase), and carbon-hydrogen bonds. Furthermore, mechanistic studies have shown that these rearrangements have essentially no counterparts in synthetic organic chemistry (Okabe *et al.*, 1981; Rudakova, 1978).

The generally accepted mechanism of adenosylcobalamin-dependent rearrangements is summarized in Figure 3. The first step is cleavage of the carbon-cobalt bond. In

principle, the carbon-cobalt bond could be cleaved in any of three ways, shown below:



Since this reaction presumably determines how hydrogen is transferred (as hydride, atom, or proton), its characteristics have been thoroughly studied. Electron spin resonance (ESR) 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 containing holoenzyme and substrate, or substrate analogues, are frozen, spectra are obtained containing resonances at g = 2.2 (a singlet assigned to cob(II)alamin), and at g$\sim$2 (a doublet attributed to an organic free radical). The appearance of these resonances requires both holoenzyme and substrate or analogue, and occurs sufficiently rapidly to represent actual catalytic intermediates (Valinsky *et al.*, 1974). These signals may be the result of a weak electrostatic exchange interaction between two unpaired electrons (Schepler *et al.*, 1975).

Spectroscopic studies provide additional evidence for the homolytic cleavage of the carbon-cobalt bond. When

diol dehydratase (Wagner *et al.*, 1966; Eager *et al.*, 1975) and ethanolamine deaminase (Joblin *et al.*, 1975) holoenzymes are allowed to react with their substrates, the ultraviolet-visible absorbtion spectra of the holoenzyme complexes change, at a rate which is consistant with catalysis, from that of adenosylcobalamin to ones very similar to cob(II)-alamin. Upon depletion of substrate, the spectrum changes to one characteristic of cob(III)alamin (hydroxycobalamin) in an oxygen-dependent process which results in inactivation of holoenzyme. When free in solution, cob(II)alamin undergoes a similar reaction in the presence of oxygen.

Subsequent to the cleavage of the carbon-cobalt bond, a hydrogen atom is transferred from C-1 of substrate to C-5' of the nucleoside moiety creating 5'-deoxyadenosine, as suggested by Ingraham (1964). Formation of 5'-deoxy-adenosine has been demonstrated with diol dehydratase (Wagner *et al.*, 1966; Finley *et al.*, 1972), methylmalonyl coenzyme A mutase (Babior *et al.*, 1973), l-β-lysine mutase (Baker *et al.*, 1973), and ethanolamine deaminase (Babior, 1970a); however, in these cases 5'-deoxyadenosine was formed during reactions with substrate analogues which irreversibly inactivated their respective holoenzymes. This suggested the possibility that 5'-deoxyadenosine was the product of a destructive side reaction and not a true catalytic intermediate. However, results obtained with ethanolamine deaminase (Babior, 1970b; Babior *et al.*, 1974a) showed that by interrupting catalysis by denaturation of the

holoenzyme, 5'-deoxyadenosine could be recovered. Moreover, the amount of 5'-deoxyadenosine recovered varied with the manner of denaturation which was interpreted to indicate the reversible formation of this moiety during catalysis. Hence, 5'-deoxyadenosine is strongly implicated as an intermediate hydrogen carrier in adenosylcobalamin-dependent rearrangements.

The next step in the mechanism is the migration of group X from C_2 to C_1. The migration is known to occur intramolecularly in the reactions catalyzed by glutamate mutase (Barker *et al.*, 1964a,b), methylmalonyl coenzyme A mutase (Kellermeyer and Wood, 1962; Wood *et al.*, 1964; Phares *et al.*, 1964), ethanolamine deaminase (Babior, 1969) and diol dehydratase (Retey *et al.*, 1966a). However, the exact nature of the migration of group X is not thoroughly understood and, given the diversity of X, may differ for the various enzymes. For example, the carbon from which X migrates undergoes a net inversion of configuration in the diol dehydratase (Retey *et al.*, 1966b; Zagalak *et al.*, 1966) and glutamate mutase (Sprecher and Sprinson, 1964) reactions, while retention was observed with methylmalonyl coenzyme A mutase (Sprecher *et al.*, 1964; Retey and Zagalak, 1975) and racemization with ethanolamine deaminase (Retey *et al.*, 1974).

There has been no success in demonstrating enzyme-catalyzed exchange between reaction products and compounds thought to represent intermediates or their analogues.

Moreover, attempts to generate active holoenzyme by allowing apoenzyme to interact with 5'-deoxyadenosine and several cobalamin derivatives postulated to represent catalytic intermediates have also failed (Carty, 1973; Eager, 1974; Krouwer and Babior, 1977; Krouwer *et al.*, 1978). Figure 4 shows some postulated mechanisms for the migration of group X based on experiments done with model reactions; these studies have been reviewed elsehwere (Schrauzer, 1971, 1976; Brown, 1973; Abeles and Dolphin, 1976; Golding and Radom, 1976). More recently, Corey *et al.* (1977) suggested a mechanism involving oxidative addition and reductive elimination with substrate and cob(I)alamin in which the corrin ring undergoes electrocyclic cleavage; however, several features of the enzymatic reactions are inconsistant with this mechanism. Most notably is the failure of the Corey mechanism to account for the cob(II)alamin resonances observed in ESR experiments.

The final stages of the mechanism involve the transfer of hydrogen from the 5'-deoxyadenosine intermediate to the product-like molecule and subsequent release of product (except in the diol dehydratase reaction where 1,1-propanediol is stereoselectively dehydrated to propionaldehyde before release (Retey *et al.*, 1966a). The migration of hydrogen is known to occur without exchange with solvent (Brownstein and Abeles, 1961; Babior, 1969; Somack and Costilow, 1973; Erfle *et al.*, 1964; Iodice and Barker, 1963; Kung and Tsai,

1971; Wood *et al.*, 1964), and all the reactions exhibit primary isotope effects when deuterium (or tritium)-labeled substrates are used, indicating that C-H bond-breaking is a significant rate-contributing step (Miller and Richards, 1969; Eager *et al.*, 1972; Weisblat and Babior, 1971; Essenberg *et al.*, 1971; Moore *et al.*., 1979).

The path of hydrogen during catalysis has been extensively studied. Early observations with diol dehydratase (Brownstein and Abeles, 1961) showed that hydrogen removed from C-1 appeared at C-2 of the product; seemingly the rearrangement was strictly intramolecular. However, experiments done by Abeles and Zagalak (1966) and Frey *et al.* (1967a) showed that when diol dehydratase holoenzyme was allowed to react with mixtures of [1-^{3}H]-1,2-propanediol and unlabeled ethylene glycol, tritium was found in both propionaldehyde and acetaldehyde on C-2. Furthermore, even in the limit of an infinite ethylene glycol to [1-^{3}H]-1,2-propanediol ratio, there was still a finite probability of tritium being found at C-2 of product propionaldehyde. They also demonstrated that tritium could be found on the 5'-carbon in adenosylcobalamin when catalysis with tritiated substrate was interrupted. Moreover, when synthetically prepared [5'-^{3}H]-adenosylcobalamin was mixed with diol dehydratase and unlabeled substrate, all of the tritium was transferred to C-2 of product. Other experiments (Retey and Arigoni, 1966; Frey *et al.*, 1967b; Miller and Richards, 1969; Switzer *et al.*, 1969; Babior, 1970; Eager

et al., 1972; Babior *et al.*, 1973; Moore *et al.*, 1979) showed that hydrogen abstracted from substrate becomes one of three equivalent hydrogens attached to C-5' of the cofactor. Conceivably, stereochemical equivalence of the three hydrogens could be achieved by rapid rotation of the C-5' methyl group once it is formed. Proton nuclear magnetic resonance experiments carried out on nucleosides bound to ethanolamine deaminase (Hull *et al.*, 1975) indicate that rotation about the C-4', C-5' bond is indeed several orders of magnitude faster than the enzymatic reaction.

While the role of the coenzyme is fairly well understood, the role of the protein is more obscure. All of the adenosylcobalamin-dependent enzymes seem to have sulfhydryl groups which are important to catalysis (Babior, 1975), and recently Kuno *et al.* (1980) showed that diol dehydratase apoenzyme could be inactivated by two arginine-specific reagents, 2,3-butanedione and phenylglyoxal, but the role played in catalysis by these residues is not understood. Moreover, the postulated mechanisms for the migration of group X (Silverman and Dolphin, 1976; Golding and Radom, 1976; Eager *et al.*, 1972; Halpern, 1974) (Figure 4) make no provision for possible effects of these residues. Presently no satisfactory picture of the rearrangements or the role of the protein in catalysis has arisen from model reactions studied thus far.

Chapter I of this thesis describes an efficient procedure for isolating pure diol dehydratase from its bacterial source. Existing procedures (Abeles, 1966; Poznanskaya *et al.*, 1979) give low yields and produce an enzyme which has undergone significant proteolysis. The improvement will greatly assist studies of the role of the protein in catalysis. Indeed, Chapters I and II deal with characterization of native diol dehydratase and of diol dehydratase which has been inactivated by substrate analogues. Results in Chapter III, which deals with isotope effects during catalysis, show that protein conformational changes are also important during catalysis. This work provides a base for future mechanistic and structural studies on diol dehydratase and other B_{12}-dependent enzymes.

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Figure 1. (a) Derivatives of cobalamin. R = CH_2CONH_2, R' = $CH_2CH_2CONH_2$

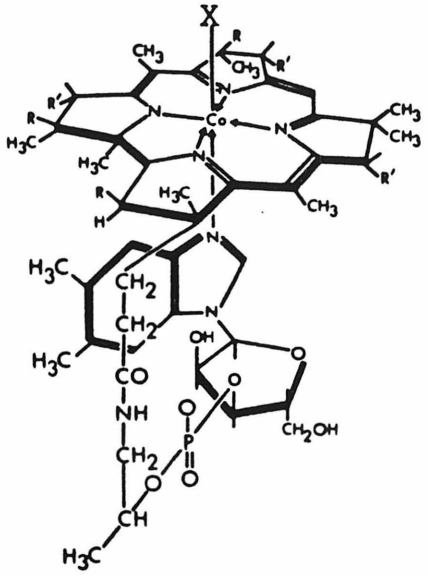
X = OH (hydroxycobalamin)

CN (cyanocobalamin)

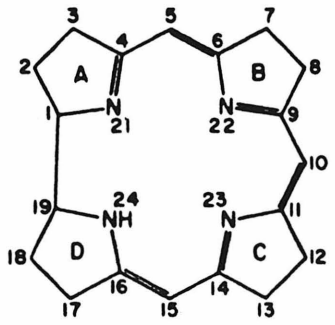
CH_3 (methylcobalamin)

5'-deoxy-5'adenosyl (adenosylcobalamin)

(b) Corrin. Letters A-D identify the individual pyrroline rings. The numbering system is the same as for porphyrin; hence the number 20 has been omitted (Hogenkamp, 1975).



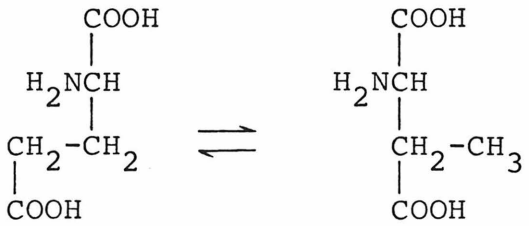
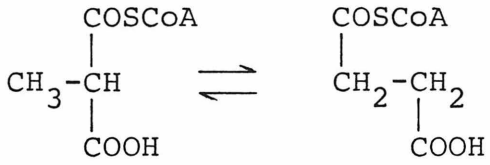
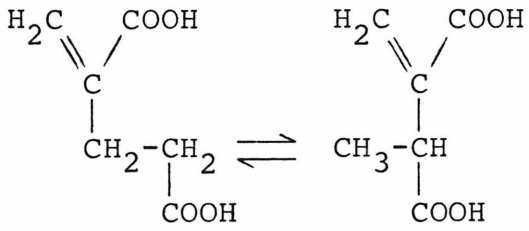
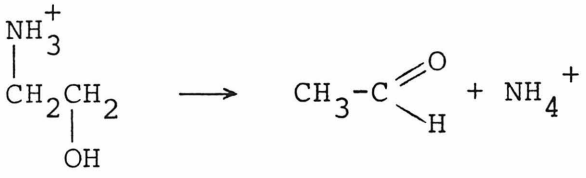
(a)

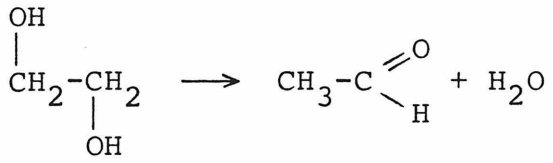
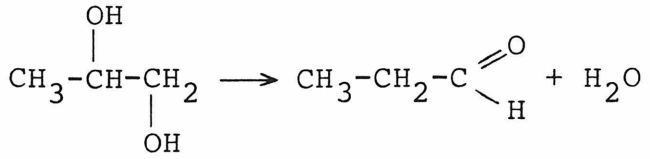
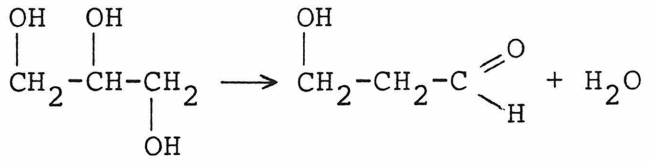
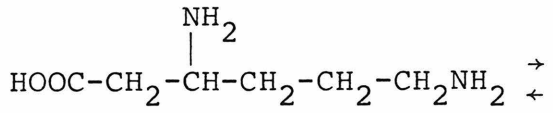
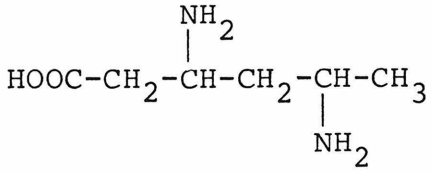
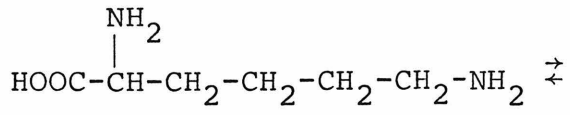
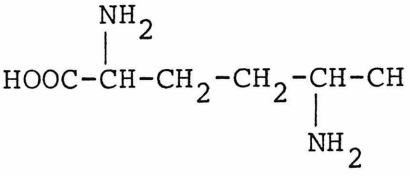


(b)

Figure 1

Figure 2. Adenosylcobalamin-Dependent Reactions. A "†" denotes enzymes that have been found in mammalian tissues.

Enzyme	Reaction
1. Glutamate mutase	
2. Methylmalonyl CoA mutase+	
3. α-Methyleneglutarate mutase	
4. Ethanolamine deaminase	

Enzyme	Reaction
5. Diol dehydratase	
6. Glycerol dehydratase	
	
7. l-β-Lysine mutase	
	
8. d-α-Lysine mutase	
	

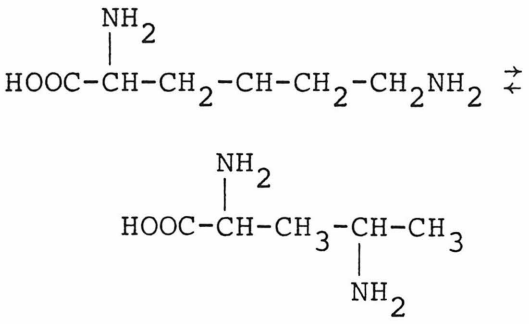
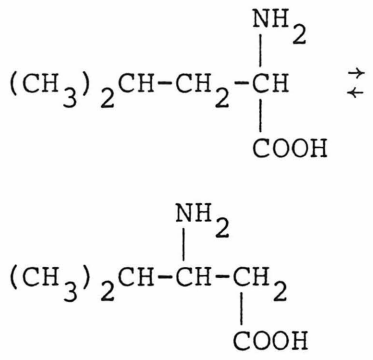
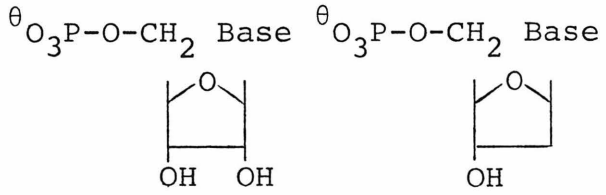
Enzyme	Reaction
9. d-Ornithine mutase	
10. Leucine 2,3-Aminomutase (Poston, 1976; 1977). †	
11. Ribonucleotide reductase	

Figure 3. Mechanism of adenosylcobalamin-dependent rearrangements. Adenosylcobalamin is represented schematically as $\begin{matrix} CH_2Ad \\ | \\ >Co< \end{matrix}$, substrate as SH, and product as PH.

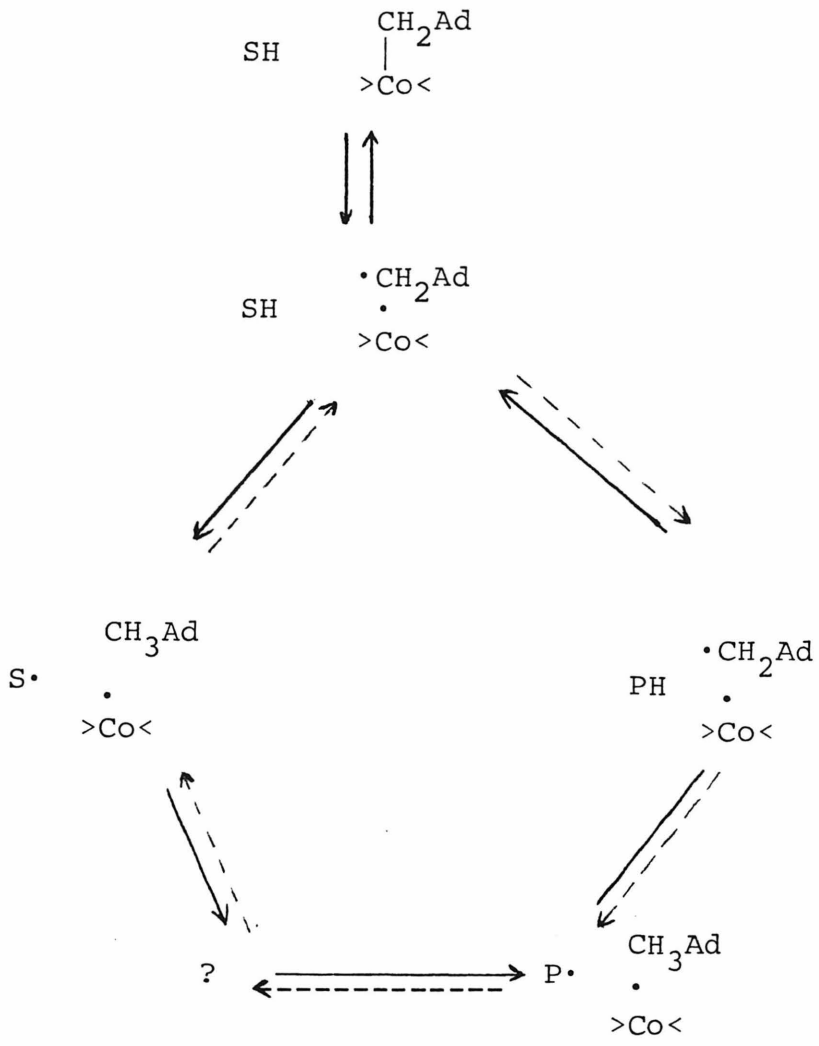


Figure 3

Figure 4. Proposed mechanism for Migration of Group X.

1. Silverman and Dolphin (1976)
2. Golding and Radom (1976)
3. Eager *et al.* (1972); Halpern (1974)

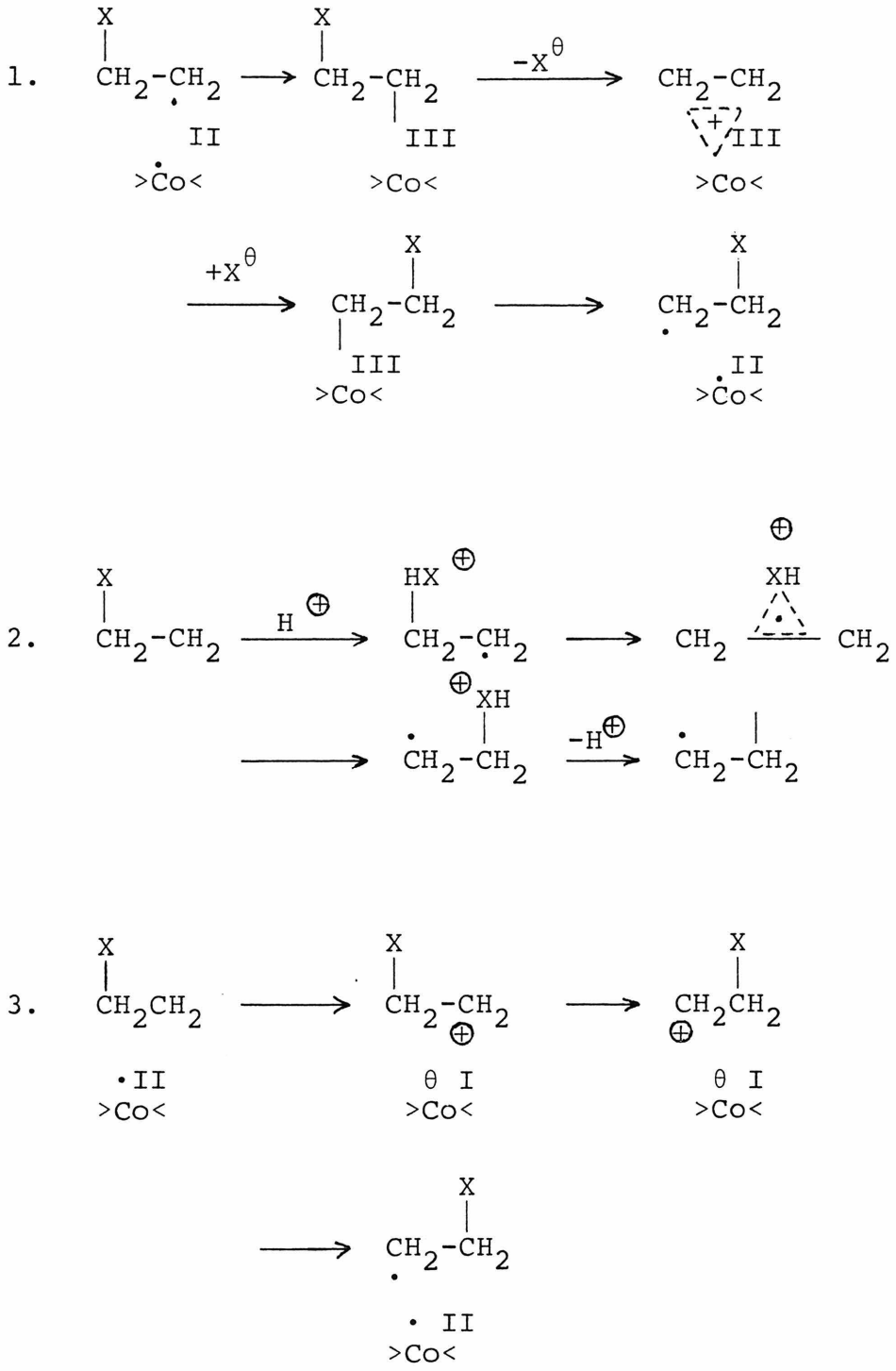


Figure 4

CHAPTER I

Purification, Subunit Stoichiometry and Characterization of Propanediol Dehydratase, a Membrane-Associated Enzyme

Introduction

Diol dehydratase[(RS)-1,2-propanediol hydrolyase; EC 4.2.1.28] from Klebsiella pneumoniae (ATCC 8724) is one of a large group of enzymes which utilize adenosylcobalamin (coenzyme B_{12}) as a specific cofactor and catalyze a variety of molecular rearrangements. In the case of diol dehydratase, the principal reaction is the rearrangement of 1,2-propanediol to 1,1-propanediol and its stereoselective dehydration to propionaldehyde (Zagalak, *et al.*, 1966; Retey *et al.*, 1966). The role of the adenosylcobalamin cofactor in the catalytic mechanism is fairly well understood (Abeles and Zagalak, 1966; Frey and Abeles, 1966; Moore *et al.*, 1979); the role of the protein in catalysis (Bachovchin *et al.*, 1977; Babior, 1979) remains obscure.

An efficient procedure for isolating the enzyme in pure form and in high yield from its bacterial source would greatly assist studies on the role of the protein in catalysis; existing procedures (Abeles, 1966; Poznanskaya *et al.*, 1979) do not give especially high yields and produce enzymes which seem to have undergone significant proteolysis during isolation. Additionally, having in hand pure, unproteolysed enzyme would facilitate further characterization of the protein and the individual subunits. Although adenosylcobalamin-dependent enzymes occur ubiquitously in nature (Babior, 1975), only the amino acid composition for

ribonucleotide reductase (Pangou *et al.*, 1972) has been reported.

Knowledge of the amino acid composition of the various subunits can be helpful in understanding their functions; for example, the sensitivity of diol dehydratase to organo-mercurials and other sulfhydryl reagents (Lee and Abeles, 1963; Toraya and Fukui, 1972; Toraya *et al.*, 1972) suggests that the subunit(s) containing cysteines may be intimately involved in catalysis. Knowledge of the amino-terminal sequence of the different subunits will reveal any sequence hologies among the subunits which have been observed in other multi-subunit protein complexes (Raftery *et al.*, 1980). Finally, comparison of the amino acid composition obtained in the individual steps of an N-terminal-sequence determination carried out on the multi-subunited enzyme allows determination of the relative subunit stoichiometry of the functional complex.

Experimental

Bacteria. *Klebsiella pneumoniae* (ATCC 8724) were grown in the presence of glycerol and 1,2-propanediol according to the procedure of Lee and Abeles (1962). Autolyzed brewer's yeast used in the growth medium was obtained from Amber Laboratories, Juneau, Wisconsin. The bacteria were harvested by using a Beckman J-21C continuous flow centrifuge equipped with a JCF-Z rotor. The pelleted cells

were weighed and washed with 0.01 M tris(hydroxymethyl)aminomethane (Tris) (5 mL/g of wet cells) and pelleted by centrifugation in a GSA rotor for one hour at 10,000 rpm. The cells were then resuspended in deionized, distilled water and lyophilized for 4 days.

Isolation of Diol Dehydratase. The lyophilized cells (5-15 g) were suspended in 10 mL/g of 10 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) adjusted to pH 8.2-8.4 with 40% potassium hydroxide. The cells were then sonicated between 5 and 15°C for a total time of 1 min/g of lyophilized cells by using one-minute bursts (240 watts) from a Brausonic 1510 sonicator, and allowing 3 minutes between each burst.

After centrifugation of the sonicated suspension at 100000 xg for one hour in a Beckman L2-75B ultracentrifuge, the supernatant was decanted and discarded. The pellet was then suspended in about 100 mL of 1% Triton X-100, 1% potassium cholate, 1 mM ethylenediamine tetraacetic acid (EDTA), and 0.1 mM PMSF, pH 8.8. Occasionally it was necessary to sonicate this suspension with 15 minute bursts (240 watts) for a total time of 5 min/g of lyophilized cells (an ice-methanol bath was used to keep the temperature below 15°C); at other times the activity could be solubilized simply by stirring the suspension for 10-15 min in the cold. After centrifugation of the suspension at 100000xg for one hour, the detergent extract was decanted and saved.

The pellet was again treated with detergents as above (no further sonication is necessary) and the centrifugation was repeated. The pooled detergent extracts were then concentrated to 50 mL with an Amicon Diaflo apparatus on a PM-30 membrane or by dialysis against 20% glycerol, 0.01 M K_2HPO_4, pH 8.0. The concentrated sample was then applied to a 5 x 81 cm Sepharose 6B gel filtration column equilibrated with 10% glycerol, 5% 1,2-propanediol, and 0.01 M K_2HPO_4, pH 8.0 (buffer G).

Fractions comprising the major activity peak (approximately 500 K daltons) of the gel filtration column were pooled (see Figure 1) and applied to a 2.5 x 15 cm Cellex-D (Bio-Rad) ion exchange column equilibrated with buffer G. After the sample was loaded, the column was washed with two bed columes of buffer G, and then a linear gradient (0-100% DBSGP) was used to elute diol dehydratase from the column, where DBSGP is a mixed-micelle detergent buffer containing 1% deoxycholate, 1% Brij 58 (Sigma), 0.1% sodium dodecyl sulfate (SDS), 0.05 M Tris, 0.02% sodium azide, and 1 mM EDTA in buffer G, pH 8.0. The mixing flask contained 250 mL of buffer G to which was added 250 mL of DBSGP. In this manner, one can elute diol dehydratase in essentially pure form; the pooled fractions have a specific activity of 95 $\pm$ 4 units/mg, as shown in Figure 2.

In order to remove substances which would interfere with the sequence analysis of "native" diol dehydratase, reagent grade acetone was added to a concentration of 75%.

The precipitate was pelleted by centrifugation for 25 min at 2800 rpm in a Sorvall GLC-2 centrifuge. The pellet was washed with acetone, dried with nitrogen and dissolved in 0.1% SDS (Bio-Rad electrophoresis grade, recrystallized from ethanol) and 0.05 M ammonium bicarbonate to yield a protein concentration of about 2 mg/mL. This sample was frozen at -70°C until ready for use.

Assays. Propanediol dehydratase activity was assayed by using yeast alcohol dehydrogenase (Sigma) and NADH (Sigma) to reduce propionaldehyde to 1-propanol (Bachovchin *et al.*, 1977). The rate of production of propionaldehyde by diol dehydratase was measured by monitoring the decrease of absorbance at 340 mm due to oxidation of NADH to NAD in the presence of excess alcohol dehydrogenase. Reaction volumes were between 2.0 and 2.5 mL, and the reaction was initiated by the addition of 30 µL of a 2 mg/mL solution of adenosylcobalamin (Sigma) in the dark). Assays were carried out on a Beckman Acta CIII. One unit of activity is defined as the amount of enzyme which will decompose 1 µmol of 1,2-propanediol per min at saturating concentrations.

Protein concentrations were determined by the method of Lowry *et al.* (1951). Bovine serum albumin was used as a standard for the protein assay, and a buffer blank was used for a zero-protein reference because the detergents present interfere with the Lowry assay.

Preparation of Diol Dehydratase Subunits. Purified diol dehydratase was heated for 5 min in gel sample buffer (15 mg/mL DTT), 3% SDS, and approximately 5 mg was electrophoresed according to Laemmli (1970) on a preparative polyacrylamide gel slab (15 x 11 x 0.6 cm), with the separating gel being 12% acrylamide and 0.32% bis(acrylamide). Electrophoresis was done at 20 mA for 18 h, after which the gel was stained in 0.25% Coomassie Brilliant Blue, 25% ethanol, and 8% acetic acid for 5-10 min. The bands were visualized over a light box, and the subunits were cut from the gel. The protein was removed from the gel by electroelution into a dialysis bag in 0.19 M glycine, 0.025 M Tris, and 0.1% SDS, pH 8.5, for 18 h. The protein solutions were dialyzed against several changes of 0.15 M sodium chloride and 0.2% SDS and then against 0.01% SDS in distilled, deionized water for 48 hours, after which they were lyophilized and aliquots were reeleectrophoresed to confirm their purity (Figure 7).

Amino Acid Analysis. The lyophilized protein was taken up in a known volume of glass-distilled deionized water and was frozen at -70°C until ready for use. Protein samples containing approximately 0.2 nmol of peptide were hydrolyzed *in vacuo* with 6N HCL for 12, 24, 48, or 72 hours or with 3 M mercaptoethanesulfonic acid for 24 or 48 hours at 106°C. After hydrolysis, the samples (still *in vacuo*) were frozen at -70°C until the analyses could be performed. Analyses were done on a Durrum D-500 amino acid analyzer.

Cysteine was determined after performic acid oxidation (Hirs, 1967). No corrections were necessary for amino acids present in buffers (determined by analyzing unhydrolyzed protein).

N-Terminal Sequence Analysis. Automated Edman degradations of the subunits were performed by Dr. M.W. Hunkapiller, Dr. M.W. Bond, or Paul Cartier with a spinning cup sequenator designed and constructed at Caltech (Hunkapiller and Hood, 1978), using a previously described computer program (Hunkapiller and Hood, 1978). Phenylthiohydantoin (Pth) derivatives of amino acids were identified by high-performance liquid chromatography (HPLC) on a Du Pont Zorbax CN column, using the procedures and standard chromatograms described elsewhere (Johnson *et al.*, 1979; Hunkapiller and Hood, 1980).

Determination of Subunit Stoichiometry. The protein sequenator was allowed to proceed through several cycles on a sample of "native" diol dehydratase. The relative amounts of amino acids released in each cycle were determined.

Each HPLC sample contained dansylglutamine as an internal standard as did a standard sample containing 250 pmol of each Pth-amino acid. Background levels of Pth-amino acids were determined in the cycle proceding the cycle of interest and subtracted to give the actual nanomolar yield. The actual yield was then corrected for repetitive yield (96% per cycle) and for pTH-amino acid recovery percentages (Hunkapiller and Hood, 1978).

Results

A number of factors influences the amount of diol dehydratase which is solubilized by sonication of the bacteria, including the length and spacing of the bursts, the pH of the suspension, and the lyophilization of the cells after harvesting. In contrast, the ionic strength of the sonicate suspension does not seem critical; the same amount of enzyme was solubilized under given conditions of pH and length of bursts at ionic strengths of 0.6 and 0.02 M. Under the conditions described, up to 93% of the diol dehydratase activity initially present in the bacteria could be pelleted by centrifugation of the sonicate suspension at 100000 x g for 1 hour. By thusly separating the diol dehydratase from proteins in the cytosol, one obtains approximately six-fold purification.

Three different methods could be used to solubilize diol dehydratase from the sonicate pellet: (i) the pellet can be resuspended and resonicated continuously for 20-30 minutes (this requires efficient cooling if thermal denaturation is to be avoided); (ii) The pellet can be extracted with detergents as described; (iii) the pellet can be washed many times with pH 9.6 buffers containing no detergents. These results suggest that diol dehydratase may be associated with the cell membrane.

Diol dehydratase as purified by this procedure contains no carbohydrate. Thus, an SDS-polyacrylamide gel of the

enzyme shows no staining using the periodic acid-Schiff reaction (Fairbanks *et al.*, 1971) to detect carbohydrate. (In this test for carbohydrate, ascites was used as a positive reference and bovine serum albumin as a negative reference.) Moreover, as shown in Table V, Asn-2 of the 51 K subunit is not glycosylated, as demonstrated by the normal yield of Pth-asparagine in cycle 2, even though the sequence Asn-X-Ser is present, which often (but not always) acts as a recognition sequence for such modification in many organisms (Sharon and Lis, 1981).

Diol dehydratase does not seem to require lipids as phospholipids for catalytic activity. Thus, extraction of the enzyme from the pellet with Triton X-100 and passage through a Sepharose 6B column preequilibrated with buffer G containing 2% Triton X-100 produced enzyme with undiminished activity (Jacobs *et al.*, 1966; Ne'eman *et al.*, 1972).

Treatment of the crude extracts with charcoal (Poznanskaya *et al.*, 1979) was unnecessary as no more than 5% of the protein was present as active holoenzyme. Accordingly, further purification of the detergent extracts involved gel filtration, which separates the larger diol dehydratase from smaller proteolytic enzymes. Omission of this step produces diol dehydratase contaminated with a very small amount of protease, which causes notable decomposition of the diol dehydratase within four weeks (see Figure 3). For the prevention of proteolysis of the

enzyme after extraction from the membrane, EDTA and PMSF were included in the detergent solutions; PMSF inhibits serine proteases such as trypsin and chymotrypsin (Fahrney and Gold, 1963), and EDTA inhibits such proteases as thermolysin which require divalent metal ions for activity (Matsubara, 1970).

In the absence of DBSGF, diol dehydratase has an unusually high affinity for Cellex-D; however, less than 5% of the activity was eluted by washing the column with 1 M potassium chloride in buffer G. This activity probably represents proteolyzed derivatives of the native enzyme.

Table I compares this new isolation procedure with previous ones. The new procedure yields about 16 times the enzyme obtained by Poznanskaya *et al.* (1979) and 44 times that reported by Abeles (1963). Figure 4 shows polyacrylamide gels of the enzyme at each step of the purification. While earlier procedures invariably yield enzyme that has been proteolysed, the same four subunits which constitute the enzyme obtained by the new procedure are observed throughout the isolation.

Molecular Weight and Subunits. Previously reported molecular weights for diol dehydratase vary from 230 K (Poznanskaya *et al.*, 1977;1979) to 250 K (Essenberg *et al.*, 1971). In the presence of DBSGP (which is similar to the detergent buffer used by Collett and Erickson (1978) to prevent protein aggregation), gel filtration on Sepharose 6B gives a relatively symmetrical elution peak

corresponding to a molecular weight of 250 K (Figure 5). In absence of DBSGP, the enzymatic activity elutes in a less symmetrical peak corresponding to an approximate molecular weight of 500 K (Figure 1). (Figure 1 also shows a smaller shoulder of activity with a molecular weight of about 80 K; due to its small amount, further studies of this apparent activity have not been undertaken.)

SDS-polyacrylamide gel electrophoresis of diol dehydratase shows four types of subunits of 60 K, 51 K, 29 K, and 15 K daltons. This composition differs somewhat from that previously reported (Poznanskaya *et al.*, 1979) of four subunits of 60 K, 23 K, 15.5 K, and 14 K daltons in "fraction S" and a fifth subunit of 26 K daltons in "fraction F", which is reported to stain poorly with Coomassie Brilliant Blue (Poznanskaya *et al.*, 1977). Figure 3 and 6 suggest that their polypeptides of 25.5 K, 23 K, and 14 K daltons may be products of proteolysis of the native subunits.

Amino Acid Analysis of Diol Dehydratase and Its Subunits. Table II collects the results of the amino acid analyses of the diol dehydratase subunits. Mole percent values were corrected for the degradation of serine and threonine and for the slow hydrolysis of valine, leucine, and isoleucine as described in the Table. Maximum values for tryptophan were taken from hydrolyses done in 3 M

mercaptoethanesulfonic acid. The other values are the averages of six determinations with standard deviations being around $\pm 3.0\%$.

The discriminating function (Z) for diol dehydratase and its constituent subunits was calculated from the amino acid data by the method of Barrantes (1975) where

$$Z = (-0.345)\ \frac{\Sigma\ (\text{charged residues})}{\Sigma\ (\text{hydrophobic residues})}\ +$$

$$H\Phi_{av}\ (6 \times 10^{-4})$$

The average hydrophobicity ($H\Phi_{av}$) (Bigelow, 1967) was determined using the relative hydrophobicities of amino acids reported by Tanford (1962). Table III shows the results of these calculations for diol dehydratase and other proteins.

Table IV shows the N-terminal sequences for the subunits of diol dehydratase. Figure 8 shows the sequence information for the 15 K, 29 K, and 51 K subunits presented in a way to demonstrate their sequence homologies.

As mentioned, Table V shows the yield of Pth-amino acids for the 51 K subunit for cycles 1, 2, 3, and 5.

Table VI shows the nanomolar yields from the various subunits when "native" diol dehydratase was sequenced. The average ratios of the subunits from two different sequenator runs are shown in Table VII. The corrections for repetitive yield and percent recovery do not affect the

stoichiometry obtained, for one gets the same result by comparing just the ratios of the Pth-amino acids in cycles 6 or 10 (where corrections for repetitive yield are unnecessary since all comparisons are made within the same cycle). Furthermore, residues selected for comparison have recovery percentages of at least 85% (Hunkapiller and Hood, 1978) making this correction relatively small. The results from Table VII suggest a minimal molecular weight of 259 K daltons for diol dehydratase, which is in agreement with the gel filtration data.

Discussion

Previous procedures for the purification of diol dehydratase (Abeles, 1966; Poznanskaya *et al.*, 1979) were developed with the tacit assumption that diol dehydratase was a cytosol enzyme, which seemed to be true, because enzymatic activity could be solubilized by rupturing the bacteria with mechanical processes. However, Table I shows that no more than 25% of the total diol dehydratase activity was solubilized by their procedures. The remaining activity can, however, be solubilized by the use of detergents and/or additional sonication which is characteristic of a membrane-associated enzyme. Accordingly, we developed conditions of sonication which allowed separation of diol dehydratase activity from the cytosol components of the cell. By these procedures, as little as 7% of the total enzymatic activity was solubilized when the bacteria were

ruptured. The insoluble components were then separated from the cytosol by centrifugation, and the bulk of the activity was liberated upon detergent treatment of the insoluble fraction. This procedure not only allows recovery of more activity (by more than an order of magnitude) than previously possible, but also produces an initial preparation of higher purity so that fewer subsequent purification steps were necessary to obtain homogenous enzyme. Moreover, by preventing proteolysis during the isolation by inhibition of serine proteases with PMSF and proteases requiring divalent metal ions with EDTA, the purified enzyme has only four different kinds of subunits (60 K, 51 K, 29 K, and 15 K) instead of five (60 K, 25.5 K, 23 K, 15.5 K, and 14 K) as previously reported (Poznanskaya *et al.*, 1979). Furthermore, the enzyme isolated by our procedure has a slightly higher specific activity than that obtained in earlier preparations, indicating, especially in light of Figures 3 and 4, that the new isolation procedure yields enzyme that more closely reflects the structure of native diol dehydratase.

The different amino acid compositions and N-terminal sequences of the four types of subunits show that the smaller subunits are unlikely to be proteolytic derivatives of the larger ones. Though the available sequence data is relatively limited, some homologies are apparent among the 51 K, 29 K, and 15 K subunits. The 15 K and 29 K subunits

both have a repeating unit (Ala-Pro-X-Ala) twice in the first 40 residues; the 51 K subunit contains a structurally similar unit (Gly-Pro-Gly-Gly). Additional homologies are delineated in Figure 8. In contrast, the 60 K subunit shows no apparent homologies with the other three subunits.

Two general classes of proteins associated with membranes have been termed "extrinsic" and "intrinsic" (Singer, 1971). In many cases, an unambiguous assignment to one of these two limiting categories is not possible (Vanderkoai, 1974). Diol dehydratase lacks at least two properties often characteristic of intrinsic membrane proteins, it apparently does not require phospholipids for catalytic activity and lacks carbohydrate. Indeed, even though the 51 K subunit contains a recognition sequence (Asn-X-Ser, residues 2-4) for glycosylation in many organisms (Sharon and Lis, 1981), the normal yield of Pth-Asn in cycle 2 (Table V) demonstrates the absence of glycosylation, for such a modification should significantly lower the yield of Pth-Asn in that cycle. On the other hand, the enzyme, as isolated by the procedure described herein, has a relatively low solubility (<<10 mg/mL) and aggregates in the absence of mixed micellular detergents; such properties often characterize intrinsic membrane proteins (Vanderkooi, 1974). The amount of diol dehydratase solubilized during sonication seems insensitive to the ionic strength of the aqueous phase, cytochrome-c, an extrinsic protein which is associated with

the membrane surface primarily through electrostatic interactions is solubilized to an appreciably greater extent at higher ionic strength (Jacobs and Sandai, 1960). Increasing the pH of the sonicate suspension increases the amount of diol dehydratase which is solubilized; the enzyme can also be removed by nondetergent buffers. All these observations taken together suggest that the enzyme is similar to other bacterial membrane proteins which, though essentially extrinsic, nevertheless have some interaction with the hydrophobic region of the lipid bilayer (Panefsky and Tzagoloff, 1971). Specific analogues might be the cytochrome c oxidase system (Poyton and Schatz, 1975a) or the protein which confers oligomycin sensitivity to the ATPase complex (MacLennon and Tzagoloff, 1968).

The average hydrophobicity values for diol dehydratase and its subunits (Table III) and a more sensitive comparison based on values for the discrimination function (Barrantes, 1975) are in agreement with the proposal that diol dehydratase is an extrinsic membrane protein which has limited contact with the lipid bilayer. The discrimination function for cytochrome c oxidase from Baker's yeast (Poyton and Schatz, 1975a) correlates interestingly with the availability of the subunits to labelling reagents. In cytochrome c oxidase, subunit 1 has a discrimination function typical of integral membrane proteins and is considerably more nonpolar than the other subunits.

Lactoperoxidase iodination, which labels those regions of peptides exposed to an aqueous environment, shows that subunit 1 of cytochrome c oxidase is almost completely inaccessible while subunit 2 was only partially accessible for protein modification. In contrast, the polar subunits 4 and 6 were readily modified (Poyton and Schatz, 1975b). In diol dehydratase, the 60 K and 29 K subunits have discrimination function values similar to that of subunit 2 of cytochrome c oxidase and are more hydrophobic than the 51 K and 15 K subunits; these observations suggest that either, or both, of the 60 K and 29 K subunits may serve to anchor the enzyme to the membrane.

Until removal of Triton X-100, the specific activity of the enzyme could not be accurately determined; the aromatic ring of this detergent interferes with the protein assay of Lowry *et al.* (1951). Chromatography on Cellex-D produced a symmetrical absorbance band which eluted at low ionic strength and at the beginning of the detergent gradient (Figure 2, fractions 75-90). This band contained no protein (by SDS gel electrophoresis) and had a UV spectrum similar to that of Triton X-100 in the same buffer. These facts suggest that this band is due to a nonionic detergent (Triton X-100) which has been displaced from the diol dehydratase by another detergent (Tanford and Reynolds, 1976).

In the absence of detergent, diol dehydratase has a molecular weight of about 500 K. This is similar to the molecular weight of ethanolamine deaminase (Kaplan and Stadtmen, 1968) which has two active sites per molecule (Babior and Li, 1969; Babior 1969). Interestingly, the enzyme isolated by Poznanskaya *et al.* (1979) does not aggregate in the absence of detergents and has a significantly higher solubility than the enzyme isolated by the procedure described herein. Proteolytic removal of hydrophobic regions could account for these properties of the diol dehydratase obtained earlier.

Tables VI and VII show that the subunit stoichiometry is $(60K)_2$ $(51K)_1$ $(29K)_2$ and $(15K)_2$ for a molecular weight of 259 K per active site which agrees well with gel filtration data in the presence of DBSGP (Figure 5). This composition can be compared with a previous report (Toraya *et al.*, 1973) showing that two protein components are necessary to bind adenosylcobalamin; one of these components (S) is sensitive to sulfhydryl reagents while the other (F) is not. Component S has a molecular weight of 200 K daltons and is, itself, composed of four different subunits of 60 K, 23 K, 15.5 K and 14 K daltons; component F has a molecular weight of 26 K daltons (Poznanskaya, *et al.*, 1979). One can attain the molecular weight of 200 K daltons for component S by two 60 K and two 23 K subunits in addition to two 15.5 K and one 14 K, or one 15.5 K and two

14 K subunits. A single 26 K subunit of component F would then account for the total observed molecular weight of about 230 K which they observed. This suggests that the 51 K subunit, of which there is only one copy per active site, gives rise, by proteolysis, to component F and either the 15.5 K or 14 K subunit of component S. Further support for this relationship between the 51 K subunit and component F comes from the way these stain with Coomassie Brilliant Blue; both component F (Poznanskaya *et al.*, 1979) and the 51 K subunit (Figures 6 and 7) stain poorly relative to the other subunits, which may, in part, reflect the subunit stoichiometry as well, for only one mole of the 51 K subunit is present for two moles of each of the other subunits. Further support for the possibility that components F and S are generated by proteolysis comes from our inability to separate the enzyme isolated by our procedure into these two components.

The 60 K peptide seems always to be associated with the diol dehydratase complex; in our work, it appears in every preparation of active enzyme and, moreover, has an amino acid composition which can account for the results of Poznanskaya *et al.* (1979) and Lee and Abeles (1963). For example, the amino acid composition indicates that this subunit contains three cysteins. Since the subunits are not held together by disulfide bonds (Poznanskaya *et al.*, 1979

and unpublished results), one cysteine, at least, of the 60 K peptide should be available for the reaction with alkylation of a free sulfhydryl (Toraya *et al.*, 1974). Furthermore, studies carried out by Carroll *et al.* (unpublished results) have shown that diol dehydratase can in fact be inactivated by alkylation of the 60 K subunit with organic mercurials.

The single 51 K subunit per active site of diol dehydratase suggests a particularly important role for the 51 K subunit in the binding of adenosylcobalamin and catalysis. For example, there is only one coenzyme binding site per 250 K daltons (Essenberg *et al.*, 1971) and incorporation of a single molecule of 2,3-butadione is sufficient to inactivate the holoenzyme (Kuno et al., 1980).

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Table 1. Purification of Diol Dehydratase

Purification Step	Protein (mg)	Activity (u)	Specific Activity (u/mg)	Yield (%)
Method #1				
Crude extract	226	54	0.24	100
Protamine sulfate	154	41	0.27	77
30% ammonium sulfate	1.19	15.4	13	28
52% K_2HPO_4	0.28	13.3	47	24
Cellex-D chromatography	0.19	11.3	60	21
Method #2				
Crude extract	350	266	0.76	100
Protamine sulfate	176	223	1.27	84
40% ammonium sulfate	70	162	2.31	61
First DEAE-cellulose column	18.9	114	6.05	43
Hydroxylapatite chromatography	5.6	82	14.8	31
Sepharose 6B chromatography	2.9	69	23.7	26
Second DEAE-cellulose column	0.52	47.8	91.5	18
Method #3				
Sonicated cells	350	1058[a]	3.02	100
Detergent extract of membrane	57.6	980	17	93
Sepharose 6B chromatography	31.8[c]	889	28[c]	84
Cellex-D chromatography	8.3	802	95	76

[a]This is the maximum amount of activity that could be solubilized by extended periods of sonication with efficient cooling. [b]The amount of activity in the detergent extracts was determined by the difference between the amount of

activity solubilized by carefully rupturing the cells and the total activity known to be present in the cells. [c]These values are uncertain due to the interfence of Triton X-100 with the protein assay.

This table summarizes the three main procedures used in purifying diol dehydratase. Method #1 is essentially that of Abeles (1963), method #2 was used by Poznanskaya *et al.* (1979), and method #3 is described in this report. In order to make meaningful comparisons the values reported have been normalized to one gram, lyophilized weight, of bacteria (this corresponds to approximately five trams of wet cells). Activity is reported in units (u) which were previously defined.

Table II. Amino Acid Composition of Diol Dehydratase

Amino Acid	Native Diol Dehydratase (mol%)[a]	Subunits (mol%)				Subunits (residues/subunit[b]			
		60K	51K	29K	15K	60K	51K	29K	15K
Asx	10.3	11.4	9.7	7.5	12.8	63	47	20	18
Thr[c]	5.0	4.8	5.9	4.5	5.8	26	28	12	8
Ser[c]	6.3	6.1	8.2	6.7	6.5	34	40	18	9
Glx	12.2	11.5	11.2	14.5	11.0	64	54	40	16
Pro	5.4	5.5	5.2	7.1	5.1	30	25	19	7
Gly	9.5	9.1	12.6	9.6	5.6	50	61	26	8
Ala	10.7	10.5	10.2	9.9	14.2	58	49	27	20
Cys	0.3	0.5	0.3	0.0	0.0	3	2	0	0
Val	6.7	7.8	5.5	6.2	5.3	43	27	17	8
Met	2.6	3.7	1.8	1.1	2.5	20	9	3	4
Ile[d]	5.2	5.1	5.0	7.1	3.8	28	24	19	5
Leu[d]	8.0	7.0	8.6	8.3	9.4	39	41	23	14
Tyr	2.4	2.7	2.0	2.0	2.6	15	10	6	4
Phe	2.9	3.2	3.0	2.9	1.9	18	14	8	3
His	1.5	1.4	1.8	1.7	0.9	8	9	5	1
Lys	4.9	4.4	4.8	5.9	5.7	24	23	16	8
Arg	5.9	4.9	4.1	4.6	6.6	27	20	13	10
Trp[d]	0.7	0.4	0.6	1.0	1.3	2	3	3	2

[a]Calculated from the subunit stoichiometry and the compositions of the individual subunits. [b]Calculated on the basis of the given molecular weights and mole percentages and founded to the nearest integral value. [c]Mole percent values were obtained by linear extrapolation to zero time from values obtained after 12, 24, 48, and 72 hours of hydrolysis. [d]Maximum value taken.

Table III. Hydrophobicities and Discrimination Function Values for Various Proteins[a]

Protein	$H\Phi_{av}$(cal)	Z
Diol Dehydratase[b]	1024	0.21
60K subunit	1022	0.22
51K subunit	961	0.16
29K subunit	1091	0.24
15K subunit	1026	0.16
Cytochrome c oxidase[c]	1185	0.45
subunit 1	1185	0.51
subunit 2	1011	0.25
subunit 4	1033	0.16
subunit 6	1029	0.14
Membrane proteins[d]	1197 $\pm$ 98	0.51 $\pm$ 0.11
Globular proteins[d]	996 $\pm$ 98	0.16 $\pm$ 0.11

[a]Average hydrophobicities (H_{av}) are calculated from published amino acid data (Bigelow, 1967), and reported in calories. Discriminant function values (Z) are computed as described by Barrantes. [b]This study. [c]Poyton and Schatz, 1975a. [d]Barrantes, 1975.

Table IV. N-Terminal Sequences for the Diol Dehydratase Subunits[a]

Subunit	5	10	15	20	25	30	35	40
60K	M R S K R F E A L A K R P V N Q D G F V K E W I E E G F I A M E S P N D X X(K)X							
51K	M N T S E L E T L I R T I L S E G L T P G S T P V G P G G K G I F G(S)V P E A I							
29K	M E I N E K L L R Q I I E D V L S E M K G S D K P V S F N A P A A S A A P Q A S							
15K	M N T D A I E S M V R D V L S R M N S L Q G E A P A A A P A A G G A S R S A R V							

[a]Parentheses ( ) denote uncertainty of a specific residue. The letter "X" signifies an undetermined residue.

Table V. Pth-Amino Acid Yields for the 51K Subunit[a]

Cycle	Amino Acid	Actual Yield (rel. units)[b]	% recovery[c]	Corrected Yield[d]
1	Met	1.17	90 ± 5	1.3 ± 0.1
2	Asn	0.88	90 ± 5	1.1 ± 0.1
3	Thr	0.16	12 ± 5	1.4 ± 0.4
5	Glu	1.07	95 ± 5	1.3 ± 0.1

[a]Values obtained from the HPLC data on the corresponding sequenator cycles. [b]Peak areas were normalized to HPLC standards containing equimolar amounts of Pth-amino acids. [c]Hunkapiller and Hood, 1978. [d]Yields were corrected for repetitive yield and % recovery. These samples did not contain internal standards so it was not possible to correct the differences in injection volumes; these differences should be less than 10%.

Table VI. Sequenator Yields of Pth-Amino Acids from Native Diol Dehydratase

Subunit	Cycle	Amino Acid	n mole[a]	n mole[b]	Average
60K	4	Lys	0.46	0.57	0.573 ± 0.015
	6	Phe	0.43	0.56	
	8	Ala	0.42	0.59	
	10	Ala	0.37	0.57	
51K	6	Leu	0.21	0.28	0.285 ± 0.013
	10	Ile	0.19	0.27	
	16	Glu	0.15	0.30	
	18	Leu	0.14	0.29	
29K	3	Ile	0.49	0.57	0.568 ± 0.041
	6	Lys	0.39	0.52	
	10	Gln	0.35	0.62	
	14	Asp	0.30	0.56	
15K	4	Asp	0.41	0.51	0.558 ± 0.036
	6	Ile	0.44	0.58	
	9	Met	0.34	0.55	
	10	Val	0.38	0.59	

[a]Corrected for background. [b]Corrected for % recovery and repetitive yield (Hunkapiller and Hood, 1978).

Table VII. Subunit Stoichiometry of Diol Dehydratase[a]

Subunit	Subunits/monomer	
	Run #1[b]	Run #2
60K	2.01 ± 0.05	1.98 ± 0.29
51K	1.00 ± 0.05	1.00 ± 0.17
29K	1.99 ± 0.14	2.00 ± 0.27
15K	1.96 ± 0.13	1.95 ± 0.21

[a]Determined from two samples of diol dehydratase as described in Methods. [b]Calculated from data shown in Table V.

Figure 1. Activity-elution profile from a 5 x 81 cm Sepharose 6-B column equilibrated with buffer G. Circles denote absorbance and triangles denote activity. The K_{av} of the major activity peak was 0.30 which corresponds to a molecular weight of 500,000 daltons. The minor activity peak has a K_{av} of 0.55 corresponding to a molecular weight of 80,000 daltons. Fractions 110-150 were pooled (Pool I) and purified further on Cellex-D, an SDS polyacrylamide gel of Pool I is shown in Figure 5, sample 5. Pool II (fractions 151-180) was not purified further; however, an SDS gel is shown in Figure 5, sample 6.

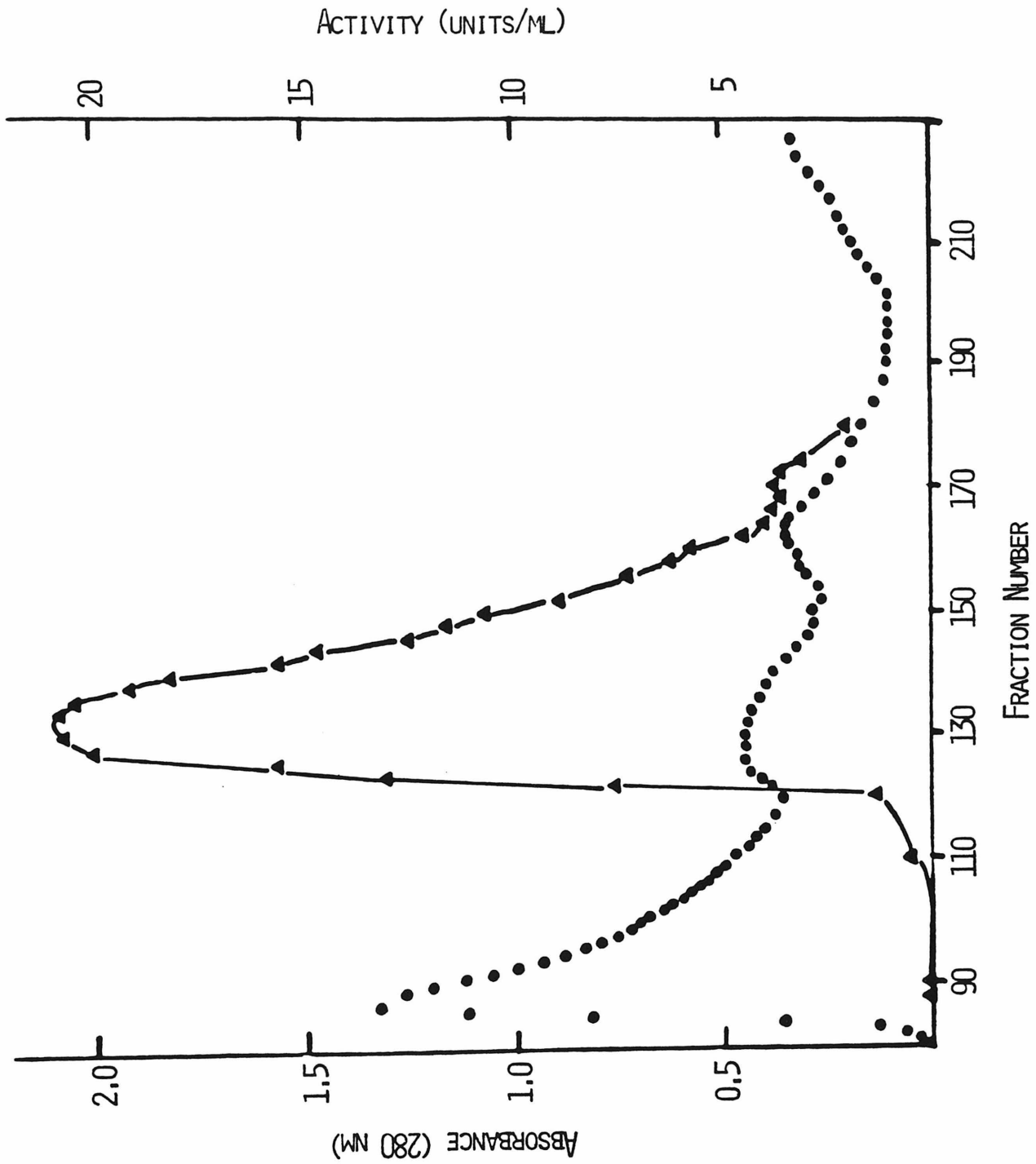

ACTIVITY (UNITS/ML)
20
15
10
5
2,0
1,5
1,0
0,5
ABSORBANCE (280 NM)
90
110
130
150
170
190
210
FRACTION NUMBER

Figure 1

Figure 2. Activity-elution profile from a 2.5 x 15 cm Cellex-D anion exchange column. Circles denote absorbance and triangles denote activity. Fractions were not collected until the buffer reservoir was switched to 1 M KCl in buffer G. The upper reservoir was switched to buffer G while fraction 41 was being collected, and the gradient was started while fraction 58 was being collected. The gradient flasks were nearly empty while fraction 136 was collected, so DBSGP was added to the upper reservoir to complete the elution. Fractions 136-170 were pooled together and the SDS gel of the enzyme is shown in Figure 5, sample 7.

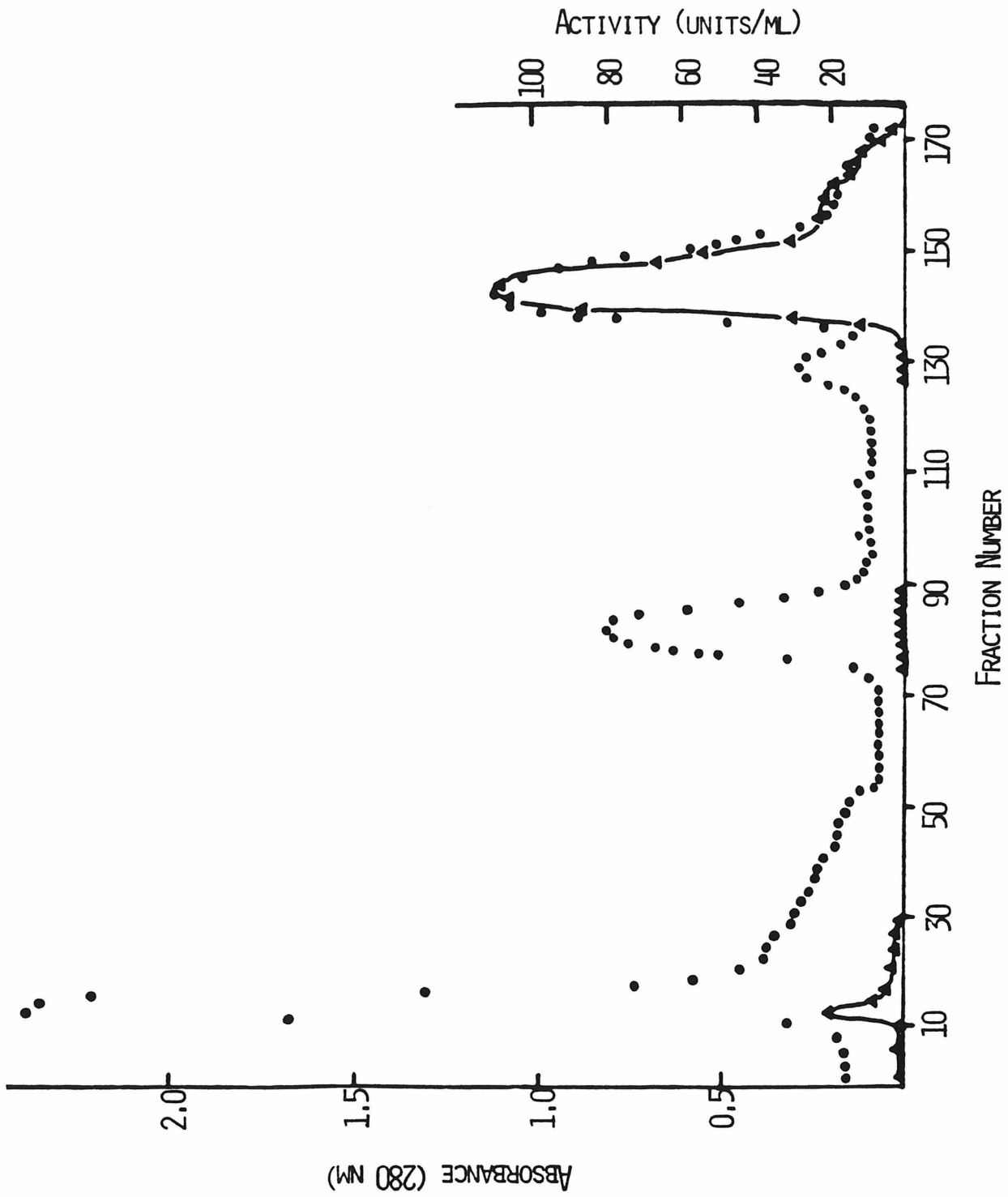

ACTIVITY (UNITS/ML)
100
80
60
40
20
170
150
130
110
90
70
50
30
10
FRACTION NUMBER
2.0
1.5
1.0
0.5
ABSORBANCE (280 NM)

Figure 2

Figure 3. Gel scans of diol dehydratase. The scan depicted by the solid line represents freshly purified diol dehydratase in which the Sepharose 6-B gel filtration was omitted; the lower scan is of the same sample four weeks later. The vertical axis measures the absorbance at 550 nm. Bromophenol Blue was the dye front marker. The approximate molecular weights of the bands in the proteolysed sample are 60,000, 51,000, 25,000, 23,000, 15,500 and 14,000 daltons. To improve resolution, another aliquot of the proteolysed sample was rerun on an SDS expotential gradient (8-18% acrylamide) gel, see Figure 7.

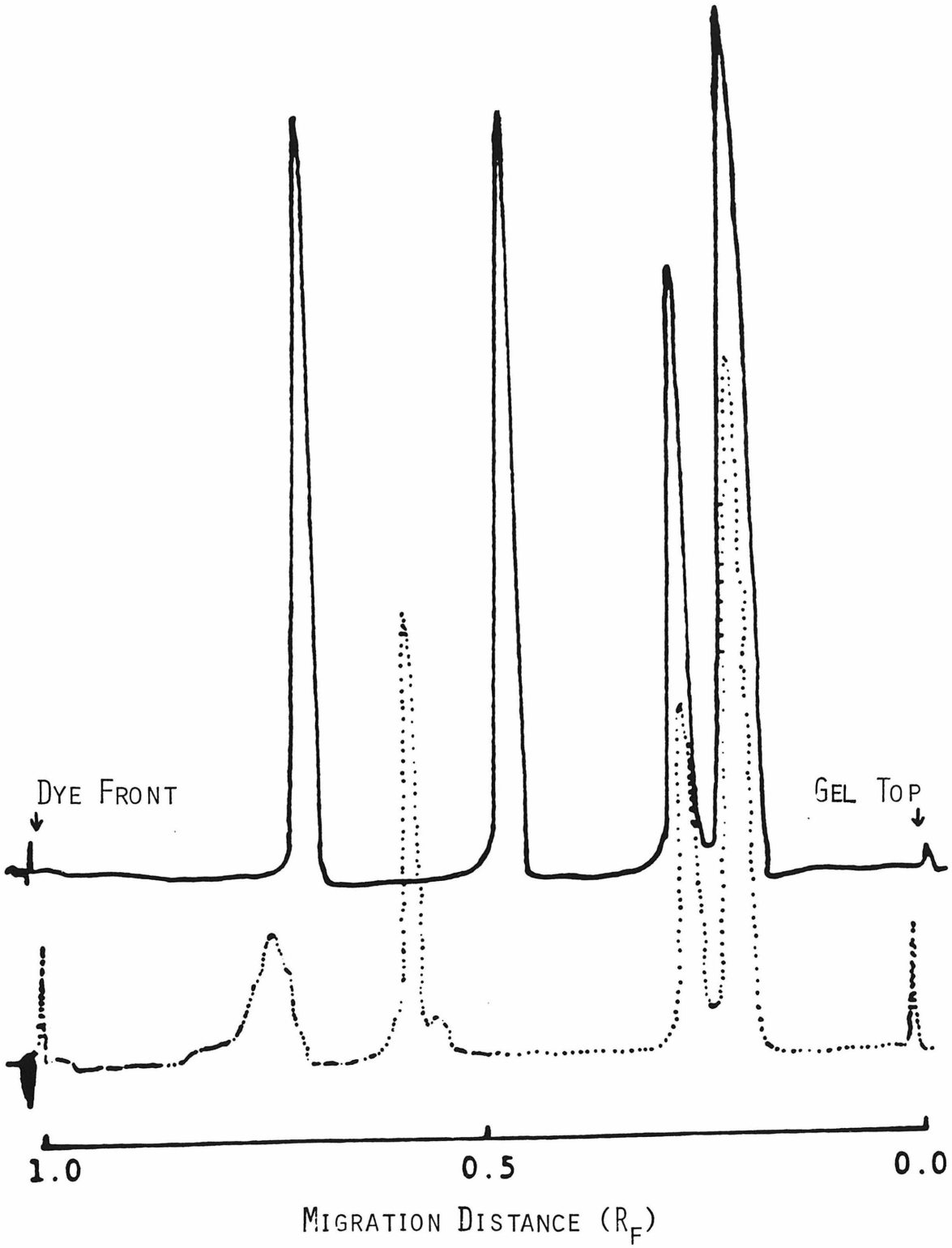


Figure 3

Figure 4. Activity-elution profile from a 5 x 81 cm Sepharose 6-B column equilibrated with DBSGP. Circles denote absorbance and triangles denote activity. The K_{av} of the activity peak was 0.39 which corresponds to a molecular weight of about 250,000 daltons. This sample came from a sonicate pellet that was extracted with DBSGP.

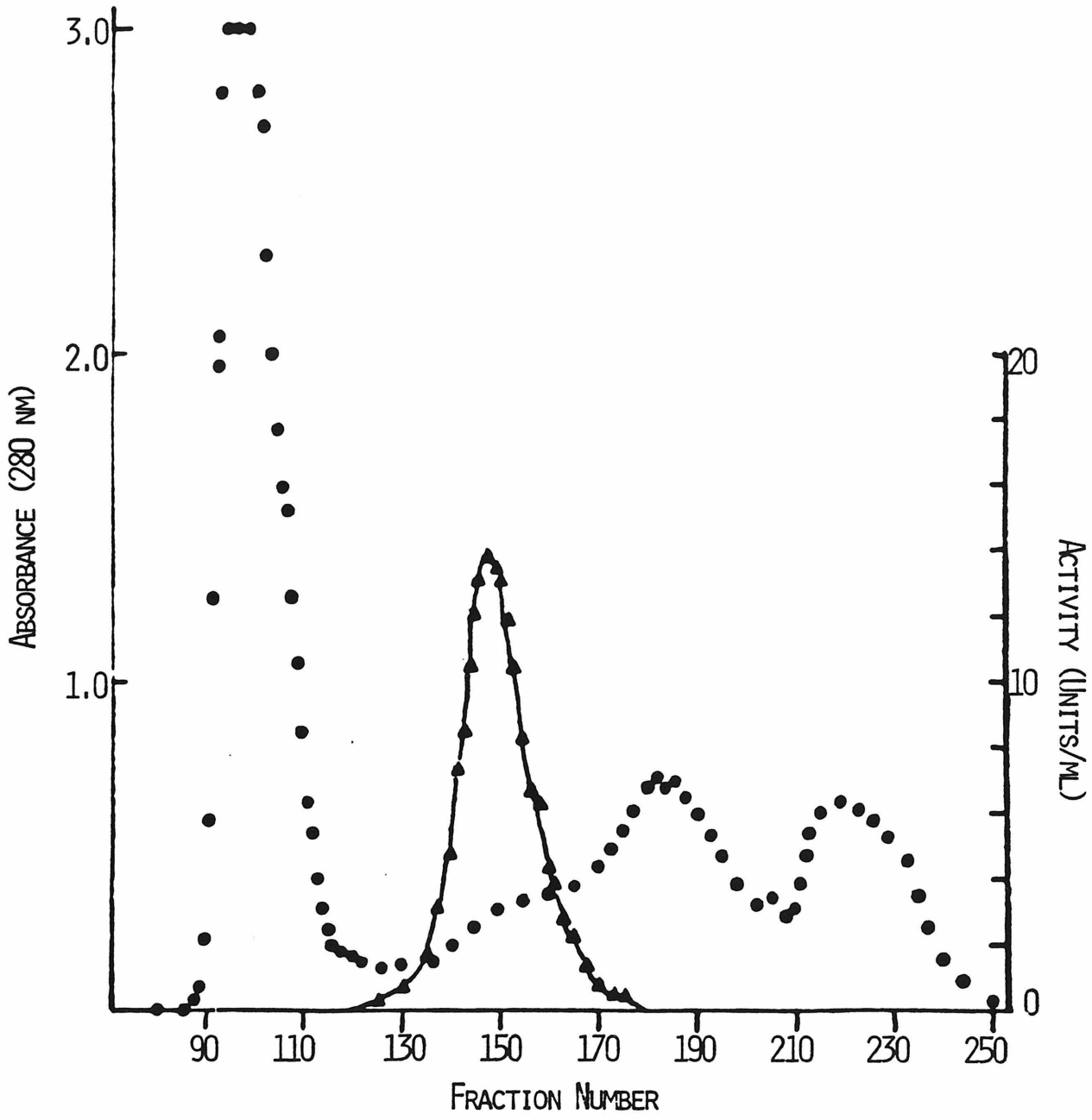

3.0
2.0
1.0
ABSORBANCE (280 NM)
20
10
0
ACTIVITY (UNITS/ML)
90
110
130
150
170
190
210
230
250
FRACTION NUMBER

Figure 4

Figure 5. SDS polyacrylamide slab gel (12% acrylamide). Sample 1, crude sonicate (supernatant of 25,000 x g spin); samples 2, 3, and 4, detergent extracts of the membrane; sample 5, Pool I (Figure 1); sample 6, Pool II (Figure 1); Sample 7, purified diol dehydrase, sample 8, diol dehydrase purified by the method of Abeles (1966). The dashed line depicts the migration of bromophenol blue.

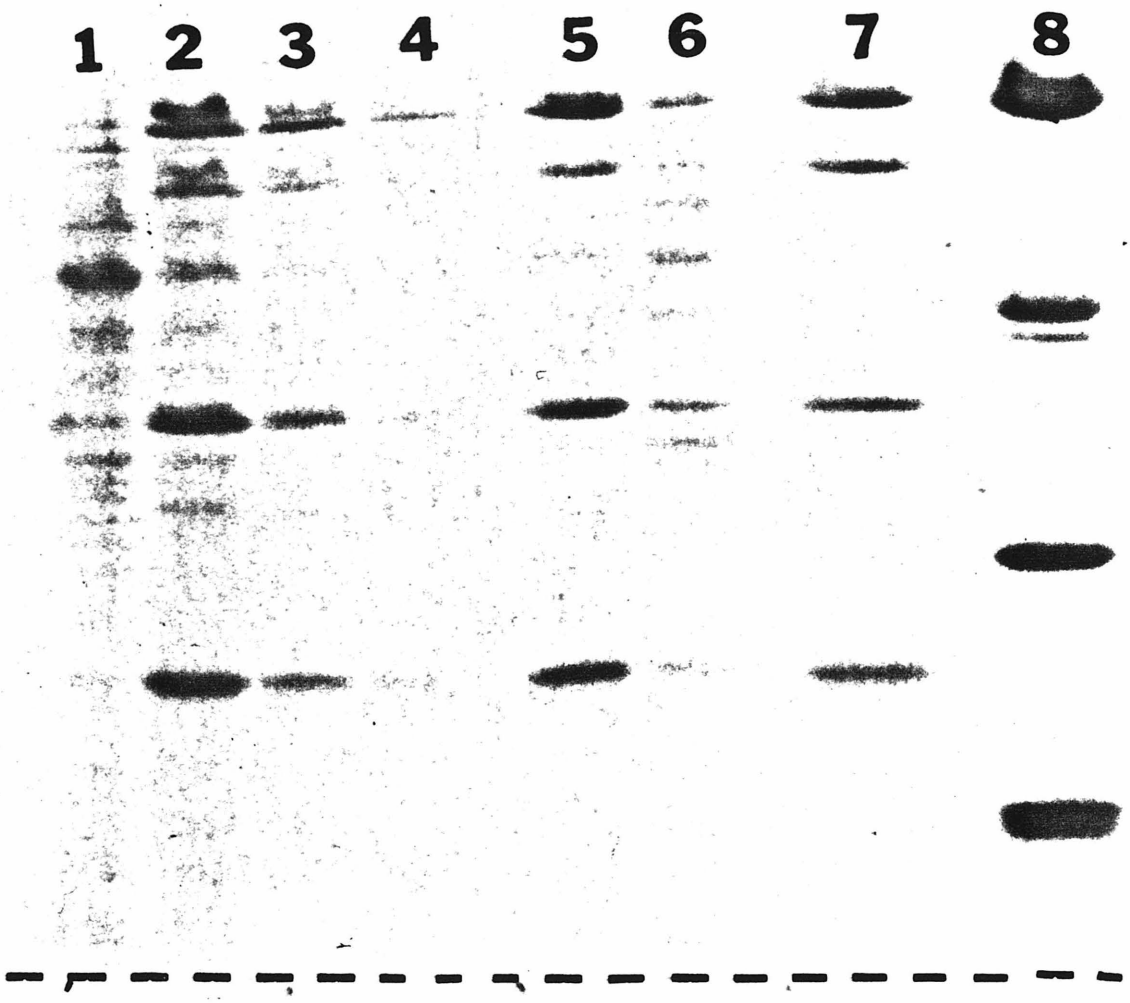


Figure 5

Figure 6. Gel scan of an SDS 8-18% polyacrlyamide expotential gradient slab gel. The sample is the same proteolysed enzyme that is shown in Figure 3. The scans at 280 and 550 nm demonstrate that 25,000 peptide does stain very well with Coomassie Brilliant Blue. The gradient also helps to resolve the 15,000 peptide from the 14,000 peptide.

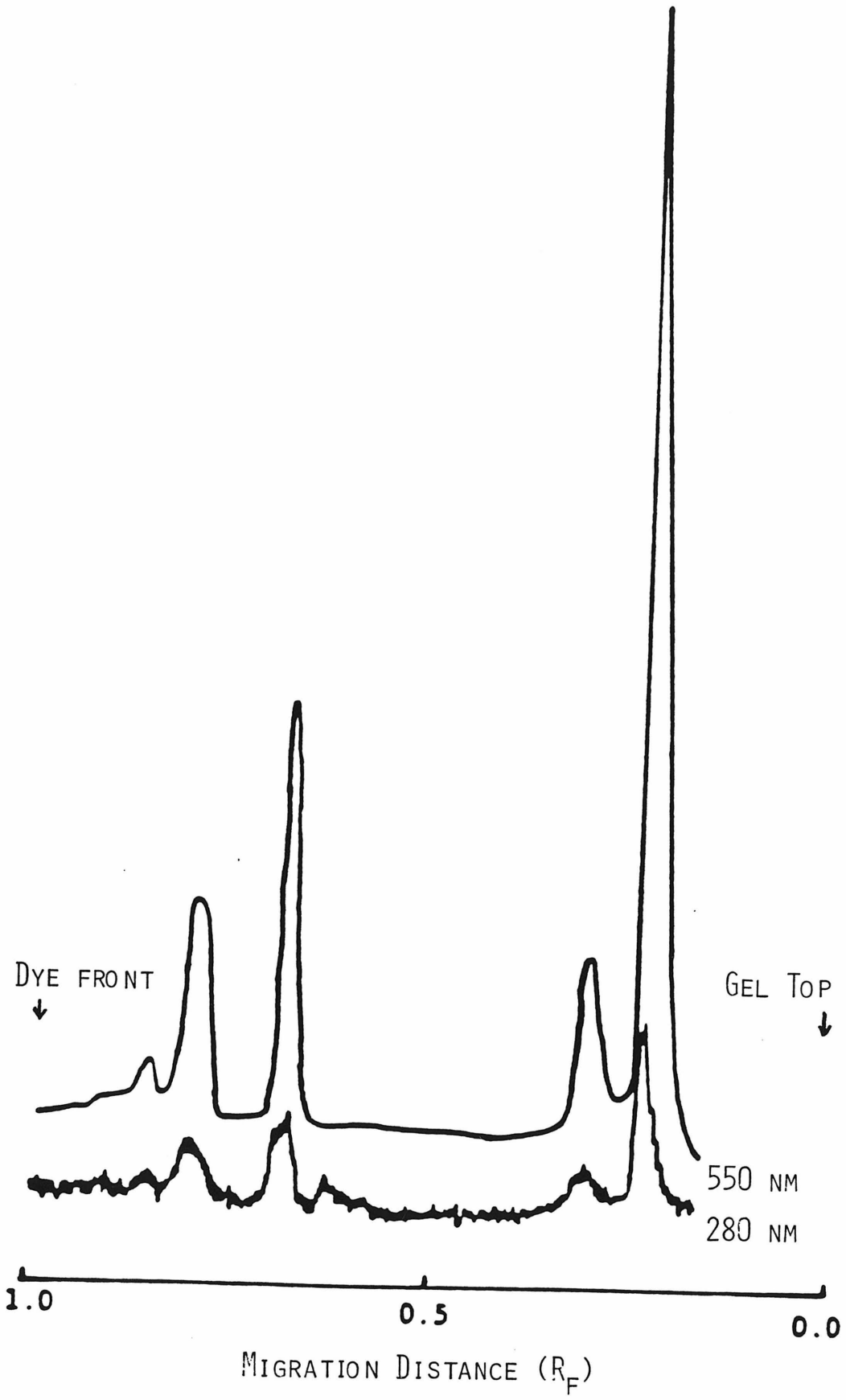


Figure 6

Figure 7. SDS polyacrylamide gel electrophoresis of diol dehydratase and its subunits. Samples (from left to right) are the 51K, 29K, 15K, 60K subunits, and native enzyme.

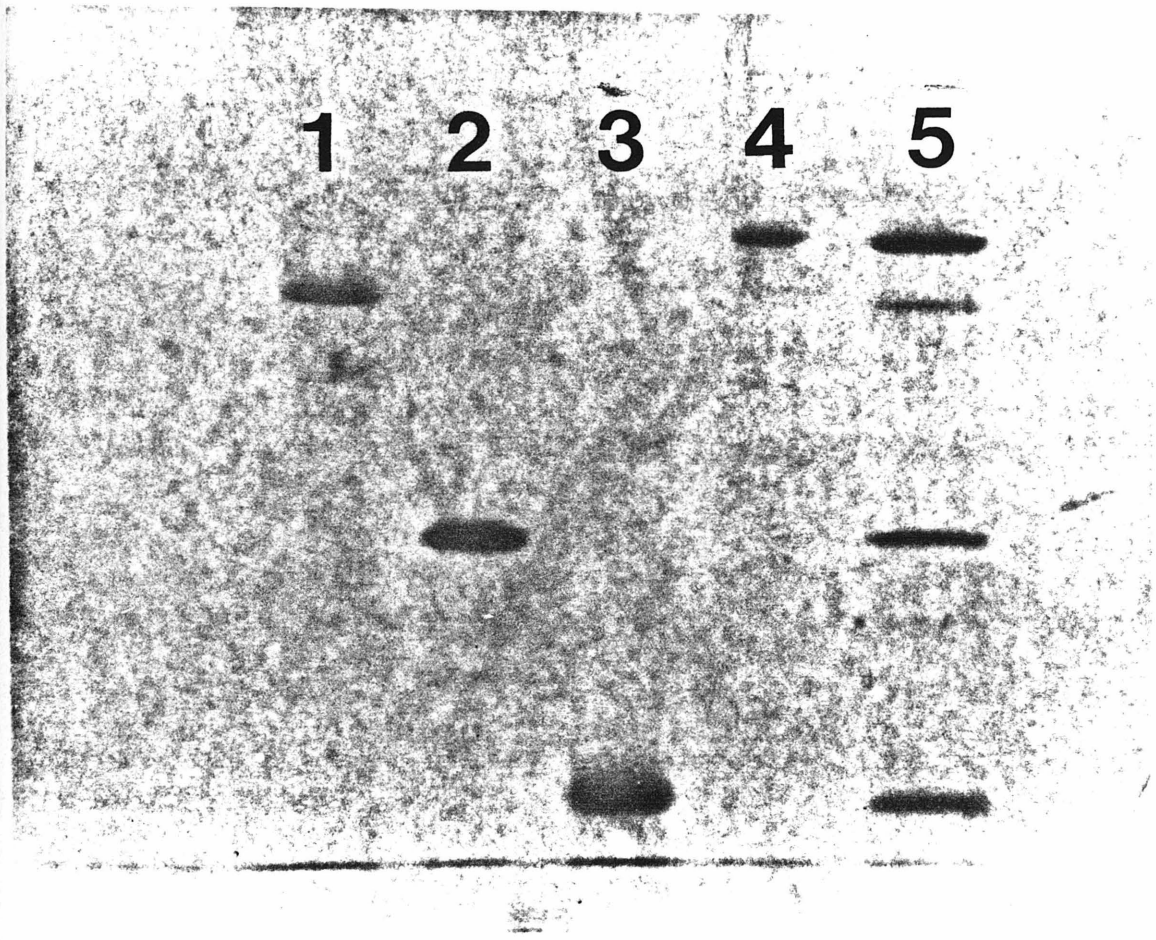
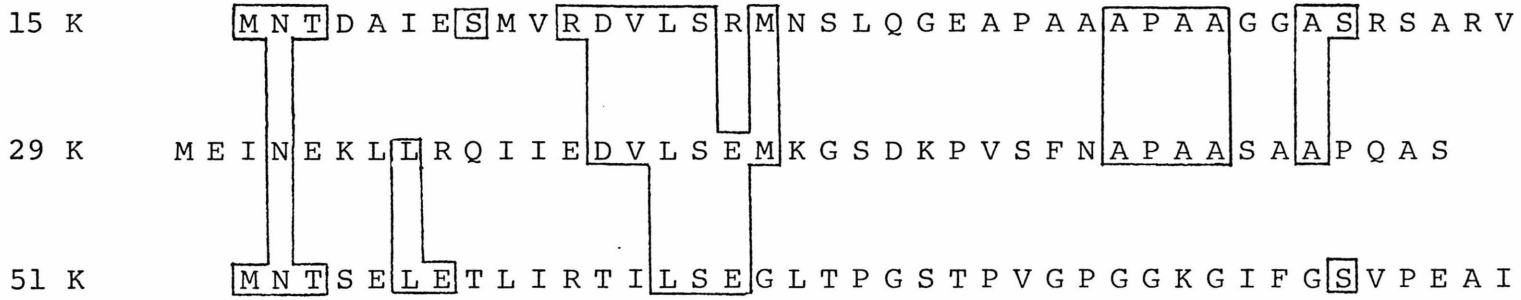


Figure 7

Figure 8. Comparison of sequences for 15K, 29K and 51K subunits. The 29K subunit is offset two residues to maximize homology. The boxed residues are the same in at least two of the subunits.

Figure 8


15 K M N T D A I E S M V R D V L S R M N S L Q G E A P A A A P A A G G A S R S A R V
29 K M E I N E K L L R Q I I E D V L S E M K G S D K P V S F N A P A A S A A P Q A S
51 K M N T S E L E T L I R T I L S E G L T P G S T P V G P G G K G I F G S V P E A I

CHAPTER II

Diol dehydratase catalyzes the conversion of both (R)- and (S)-1,2-propanediol to propionaldehyde (Retey *et al.*, 1966); however, seemingly minor structural changes of substrate-analogues result in the inhibition or destruction of catalytic activity. Eager *et al.* (1975) found that 3-fluoro-1,2-propanediol is a substrate for diol dehydratase, being converted to acrolein and hydrogen fluoride at a rate comparable to the reaction rate of 1,2-propanediol; however, Bachovchin *et al.* (1977) found that 3-chloro-1,2-propanediol, in addition to many other vicinal diols, was a fairly potent inactivator of the enzyme as well as being a substrate. Moore and Richards (1979) found that, while meso-2,3-butanediol was a substrate and inactivator of the enzyme, dl-2,3-butanediol was a purely competitive inhibitor which protected the holoenzyme from light and oxygen inactivation. Toraya and Fukui (1972) found that styrene glycol, although catalytically inert, was a weak competitive inhibitor. The inactivation reactions require both apoenzyme and coenzyme, and are independent of the presence of oxygen.

Toraya *et al.* (1976) and Bachovchin *et al.* (1977) reported that glycerol functions as both a substrate and as an inactivator of diol dehydratase. This inactivation reaction has the same requirements of apoenzyme and coenzyme as do those with other diol-inactivators, and neither the product of the reaction (β-hydroxypropionaldehyde), nor its

dehydration product (acrolein) are responsible for the inactivation (Bachovchin *et al.*, 1977; Eager *et al.*, 1975). The inactivation mechanism for glycerol is of particular interest, because glycerol is present in many biological environments (for example, glycerol induces the production of diol dehydratase in *Klebsiella pneumoniae* (Toraya *et al.*, 1978), and is structurally similar to 1,2-propanediol. Moreover, an immunochemically different adenosylcobalamin-dependent glycerol dehydratase (Toraya and Fukui, 1977) has been reported to be inactivated by its substrate, glycerol (Schneider and Pawelkiewicz, 1966). The inactivation is stereospecific, occurring as a result of glycerol binding in the "pro-(S)" configuration, with binding in the "pro-(R)" configuration leading to the product-forming reaction (Bachovchin *et al.*, 1977). This specificity may be metabolically significant in light of the results of Honda *et al.* (1980) which showed that the glycerol inactivation could be reversed in toluene-treated bacterial cells by the addition of ATP and Mn^{2+} or Mg^{2+} (but only in bacteria that were grown in a glycerol-containing medium). These results suggest that the stereospecific inactivation observed *in vitro* with pure diol dehydratase is either a destructive side reaction, for which the bacteria have a repair mechanism, or that a specific derivative of glycerol is produced *in vitro*, with an alkylated diol dehydratase being an intermediate in the biological pathway. In either case, the protein or cofactor might be

covalently modified in a specific fashion.

To determine if the protein or cofactor is covalently modified during the inactivation reaction, radiolabeled glycerol and isobutylene glycol were reacted with holoenzyme. A technique was developed to minimize non-specific labeling by glycerol and product aldehyde (Bachovchin *et al.*, 1977; Moore, 1979), and, when these precautions were taken, approximately one mole of glycerol was found to be strongly associated with the inactivated holoenzyme complex. Unfortunately, these techniques were not as effective in controlling the stoichiometry of enzyme-bound inactivator for the isobutylene glycol-dependent inactivation.

As described in Chapter I, early isolation procedures (Abeles, 1966; Poznanskaya *et al.*, 1979) yielded enzyme which had been proteolysed. For this reason some of the inactivation studies of Bachovchin *et al.* (1977) and Moore (1979) were repeated to see what effects (if any) proteolysis of the enzyme has on the inactivation reactions. Additionally, inactivation studies with 2-d-glycerol, 1,1,3,3-d_4-glycerol, 1,1-d_2-isobutylene glycol, d_6-isobutylene glycol, and a new class of inactivators, hydroxyacetone and dihydroxyacetone were carried out.

Experimental

Enzyme Preparations. Diol dehydratase was obtained as described in Chapter I. Enzyme was prepared for inactivation kinetics experiments by continuous-flow dialysis against 2 mM dl-2,3-butanediol (Moore, 1979). The presence of this competitive inhibitor (or substrates) in the dialysis buffer prevents irreversible denaturation of the apoenzyme by the detergents present in DBSGP (the detergent buffer used to elute diol dehydratase from the final column in the purification procedure, see Chapter I). The dl-2,3-butanediol and enzyme concentrations were such that the concentration of the dl-butanediol in a given inactivation assay was only about 2 μM. Since the Michaelis constant of dl-2,3-butanediol is about 100 μM (Moore, 1979), the observed inactivation values will not be measurably affected.

Assays. Activity assays were carried out as described in Chapter I. Inactivation rates were determined using the method of Bachovchin *et al.* (1977). Reaction mixtures contained NADH (0.2 mM), alcohol dehydrogenase (15 units), and K_2HPO_4 (20 mM), pH, 8.0 in a total volume of 2.0 mL. The reaction mixture was incubated with stirring at 37°C for 15 min, then the inactivation reaction was initiated by adding 0.03 mL of an adenosylcobalamin solution (2 mg/mL) and stopped by adding 0.05 or 0.10 mL of 7 M 1,2-propanediol. Enzymatic activity (determined by the relative rates of propionaldehyde production) was measured at four different inactivation times; the slope

of the resulting semilog plot (equation 1) represents the observed inactivation rate constant ($k_{i,obsd}$) for the given concentration of inactivator.

Protein concentrations were determined using the Lowry assay (Lowry *et al.*, 1951) as described in Chapter I.

Radioactive samples were counted in Aquasol-2 on a Beckman LS-250 scintillation spectrometer.

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

[1,3-^{14}C]-Glycerol and [2-^{3}H]-glycerol were purchased from ICN.

(R)-, (S)-, and (RS)-1,1-dideuterioglycerol (d_2-glycerol) were obtained as previously described (Moore, 1979).

1,1,3,3-Tetradeuterioglycerol (d_4-glycerol) was prepared by reducing dimethyltartronate with $LiAlD_4$ (99%-d, KOR Isotopes) using the workup procedure of Gidez and Karnovsky (1952). Dimethyl tartronate was prepared by refluxing tartronic acid (Sigma) in a methanolic suspension of Dowex 50W-X8 (H^+ form) followed by distillation of the product.

2-Deuterioglycerol (2-d-glycerol) was synthetized by the reduction of dihydroxyacetone (Sigma) with $NaBD_4$ (Sigma) by the procedure of Aasen *et al.* (1970) and purified by vacuum distillation.

[^{14}C]-Isobutylene glycol was prepared by stirring glycolide (Sporzynski *et al.*, 1949) in an ethereal solution

of [^{14}C]-CH_3MgI overnight. The Grignard reagent was prepared from [^{14}C]-methyl iodide (New England Nuclear). After quenching the reaction with a 10% aqueous solution of ammonium chloride, the solution was extracted three times with six volumes of ether. After drying over anhydrous magnesium sulfate, the pooled ether extracts were evaporated at room temperature on a rotary evaporator. The residue was isobutylene glycol which was about 90% pure (by proton magnetic resonance) and had a specific activity of 1.74×10^7 cpm/mmol. The product was not purified further.

1,1-Dideuterioisobutylene glycol (d_2-isobutylene glycol) was prepared by the reduction of methyl 2-hydroxyisobutyrate (Aldrich) with $LiAlD_4$. The product was purified by vacuum distillation.

2-Trideuteriomethyl-3,3,3-trideuterio-1,2-propanediol (d_6-isobutylene glycol) was prepared by reacting CD_3MgI (from CD_3I obtained from Sigma) with acetylated n-butyl glycolate. The n-butyl glycolate was synthesized according to the procedure of Kuznetsov *et al.* (1964) and was acetylated by the addition of excess acetyl chloride to the hydroxyester. The acetylated n-butyl glycolate was purified by fractional distillation under vacuum using a thermally controlled column (10 cm) packed with glass helices. The d_6-isobutylene glycol obtained from the Grignard reaction was purified by vacuum distillation and characterized by proton and ^{13}C nuclear magnetic resonance.

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

Labeling of Diol-Dehydratrase by Radioactive Inactivator. Freshly isolated diol dehydratase (200 units) was dialysed against 0.01 M $(NH_4)_2HPO_4$, 10 mM 1,2-propanediol, pH 8.0 to remove glycerol from the protein solution. The specific catalytic activity of the protein was then determined to allow correction for any denaturation which might have occurred. To this solution was added 0.2 mL of 7 M 1,2-propanediol and the mixture was incubated at 37°C for 10 min. Then excess adenosylcobalamin (1-6 mg) was added in the dark with stirring. After 30 sec, potassium borohydride (0.1 g) was added, and 30 sec later, the solution was made 60 mM in dl-2,3-butanediol. After 10 min, sufficient [^{14}C]-glycerol, [2-^{3}H]-glycerol, or [^{14}C]-isobutylene glycol to completely inactivate the holoenzyme was added. Within one hour greater than 96% of the activity was gone. Since dl-2,3-butanediol protects diol dehydratase from oxygen inactivation (Moore and Richards, 1979; Chapter III results), and KBH_4 does not affect the enzymatic activity (Essenberg *et al.*, 1971), the inactivation should have been due solely to the glycerol or isobutylene glycol present. The reaction was allowed to proceed overnight to ensure complete inactivation.

The reaction mixture was quantitatively transferred to a dialysis bag and dialysed in the dark versus three changes

of 0.01% SDS, 10% glycerol, 0.01% sodium azide over a 24-hour period and then an additional five days against 10% glycerol, 5% 1,2-propanediol and 0.01% sodium azide, and finally against deionized water to remove the glycerol and 1,2-propanediol. Aliquots of the protein sample were then analyzed for protein content and radioactivity to determine the number of inactivator molecules per active site. With glycerol-inactivated holoenzyme, it was also possible to determine the number of coenzyme sites present by measuring the absorbance of enzyme-bound hydroxycobalamin at 362 nm. The hydroxycobalamin is completely formed within 24 hours by the air oxidation of the alkylcobalamin produced when the enzyme is inactivated by glycerol (Bachovchin *et al.*, 1977). Both the protein assay and spectrophotometric measurements yielded similar results.

Results

In the presence of glycerol and many other glycols (Bachovchin *et al.*, 1977) diol dehydratase holoenzyme undergoes rapid, irreversible inactivation such that catalytic activity cannot be regenerated by addition of either 1,2-propanediol, adenosylcobalamin, or by extensive dialysis. However, the inactivation process is instantly stopped if saturating amounts of 1,2-propanediol are added. Hence, the kinetics of the inactivation reaction can be studied by adding saturating amounts of 1,2-propanediol after variable periods of time to a holoenzyme-inactivator mixture; the amount of catalytically active enzyme remaining is then determined by measuring the subsequent rate of propionaldehyde production.

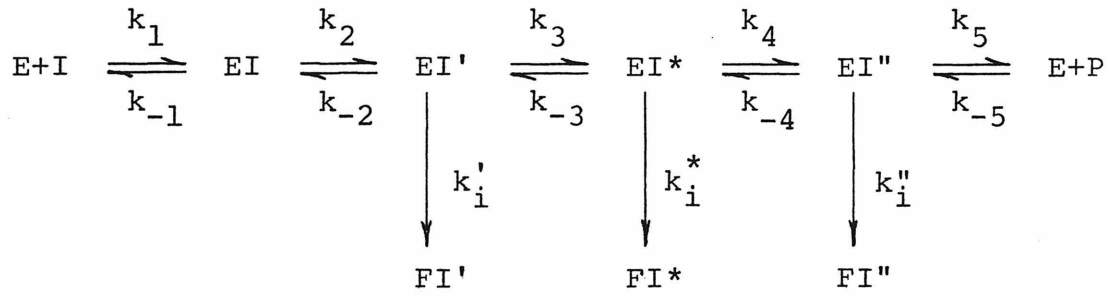
The inactivation follows a first-order rate law according to the relation:

$$(E_a) = (E_o) \exp (- k_{i,obs} t) \qquad (1)$$

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. Therefore, a plot of ln (E_a) as a function of time is linear with the slope yielding a value for $k_{i,obs}$ for a particular inactivator concentration. Bachovchin *et al.* (1977) showed

that Michaelis-Menten saturation kinetics apply to the inactivation process. Accordingly, the following kinetic scheme can be used to describe the inactivation process:

Scheme I



E is the holoenzyme; I represents the molecule which can act either as a substrate or an inactivator; EI is the inactivator-holoenzyme complex; EI' is the complex at some point after the abstraction of hydrogen from the inactivator; EI* is the intermediate immediately prior to the transfer of hydrogen from cofactor to product; and EI" is the holoenzyme-inactivator complex at some point after the second hydrogen transfer but before the release of product, P. All of these intermediates describe catalytically active enzyme. The rate constants k_2, k_{-2}, k_4, and k_{-4} describe hydrogen transfer reactions, but are not necessarily microscopic rate constants. For example, k_2 might describe a hydrogen transfer followed by an oxidation-reduction reaction or protein conformational

change in going from EI to EI'. If, say, the protein conformational change occurred much more slowly than the hydrogen transfer, then k_2 might not exhibit a significant primary isotope effect (Rose, 1976).

The species FI', FI*, and FI" represent inactivated holoenzyme complexes which are formed according to their respective rate constants k_i', k_i^*, or k_i''. In all likelihood, a given inactivator will deviate from the normal catalytic pathway at only one specific point. By examining the rate laws governing the inactivation processes and experimental data, it may be possible to determine the point of deviation.

Since inactivation events are rare relative to catalytic events (Bachovchin *et al.*, 1977), the active enzyme intermediates will have a steady-state distribution even though the amount of active enzyme is decreasing at a first-order rate. The steady-state distribution of the various active species is easily derived by the method of King and Altman (1958) and is shown below. In the derivation, the concentration of P was assumed to be negligible; hence, terms containing $k_{-5}[P]$ do not appear in the rate law.

$$\frac{(E)}{(E_a)} = \frac{k_4k_5(k_2k_3 + k_{-1}k_3 + k_{-1}k_{-2}) + k_{-1}k_{-2}k_{-3}(k_5 + k_{-4})}{\Delta} \quad (2)$$

$$\frac{(EI)}{(E_a)} = \frac{k_1k_4k_5(I)(k_3 + k_{-2}) + k_1k_{-2}k_{-3}(k_5 + k_{-4})(I)}{\Delta} \quad (3)$$

$$\frac{(EI')}{(E_a)} = \frac{k_1k_2(I)(k_4k_5 + k_{-3}k_5 + k_{-3}k_{-4})}{\Delta} \quad (4)$$

$$\frac{(EI^*)}{(E_a)} = \frac{k_1k_2k_3(I)(k_5 + k_{-4})}{\Delta} \quad (5)$$

$$\frac{(EI'')}{(E_a)} = \frac{k_1k_2k_3k_4(I)}{\Delta} \quad (6)$$

The denominator (Δ) is equal to the sum of the numerators in equations 2-6.

The inactivation rate laws have the form

$$d(E_a)/dt = -k_i''(EI'') \quad (7)$$

with the specific rate constant and intermediate in equation 7 being determined by the point of deviation from the normal catalytic pathway. When the appropriate substitutions are made into equation 7, the rate laws then take the form

$$d(E_a)/dt = -k_{i,obs}(E_a) \quad (8)$$

where

$$k_{i,obs} = \frac{k_i}{1 + \frac{K_I}{(I)}} \tag{9}$$

Integration of equation 8 yields equation 1. The parameter K_I is identical to the Michaelis constant describing the product-forming pathway (Segel, 1975) and is independent of the pathway of inactivation; it is equal to

$$K_I = \frac{(I)(\text{numerator of equation 2})}{(\text{the sum of the numerators in equations 3-6})} \tag{10}$$

The rate constant of inactivation, k_i, does depend upon the pathway of inactivation and can be described by one of the equations shown below:

$$k_{3,i} = k_i' \frac{(\text{numerator of equation 4})}{(\text{the sum of the numerators in equations 2-6})} \tag{11}$$

$$k_{4,i} = k_i^* \frac{(\text{numerator of equation 5})}{(\text{the sum of the numerators in equations 2-6})} \tag{12}$$

$$k_{5,i} = k_i'' \frac{(\text{numerator of equation 6})}{(\text{the sum of the numerators in equations 2-6})} \tag{13}$$

Hence,

$$-d(E_a)/dt = \frac{k_{3,i}}{1 + \frac{K_I}{(I)}} (E_a) \qquad (14)$$

when inactivation occurs after the formation of EI' through the pathway described by k_i'. The numerical subscripts in the constants $k_{3,i}$, $k_{4,i}$, and $k_{5,i}$ denote the step in the product-forming pathway which competes with the inactivation reaction, thus k_3 in the product-forming pathway competes with the inactivation pathway described by $k_{3,i}$. Unless the distinction is necessary, $k_{3,i}$, $k_{4,i}$, and $k_{5,i}$ will all be referred to simply as k_i or the zero-order inactivation rate constant.

Equation 9 predicts that a plot of $1/k_{i,obs}$ versus $1/[I]$ should yield a straight line, with the ordinate intercept being equal to $1/k_i$ and the abscissa intercept being equal to $-1/K_I$. For glycerol, and its deuterated derivatives, and isobutylene glycol this appears to be the case (Figures 1 and 2 and Table I); however, for the deuterated derivatives of isobutylene glycol, hydroxyacetone, and dihydroxyacetone (Figures 2, 3, and 4, respectively), the double-reciprocal plots appear to be nonlinear. Interestingly, dihydroxyacetone appears to have two concentration regions where the double-reciprocal plots are approximately linear. Table I collects the relevant data for the different inactivators tested.

The nonlinear double reciprocal plots suggest that either two substrate binding sites are being acted upon or, possibly, two forms of the inactivator are present with the ratio of the two forms being dependent on the inactivator concentration. Although carbon-13 magnetic resonance spectra of dihydroxyacetone and hydroxyacetone indicate the presence of the hydrated ketones (1,2,2,3-tetrahydroxypropane or 1,2,2-trihydroxypropane) as well as the carbonyl species, the ratio of the various species should always be constant. Hence one should obtain a linear double-reciprocal plot if there is only one binding site being acted upon. Furthermore, proton and carbon magnetic resonance measurements on solutions of d_2- and d_6-isobutylene glycol indicate the presence of only a single species. Consequently, there must be two substrate binding sites being acted upon.

Spectral Observation of Holoenzyme-Inactivator Complexes. The visible absorption spectra for holoenzyme that has been inactivated by glycerol or isobutylene glycol have been reported previously (Bachovchin *et al.*, 1977). When glycerol inactivates diol dehydratase, little change in the alkylcobalamin absorption spectrum of the holoenzyme is observed. However, after about 24 hours, the alkylcobalamin-type spectrum gradually changes to one resembling hydroxycobalamin in an oxygen-dependent process (Bachovchin *et al.*, 1977, 1978). In contrast, isobutylene glycol-inactivated holoenzyme changes the holoenzyme spectrum from that of an alkylcobalamin to

one which resembles cob(II)alamin. Moreover, the inactivated complex retains its cob(II)alamin-type spectrum for at least five days, even after dialysis against buffers containing glycerol. However, upon denaturation of the complex in 8 M urea, 0.1% SDS, 1 mM EDTA, pH 4, a cobalamin is released from the protein having a spectrum identical to hydroxycobalamin (Figure 5). This suggests that the enzymatically modified isobutylene glycol protects the cob(II)alamin from oxygen, while the protein is in its native state, much like substrate protects active holoenzyme from oxygen inactivation (Moore and Richards, 1979).

Both hydroxyacetone and dihydroxyacetone (and/or their hydrates) induce a cob(II)alamin-type spectrum when added to holoenzyme (Figure 6). Because of the biphasic nature of the double-reciprocal plots (Figures 3 and 4), it was of interest to see if the same spectrum resulted from inactivation at high concentrations of inactivator as for low concentrations of inactivator. Figure 7 shows the "titration" of holodiol dehydratase with small aliquots of dihydroxyacetone in the presence of 2 mM dl-2,3-butanediol. The dl-2,3-butanediol acts as a purely competitive inhibitor and protects the enzyme from oxygen inactivation during the titration; furthermore, the adenosylcobalamin-type spectrum remains in the presence of this competitive inhibitor (Moore and Richards, 1979) making the observation of the spectral changes due to the inactivator straightforward. Comparison

of Figures 6 and 7 shows that dihydroxyacetone produces a cob(II)alamin-type spectrum regardless of the concentration used to totally inactivate diol dehydratase holoenzyme.

From the amount of dihydroxyacetone needed to completely inactivate the enzyme, it was determined that one molecule of enzyme will turn over about 600 molecules of dihydroxyacetone (and/or its hydrate) before an inactivation event occurs (in the low concentration region). In comparison, diol dehydratase will turn over about 19,000 molecules of glycerol and 2300 molecules of isobutylene glycol per inactivation event (Moore, 1979), showing that dihydroxyacetone is a more potent inactivator. Because of the low number of turnovers per inactivation event, it could be very difficult to determine the product enzymatically derived from dihydroxyacetone. Moreover, the presence of the hydrate and keto forms presents the complication that two or more products might be produced. Furthermore, glyceraldehyde (a likely product from the rearrangement of the hydrate of dihydroxyacetone) might react with diol dehydratase as well.

Labeling of Diol Dehydratase Holoenzyme by Radioactive Inactivators. Table II collects the results for the attempted labeling of diol dehydratase holoenzyme by the radioactive inactivators [^{14}C]-isobutylene glycol and [2-^{3}H]- or [^{14}C]-glycerol. Nonspecific labeling by glycerol (Bachovchin *et al.*, 1977) was overcome by first reacting the holoenzyme with propanediol to generate propionaldehyde which

can form Schiff's base adducts with lysine residues and N-termini and thereby prevent such a reaction with β-hydroxypropionaldehyde. Furthermore, since Schiff's base adducts form and decompose reversibly, it would be possible for propionaldehyde in an adduct to exchange with free radioactively labeled β-hydroxypropionaldehyde, thus introducing spurious labels into the protein. To prevent such an exchange reaction, KBH_4 was added to the reaction mixture before adding the radioactive glycerol (or isobutylene glycol). This reduces the Schiff's base adducts with propionaldehyde to stable N-propylamines. Also, it appears that carrying out the reaction in $(NH_4)_2HPO_4$ helps reduce spurious labeling; presumably, the ammonium ion interacts with the aldehydes as well. Glycerol itself appears to bind very strongly to diol dehydratase even in the absence of coenzyme (Bachovchin *et al.*, 1977); hence, dialysis against concentrated solutions of glycerol for several days was necessary to bring the number of counts in a given protein sample to a constant level. These precautions reduced the number of radioactive labels per enzyme molecule from between 4 and 50 (Moore, 1979) to approximately one (Table II) when [^{14}C]-glycerol was the inactivator. In contrast, when [2-^{3}H]-glycerol was the inactivator, only about 0.15 labels became associated with each molecule of enzyme, suggesting that there might be a loss of hydrogen at C-2 during the inactivation process. Unfortunately, these blocking techniques did not work as well for isobutylene glycol, for about 10

labels became associated with the holoenzyme complex.

Figure 8 shows a histogram for the distribution of counts when [^{14}C]-glycerol-inactivated holoenzyme was run on an SDS-polyacrylamide gel. As one can see, the bulk of the counts ran ahead of the protein, indicating that the protein subunits were not covalently modified as a result of the inactivation process. Subsequent gel filtration on Bio-Gel P-4 showed that the radioactivity was of low molecular weight, but it was not associated with cobalamin.

Similarly, when holoenzyme that had been inactivated by radioactive isobutylene glycol was denatured with urea and passed down a Bio-Gel P-4 column, a radioactive molecule was released from the protein complex. It was difficult to quantitate the amount of radioactivity, because the peak was only about twice background radiation; however, the amount was at the approximate level expected for a single label per holoenzyme complex. This molecule migrated ahead of urea, but behind hydroxycobalamin, indicating that its molecular weight was on the order of substrate.

Discussion

Glycerol, as well as certain vicinal diols (Toraya *et al.*, 1976; Bachovchin *et al.*, 1977), can be termed "suicide substrates" (Maycock and Abeles, 1976; Walsh, 1977), because they are chemically unreactive until bound at the enzyme site and undergo at least one step of the catalytic process,

thus exposing or creating a reactive functional group. However, unlike some other suicide substrates which react in a stoichiometric fashion (Batzold and Robinson, 1975; Corey and Robinson, 1976), diol dehydratase turns over several glycerol, or diol, molecules before an inactivation event occurs. Moreover, even the natural substrates for the enzyme ((R)- and (S)-1,2-propanediol) inactivate the holoenzyme (Jensen and Neese, 1975), albeit quite slowly.

As shown in Results, the inactivation process can be described in a manner analgous to classical Michaelis-Menten kinetics. For example, equations 9 and 10 show that K_I is identical to the Michaelis constant for the product-forming pathway, for when $k_{i,obs}$ is one half of the zero-order k_i, the inactivator concentration will equal K_I. When $1/k_{i,obs}$ versus the reciprocal of the inactivator concentration was nonlinear, K_I and k_i were estimated by linear extrapolation from the low and high concentration regions as shown in Figure 4.

The values in Table I for k_i and K_I for glycerol, (R)-, (S)- and (RS)-d_2-glycerol, and d_5-glycerol (using native enzyme) are remarkably similar to those reported by Bachovchin *et al.* (1977) and Moore (1979) using proteolysed enzyme when one considers that k_i and K_I for isobutylene glycol are both larger than the reported values for proteolysed enzyme (Bachovchin *et al.*, 1977) with k_i/K_I being about 2.7 times larger with native enzyme. This suggests that there is

less nonproductive binding of the alternate substrate (isobutylene glycol) in the native enzyme relative to enzyme that has been proteolysed. Since methyl groups are important in binding and properly orienting the substrate in the active site (Moore and Richards, 1979), the methyl groups of isobutylene glycol could be binding in the methyl-group binding pocket(s) of the proteolysed enzyme, but with an improper orientation for catalysis to occur. In native enzyme, where the shape of the binding pocket might be more restrictive, there would be less chance of an improper binding orientation; hence, nonproductive binding would decrease. It appears that the essential binding features for glycerol (a biological substrate of diol dehydratase (Toraya *et al.*, 1978)) are present in both native and the proteolysed enzyme, and they are such that the steric changes in the binding site due to proteolysis do not affect the binding of glycerol.

Binding Site Interactions. The nonlinear plots of Figures 2, 3 and 4 suggest that two substrate binding sites are being acted upon by isobutylene glycol, hydroxyacetone and dihydroxyacetone as the concentration of the inactivator is varied. In theory, there seem to be three possibilities: (i) There could be a mixture of isozymes which have different affinities for the inactivators used; (ii) there could be one binding site per 250,000 molecular weight, but the enzyme is a "dimer" of 500,000 daltons which exhibits noncooperativity with respect to certain inactivators; and (iii) there might

be two substrate binding sites per 250,000 daltons with different binding affinities, and the apparent value of k_i depends upon whether one or both sites are occupied.

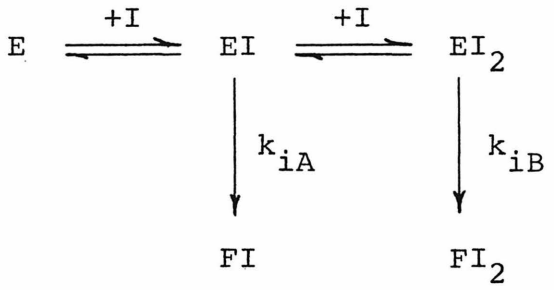
In view of the results of Chapter I, it seems unlikely, although not impossible, that isozymes are present. The isozymes would have to have identical N-terminal amino acid sequences (at least 40 residues into each of the four different subunits) and have identical affinities for Cellex-D. Furthermore, Bachovchin *et al.* (1977) did not observe any unusual kinetic behavior with the large number of vicinal diols that they tested. Hence, it would be highly fortuitous for the isozymes to behave identically with so many inactivators, and then differ on the few tested here.

Explanation (ii) also seems unlikely, even though native diol dehydratase appears to be a "dimer" of about 500,000 daltons, as described in Chapter I. Again, it would seem odd that subunit interactions would be observed for only some inactivators and not others, especially for the case in Figure 2 where the only difference between inactivators is isotopic substitution.

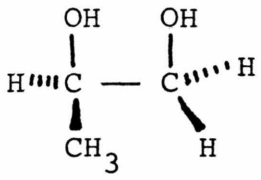
Explanation (iii) holds more promise and has more precedence than the previous two explanations. Unpublished experiments by Carroll *et al.* have shown that the kinetic behavior of diol dehydratase is subject to the stereochemistry of the substrates present. For example, it appears that both

the first and second hydrogen transfer reactions are rate influencing when only (R)- or (S)-1,2-propanediol is present in the solution; however, when both stereoisomers are present in equal amounts, the second hydrogen transfer is exclusively rate limiting. Hence, there may be binding sites for both the (R) and (S) isomers of 1,2-propanediol juxtaposed to a single catalytic site. It may be that hydroxyacetone, dihydroxyacetone and/or their hydrates bind to both of these sites, with different affinities for each site. As with catalysis, the apparent zero-order k_i might change from k_{iA} to k_{iB} as the inactivator concentration is increased according to Scheme II below.

Scheme II

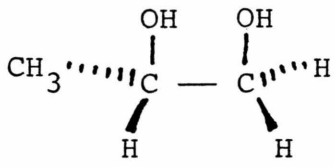


A similar situation could be the case for isobutylene glycol, which contains the structural elements of both (R)- and (S)-1,2-propanediol as shown below:



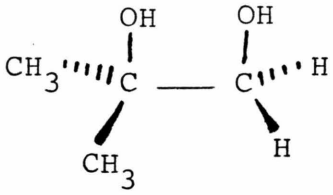
(R)-1,2-Propanediol

I



(S)-1,2-Propanediol

II



Isobutylene Glycol

III

It may be that, unlike glycerol, which binds in two configurations but only inactivates in one of them (Bachovchin *et al.*, 1978), isobutylene glycol, dihydroxyacetone, and hydroxyacetone can bind to two sites and cause inactivation or undergo catalysis from both. The observed nonlinearity in Figure 2 upon isotopic substitution in isobutylene glycol could stem from different sensitivities of the different catalytic and/or inactivation pathways (described by k_{iA} and k_{iB} in Scheme II) to isotopic substitution.

Spectral Properties of Enzyme-Bound Cofactor. Substrate-induced transformation of enzyme-bound cofactor to a species spectrophotometrically the same as cob(II)alamin has been observed with both diol dehydratase and ethanolamine deaminase (Wagner *et al.*, 1966; Abeles and Lee, 1964; Babior, 1969; Bachovchin *et al.*, 1977). This transformation is associated with catalysis and/or inactivation, and presumably involves homolytic cleavage of the carbon-cobalt bond.

Glycerol and meso-2,3-butanediol do not generate a detectable cob(II)alamin species (Bachovchin *et al.*, 1977; Moore and Richards, 1979) when reacted with diol dehydratase. Glycerol produces an alkylcobalamin-type spectrum very similar to that of adenosylcobalamin which gradually changes to a spectrum similar to hydroxycobalamin in an oxygen-dependent process, and meso-2,3-butanediol produces an hydroxycobalamin-type spectrum at a rate similar to that of inactivation. The absence of a cob(II)alamin species with glycerol and meso-2,3-butanediol as substrates probably represents a change in the distribution of the enzyme intermediates, resulting in the predominance of an intermediate other than cob(II)alamin, rather than a change in the mechanism of catalysis, for Bachovchin *et al.* (1978) isolated 5'-deoxyadenosine that contained tritium from diol dehydratase that had been inactivated with [1-^{3}H]-glycerol. Hence, the cob(II)alamin species still intervenes, but was not detected by the techniques used.

Like most of the vicinal diols tested by Bachovchin *et al.* (1977) isobutylene glycol (Figure 5), hydroxyacetone and dihydroxyacetone (Figure 6), produce a cob(II)alamin-type spectrum with the inactivated diol dehydratase. Figure 7 shows that when diol dehydratase is inactivated by low concentrations of dihydroxyacetone (corresponding to Region 1 in Figure 4) a cob(II)alamin spectrum is also obtained once the holoenzyme has been completely inactivated.

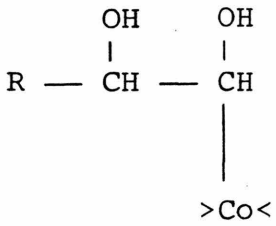
Unfortunately, these results do not help determine the point of deviation from the normal catalytic pathway, for all the points of deviation suggested in Scheme I could conceivably contain a cob(II)alamin species.

Inactivation of Diol Dehydratase by Radiolabeled Glycerol or Isobutylene Glycol. Table II shows that when diol dehydratase is inactivated by glycerol, approximately one molecule of enzymatically modified glycerol becomes tightly associated with the holoenzyme complex, with the modification involving a significant loss of tritium from C-2. Figure 8 shows that the protein does not become covalently modified in the process. Interestingly, the radioactive molecule migrates in the electrostatic field as if it were negatively charged in the presence of sodium dodecyl sulfate (SDS); however, it is possible that SDS interacts with the modified glycerol molecule causing it to migrate thusly. For example, Pyronin Y is a positively charged dye which migrates as if it were negatively charged in the presence of SDS; this dye is commonly used as a marker when running SDS-polyacrylamide gels.

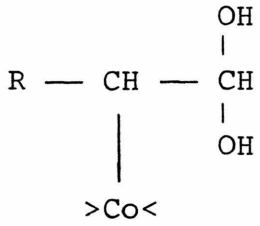
When radioactively labeled isobutylene glycol is used to inactivate the enzyme, a small radioactive molecule is also released from the protein upon denaturation. Unfortunately, several other labels were still attached to the protein (see Table II), making it difficult to say whether or not the molecule that was released had anything to do

with the inactivation reaction. However, as shown in Figure 5, the cob(II)alamin initially produced during the inactivation was stable for five days, but became oxidized when the protein was denatured. Since enzyme-bound substrate or competitive inhibitor protects diol dehydratase from oxygen inactivation (Moore and Richards, 1979), one might expect that enzyme-bound inactivator could protect cob(II)-alamin from being oxidized to hydroxycobalamin. Hence, it is possible that the small molecule that was released from the inactivated complex was involved in the inactivation process, especially in view of the results obtained with glycerol.

Bachovchin *et al.* (1977) proposed that the alkylcobalamin-type spectrum of glycerol-inactivated diol dehydratase may have been due to "trapped" intermediates, such as IV or V.



IV



V

The inability of Bachovchin *et al.* (1977) to isolate the light-sensitive alkylcobalamin from the inactivated complex

favors intermediate V, because secondary alkylcobalamins are very unstable (Hogenkamp, 1975). Moreover, oxidation of V could easily account for the low level of labeling observed when [2-^{3}H]-glycerol was used to inactivate the enzyme (Table II), because homolysis of the carbon-cobalt bond in the presence of oxygen results in the formation of hydroxycobalamin and a carbonyl functionality on the carbon (Abeles, 1972). Since it is necessary to dialyze the inactivated complex for several days in order to remove spurious labels, oxidation of the complex during this treatment is unavoidable; hence, it could be very difficult to determine the extent of labeling by [2-^{3}H]-glycerol on the unoxidized complex.

However, the formation of V alone does not account for the observed inactivity of apoenzyme upon removal of cobalamin. Continued dialysis versus solutions containing SO_3^{2-} beyond necessary to remove enzyme-bound cobalamin does restore some activity. Presumably, the SO_3^{2-} reduces a reactive group on the apoenzyme that became oxidized during the inactivation process (Bachovchin *et al.*, 1977).

Isotope Effects on the Glycerol Inactivation Reaction. Interpreting isotope effects on inactivation is a complicated task, because it is difficult to distinguish between isotope effects which pertain to the product-forming pathway and those unique to the inactivation pathway. For example, d_5-glycerol has a large isotope effect on the inactivation reaction (Table I); however, the rate constants governing one

or more of the hydrogen transfers in the product-forming pathway also appear in the possible definitions of the zero-order inactivation rate constant (equations 11-13) when derived according to Scheme I. Therefore, an isotope effect on a step in the catalytic pathway could also appear as an isotope effect on the zero-order inactivation rate constant even if there is no isotope-sensitive step unique to the inactivation pathway. In other words, if the rate-limiting step in catalysis is sensitive to isotopic substitution, one will see this isotope effect expressed in one of two ways on the observed zero-order inactivation rate constant, depending upon where the inactivation pathway deviates from catalysis.

(i) For a moment, let us assume that EI* in Scheme I can continue along the catalytic pathway via the rate-limiting hydrogen transfer (described by k_4) or divert to the inactivation pathway via the rate constant k_i^*. Also, assume that k_i^* is not sensitive to isotopic substitution. Under these circumstances, one would expect to see an inverse isotope effect on inactivation if the step described by k_4 involved the transfer of deuterium, because slowing k_4 would have the effect of increasing the steady-state concentration of EI*. Since the observed inactivation rate is proportional to $(EI^*)\cdot k_i^*$, the observed inactivation rate will increase, yielding an apparent inverse isotope effect on the zero-order inactivation rate constant. As will be discussed, d_6-isobutylene glycol does exhibit an inverse isotope effect on activation.

(ii) Now let us assume that the same conditions described in (i) prevail except that inactivation now deviates from catalysis at a rate which is proportional to $(EI') \cdot k_i'$. Also, assume that k_{-3} (the rate constant describing the conversion of EI* to EI') is small relative to k_4 (which can describe the transfer of protium or deuterium). Again slowing k_4 by the presence of a heavy isotope would increase the steady-state concentration of EI*, thus (since the total enzyme concentration is a constant) the steady-state concentration of EI' (and other intermediates) will decrease accordingly. Therefore, the observed inactivation rate will decrease even though k_i' is not sensitive to isotopic substitution.

Thus, case (ii) appears to account for the conclusion of Bachovchin *et al.* (1978) that the transfer of deuterium from 5'-deoxyadenosine to either the glycerol skeleton or some other species was responsible for the large observed isotope effect on inactivation with d_5-glycerol. Moreover, case (ii) is also consistent with their observation that 5'-deoxyadenosine (a catalytic intermediate [Frey *et al.*, 1967]) can be isolated from glycerol-inactivated diol dehydratase in amounts proportional to the amount of enzyme that was inactivated. Therefore, it appears that the inactivation pathway for glycerol deviates from catalysis at some point after the first hydrogen transfer; but before the second hydrogen transfer, for example, in Scheme I, by

the microscopic rate constant k_i'. The nature of the reaction described by k_i' will be discussed later.

Bachovchin *et al.* (1978) and Moore (1979) observed a large difference in the isotope effect on inactivation between d_5-glycerol and (R)-, (S)-, or (RS)-1,1-d_2-glycerol (see Table I). They were able to account for most of the differences by assuming the intervention of a three-hydrogen reservoir (the C-5' methyl group of 5'-deoxyadenosine). They pointed out that the reservoir will not be fully deuterated when the d_2-glycerols are substrates as it is when d_5-glycerol is the substrate; hence, only a fraction of the inactivation (actually product-forming) events will involve the heavy isotope. An alternate explanation, although it is without precedence in the diol dehydratase system, is that hydrogen at C-2 of glycerol was being abstracted in the inactivation reaction in the rate-limiting step.

To distinguish between these two possibilities, inactivation experiments were conducted with d_4-glycerol and 2-d-glycerol. According to the model presented by Moore (1979) and Bachovchin *et al.* (1978), d_4-glycerol should exhibit an isotope effect on inactivation similar to that of d_5-glycerol with the isotope effect for 2-d-glycerol being small or nonobservable. The alternate model would predict the opposite relative magnitudes for the two isotope effects. As shown in Table I, the observed isotope effects accord with the model presented by Bachovchin *et al.* (1978) and Moore (1979)

with 2-d-glycerol having an isotope effect on inactivation of 1.46 and that for d_4-glycerol being about 15.

The isotope effect on inactivation of 1.46 by 2-d-glycerol (Table I and Figure 1) suggests that there could be an abstraction or elimination of hydrogen from C-2 of glycerol, although not in the rate-limiting step of the overall pathway. Moreover, this would be qualitatively consistent with the observed loss of tritium from C-2 when [2-^{3}H]-glycerol was used to label holoenzyme.

Alternately, the observed isotope effect with 2-d-glycerol could reflect a secondary isotope effect on k_i' (the microscopic rate constant describing the point at which inactivation deviates from catalysis, as discussed earlier), for, as shown by equation 11, the observed zero-order inactivation rate constant is directly proportional to k_i'. According to Streitwieser *et al.* (1958) secondary isotope effects can result from a change in hybridization at the α-carbon (in this case C-2 of glycerol) from sp^3 in a reactant to sp^2 in the product if deuterium is bound to the α-carbon. Although experimentally measured α-deuterium isotope effects on solvolysis reactions are usually less than 1.25 (Melander and Saunders, 1980), the theoretical estimate of such a change in hybridization is about 1.4. The formation of the secondary alkylcobalamin (compound V) from a secondary radical (Eager *et al.*, 1972) is one possibility that could account for an α-deuterium isotope effect with 2-d-glycerol. Alkyl radicals

generally have a tetrahedral (sp^3) geometry (Lowry and Richardson, 1976) and in alkylcobalamins, the carbon bonded to the cobalt can exhibit much sp^2 character even though this carbon is four coordinate (Hogenkamp, 1975). For example, Co- C-5'- C-4' bond angle in adenosylcobalamin is 125° (Lenhert and Hodgkin, 1961) which is an even larger bond angle involving the C-5' carbon than a normal sp^2 carbon. Conceivably, a compound such as V could also have a large Co- C-2- C-1(or C-3) bond angle, thus giving much sp^2 character to C-2 (to which deuterium is bonded in 2-d-glycerol) in the product of the inactivation reaction.

There are some attractive features to postulating the formation of compound V as the departure from the normal catalytic pathway (which might be followed by or occur in concert with, other reactions which modify the protein in some way). The oxidation of V can account for the substantial loss of tritium from [2-^{3}H]-glycerol in the inactivated complex. The fact that one mole of [^{14}C]-glycerol becomes associated with each 250,000 daltons of protein is consistent with the observation of Essenberg *et al.* (1971) that only one coenzyme molecule is bound per 250,000 daltons. Moreover, one can also account for the α-deuterium isotope effect observed on inactivation of diol dehydratase by 2-d-glycerol.

If V is formed by a secondary radical (probably 1,1,3-propanetriol-2-yl formed from the rearrangement of the substrate) reacting with cob(II)alamin, this suggests that

there is at least one step that follows the formation of this enzyme-bound radical (given by EI' in Scheme I) before hydrogen is transferred to an intermediate designated as EI*, which, according to existing models of hydrogen transfer in adenosylcobalamin-dependent enzymes (Silverman and Dolphin, 1976; Golding and Radom, 1976; Eager *et al.*, 1972; Halpern, 1974), should also contain the secondary radical. A protein conformational change in the product-forming pathway could create a distinction between EI' and EI* in which both species seem to contain a secondary product radical. In EI' the radical might be favorably aligned to react with the cobalamin to form V according to the rate constant k_i', but after the protein conformational change (described by k_3), the product-radical in EI* is favorably aligned for the hydrogen transfer reaction described by k_4.

Isotope Effects on the Isobutylene Glycol Inactivation Reaction. As shown in Figure 2 and Table I, isobutylene glycol behaves differently than glycerol upon isotopic substitution for hydrogen at various positions. With deuterium in the reactive positions at C-1, d_2-isobutylene glycol shows little or no isotope effect on the zero-order inactivation rate constant but a large effect on K_I (K_I increases about 19-fold). In contrast, d_5-glycerol shows a large isotope effect on the zero-order inactivation rate constant (15) with

only about a threefold increase in K_I. As discussed, the isotope effect observed on the inactivation of diol dehydratase by glycerol might reflect the rate-limiting transfer of hydrogen in the product-forming pathway, by the same reasoning the absence of a primary isotope effect on inactivation with isobutylene glycol suggests that the rate-limiting step in the conversion of isobutylene glycol to 2-methylpropionaldehyde might be something other than hydrogen transfer. This is not surprising since diol dehydratase turns over isobutylene glycol at only about 7% of the rate that 1,2-propanediol is turned over.

The isotope effect on the zero-order inactivation rate constant of 0.56 for d_6-isobutylene glycol most likely is a secondary isotope effect and not a primary one, because there is no precedence for the abstraction of hydrogen from a methyl group (C-3) by diol dehydratase. Moreover, the secondary isotope effect has to be a β-deuterium isotope effect (reflecting changes at C-2 of substrate), because deuterium more remote than β to the reaction center tends to exhibit little effect (2-3%)(Shiner, 1970; Jewett and Dunlap, 1968). Furthermore, the observed effect must be related to the slowing of the catalytic pathway relative to the inactivation pathway, because β-deuterium isotope effects are always greater than 1.0 (Lowry and Richardson, 1976); the minimum effect on the catalytic pathway would be the reciprocal of 0.56 which is 1.79. This is very similar to

the isotope effect observed in the solvolysis of $(CD_3)_2CEtCl$ in 80% aqueous ethanol (Dewar, 1962) which is an S_N1 reaction which involves the formation of a carbonium ion β to the deuterated methyl groups. Because a full β-deuterium isotope effect is observed (actually its reciprocal) on the zero-order inactivation rate constant of d_6-isobutylene glycol, a catalytic step is slowed that is in direct competition with the step that deviates from the product-forming pathway.

According to Melander and Saunders (1980), β-deuterium isotope effects arise primarily from weakening of the carbon-hydrogen bonds that are β to an electron-deficient carbon atom by hyperconjugation. This suggests that the formation of a carbonium ion at C-2 of the substrate molecule is the step in the catalytic pathway that is being slowed. In the model for the rearrangement of substrate proposed by Eager *et al.* (1972), the formation of a carbonium ion at C-2 of substrate would coincide with the migration of the hydroxyl group from C-2 to C-1. If this is so, then the inactivation by isobutylene glycol would occur before the rearrangement of the substrate, in contrast to glycerol which appears to inactivate after the substrate has rearranged. Further studies with these two inactivators could yield information about two different aspects of the mechanism of action.

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TABLE I

Inactivator	$k_{i\,(min^{-1})}$	$K_{I\,(mM)}$	$\frac{k_{i,H}}{k_{i,D}}$[a]	$\frac{k_i}{K_I}(min^{-1}{}_{mM}{}^{-1})$
Glycerol	1.34	1.55	--	0.86
d_5-glycerol	0.083	3.9	16±2	0.021
d_4-glycerol	0.092[b]	--	15±2	--
2-d-glycerol	0.92	0.58	1.46	1.59
(S)-d_2-glycerol	0.88	2.9	1.52	0.30
(R)-d_2-glycerol	0.54[b]	--	2.48	--
(RS)-d_2-glycerol	0.80	1.6	1.68	0.50
Isobutylene glycol	2.40	1.7	--	1.41
d_2-isobutylene glycol[d]	2.40	33.0	1.00	0.073
d_6-isobutylene glycol[d]	4.30	18.0	0.56	0.239
Hydroxyacetone[d]	3.61	0.33	--	0.091
Dihydroxyacetone (1)	1.56[c]	0.25	--	6.24
Dihydroxyacetone (2)	5.10[c]	2.01	--	2.54

[a]Observed isotope effect on the zero-order inactivation rate constant.

[b]Determined at an inactivator concentration of 50 mM.

[c]Constants reported correspond to Regions 1 and 2 in Figure 4.

[d]These inactivators yielded non-linear double reciprocal plots; hence, the constants reported are from graphical approximations in the high concentration region.

TABLE II

Inactivator	Active Sites (nmol)	Labels (nmol)[c]	Labels Active Site
[2-^{3}H]-glycerol	9.12[a]	1.29	0.14
[2-^{3}H]-glycerol	0.66[a]	0.11	0.17
[1,3-^{14}C]-glycerol	0.39[a]	0.44	1.12
[1,3-^{14}C]-glycerol	0.41[a]	0.40	0.97
[1,3-^{14}C]-glycerol	0.24[b]	0.26	0.91
[^{14}C]-isobutylene glycol	3.59[a]	39.10	10.90

[a] Protein was determined by the method of Lowry *et al.* (1951). The molecular weight of pure diol dehydratase was assumed to be 259,000 daltons with a specific activity of 95 units/mg (Chapter I). If partial denaturation occurred during the preparation of the protein (determined by measuring the specific activity of the enzyme immediately before use), an appropriate correction for the amount of protein per active site was made.

[b] The holoenzyme concentration was determined by measuring the absorbance at 362 nm. The molar extinction coefficient for A_{358} (2.06×10^{4}) for hydroxycobalamin (Hogenkamp, 1975) was corrected to account for the bathochromatic shifts reported by Bachovchin *et al.* (1977).

[c] Determined from the initial specific activity (cpm/nmol) of the inactivator.

Figure 1. Inactivation data for 2-d-glycerol (dots) and glycerol (squares) were plotted as the reciprocal of the observed inactivation rate constant versus the reciprocal of the inactivator concentration.

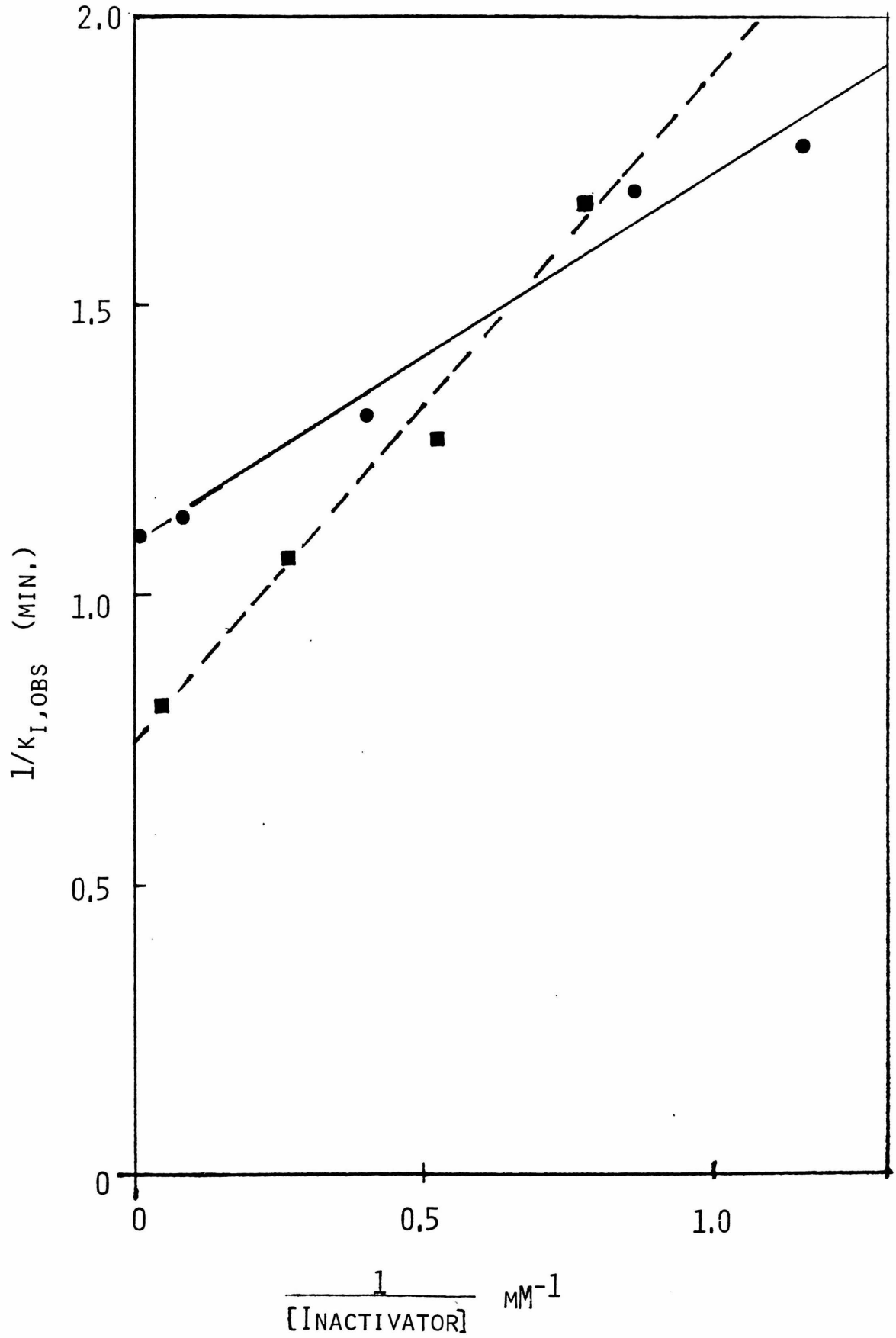


Figure 1

Figure 2. Inactivation data for d_2-isobutylene glycol (dots), d_6-isobutylene glycol (squares), and isobutylene glycol (triangles) were plotted as the reciprocal of the observed inactivation rate constant versus the reciprocal of the inactivator concentration. Non-linear results were hand fitted.

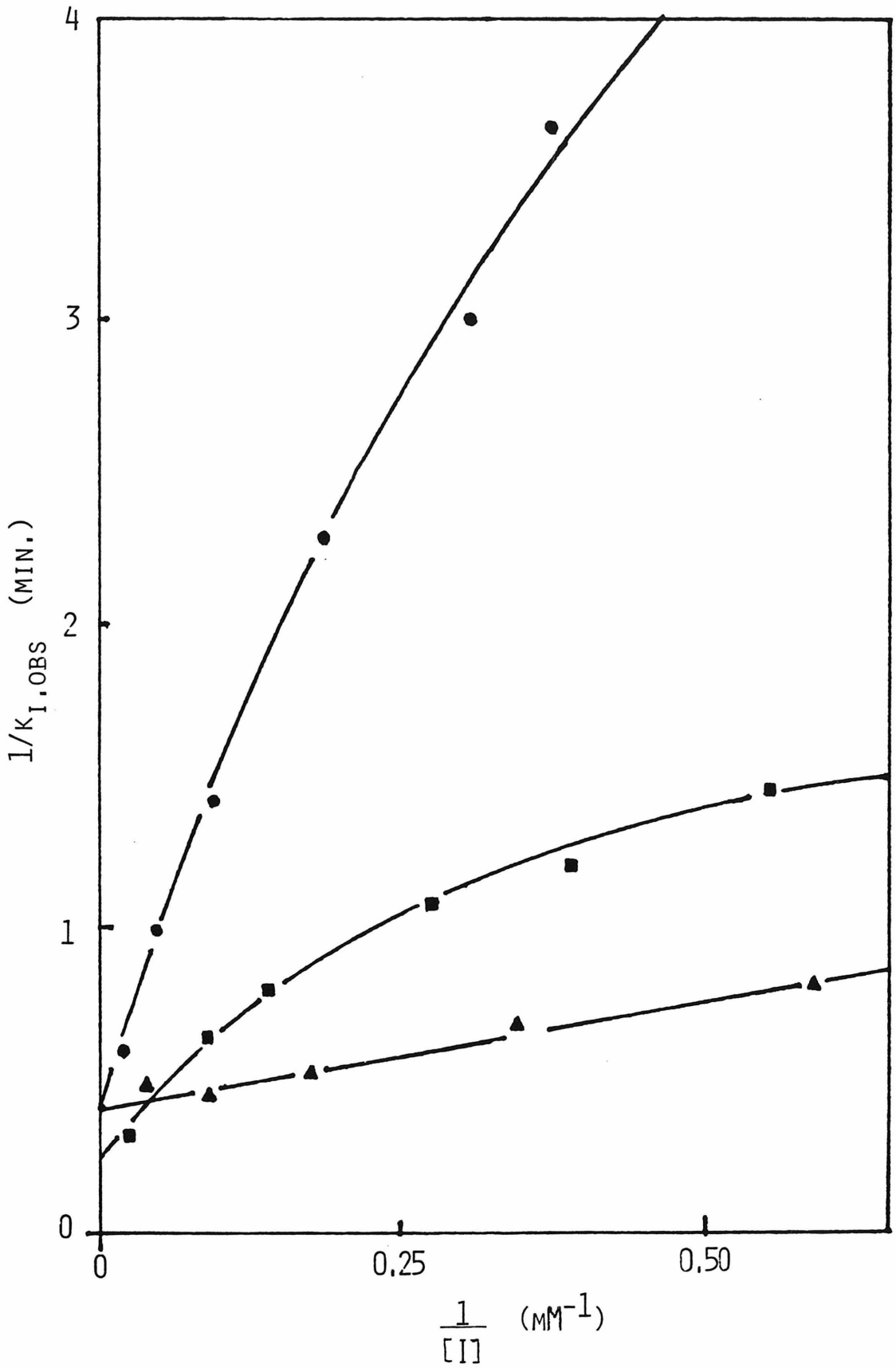


Figure 2

Figure 3. Inactivation data for hydroxyacetone were plotted as the reciprocal of the observed inactivation rate constant versus the reciprocal of the hydroxyacetone concentration. The curve shown represents a hand fit to the data.

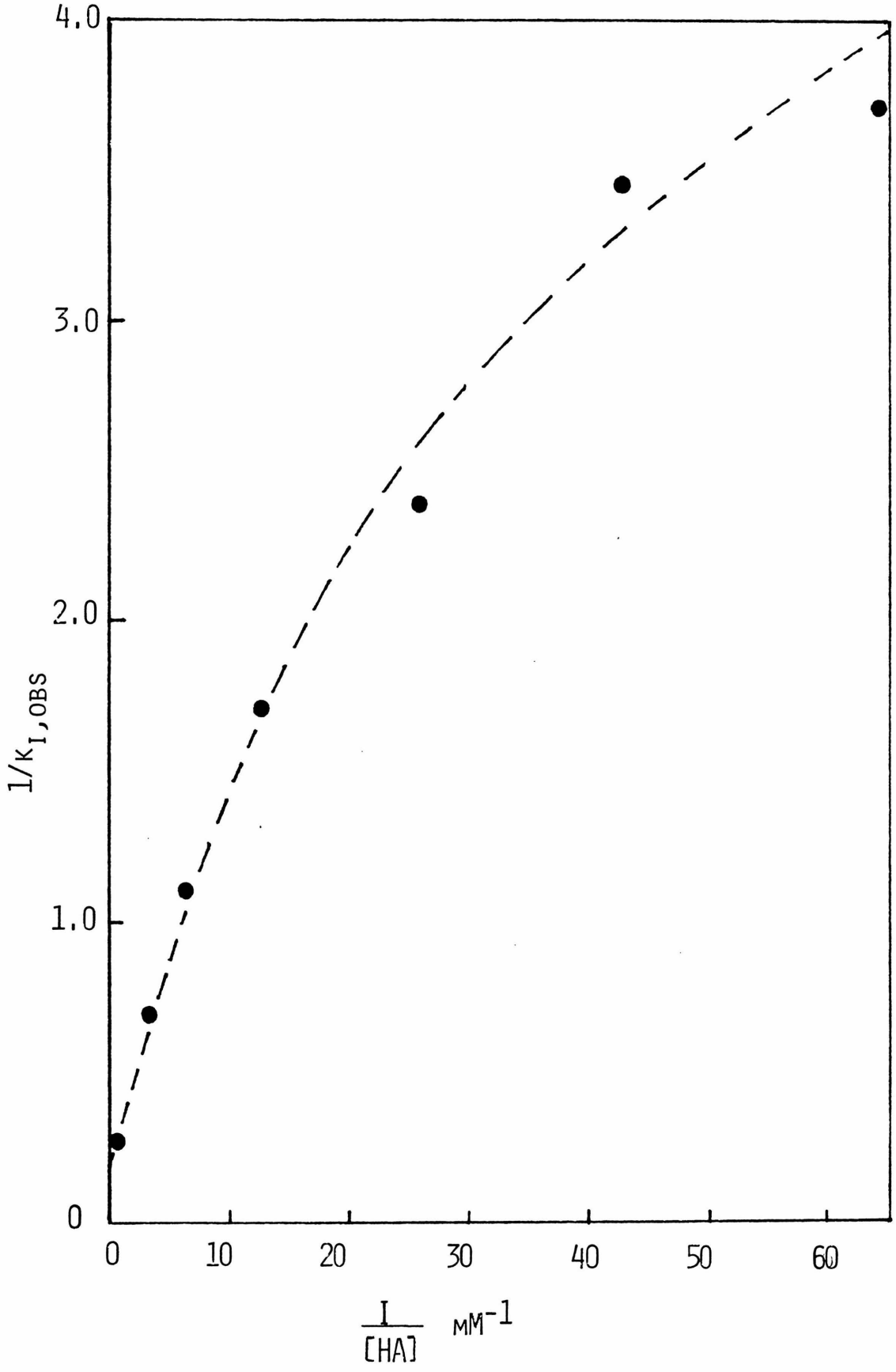


Figure 3

Figure 4. Inactivation data for dihydroxyacetone were plotted as the reciprocal of the observed inactivation rate constant versus the reciprocal of the dihydroxyacetone concentration. The lines drawn represent least squares fits to the circles (Region 1) and the squares (Region 2) with the point denoted by a triangle being common to both sets of calculations.

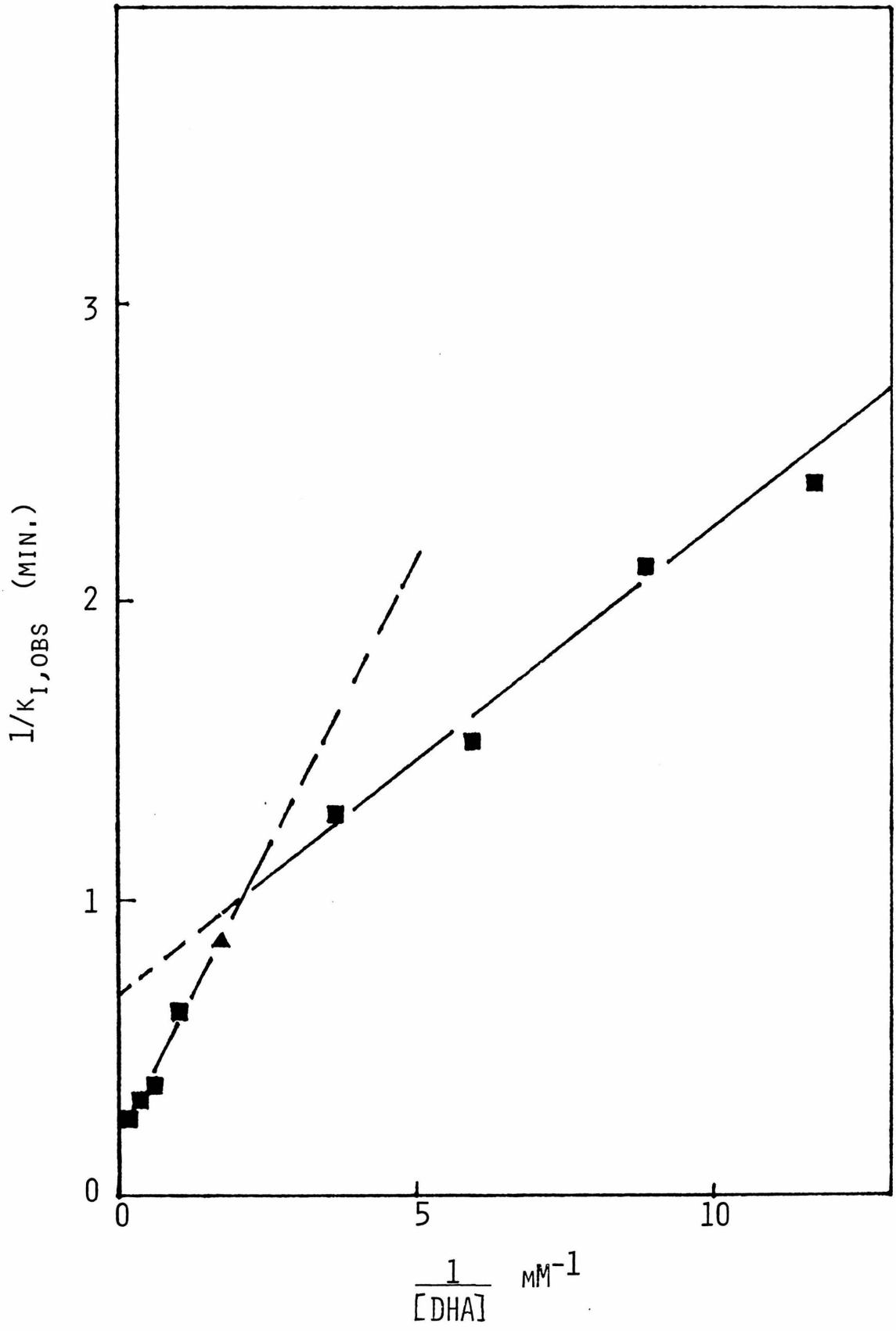


Figure 4

Figure 5. Absorption spectrum (1) was taken of diol dehydratase holoenzyme that had been inactivated by radio-labeled isobutylene glycol five days earlier and dialysed extensively as described in Experimental. Absorption spectrum (2) was taken within a few hours after denaturation of the holoenzyme complex with 8M urea. The cobalamin was separated from the protein by chromatography on Bio-Gel P-6 and the spectrum was obtained. Spectrum (1) is vertically displaced for clarity.

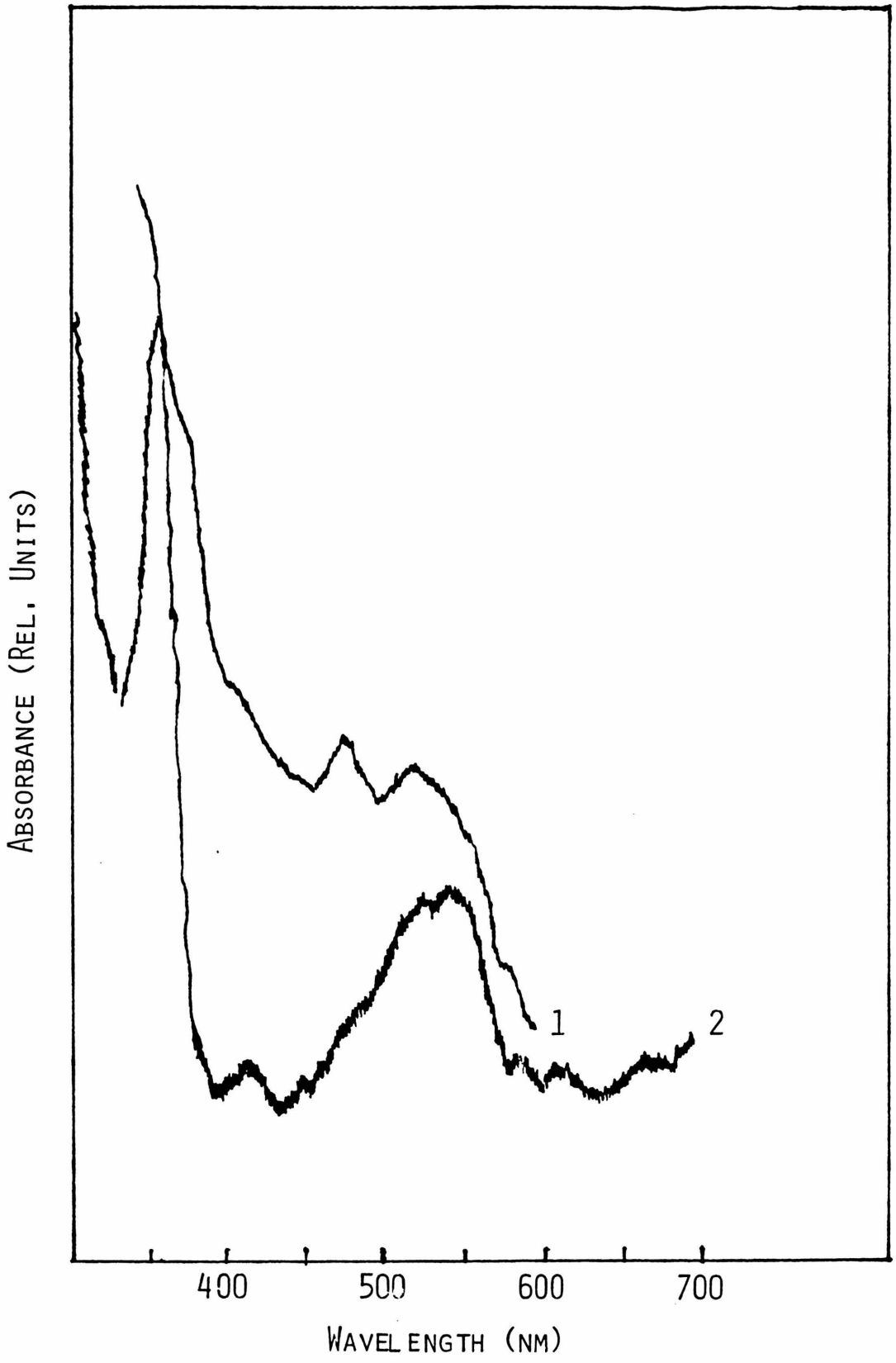


Figure 5

Figure 6: The absorption spectra were obtained by taking an enzyme versus enzyme spectrum to determine the baseline over the wavelengths scanned. Then, adenosylcobalamin (1.0 mmol in 0.010 mL) was added to 0.5 mL of enzyme solution containing 1.5 nmol of diol dehydratase and 1.0 μmol of dl-2,3-butanediol and mixed. For spectrum 1 hydroxyacetone (130 μmol in 0.010 mL) was added to the holoenzyme solution; for spectrum 2 dihydroxyacetone (10 μmol in 0.010 mL) was added. The spectra were obtained five minutes after adding the inactivator. The spectra shown were corrected for baseline variations. Spectrum 1 was obtained at a higher instrument sensitivity than spectrum 2, and is vertically displaced in the figure.

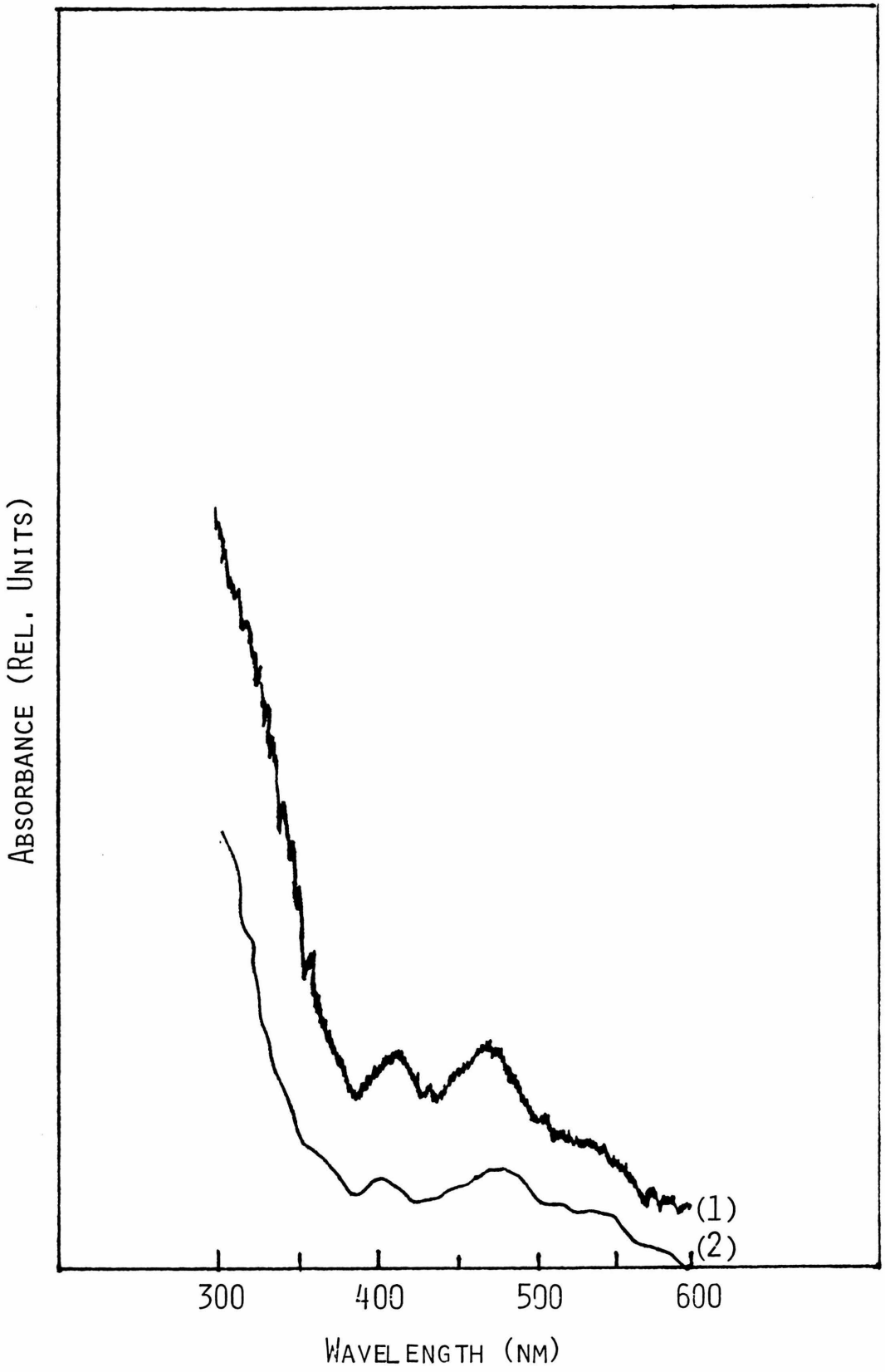


Figure 6

Figure 7. Changes in the holoenzyme absorption spectrum were recorded as small amounts of dihydroxyacetone were added. The spectra are corrected for baseline variations as described in Figure 6. The initial spectrum (1) is of 1 nmol of holoenzyme in a total volume of 0.51 mL; spectrum 2 was collected after the addition of 300 nmol of dihydroxyacetone; spectrum 3 after a total of 500 nmol of dihydroxyacetone was added; spectrum 4 after 700 nmol. Adding another 200 nmol of dihydroxyacetone to the sample from which spectrum 4 was obtained caused no change in the spectrum. During the titration, the cuvette concentration of dihydroxyacetone never exceeded 400 μM (which is the approximate concentration where the lines for Regions 1 and 2 from Figure 4 intersect). The total volume added to the holoenzyme solution was 0.014 mL. Between 5 and 10 minutes elapsed between the taking of each spectrum. As was the case in Figure 6, the dl-2,3-butanediol concentration was 2 mM to protect against oxygen inactivation during the course of the experiment.

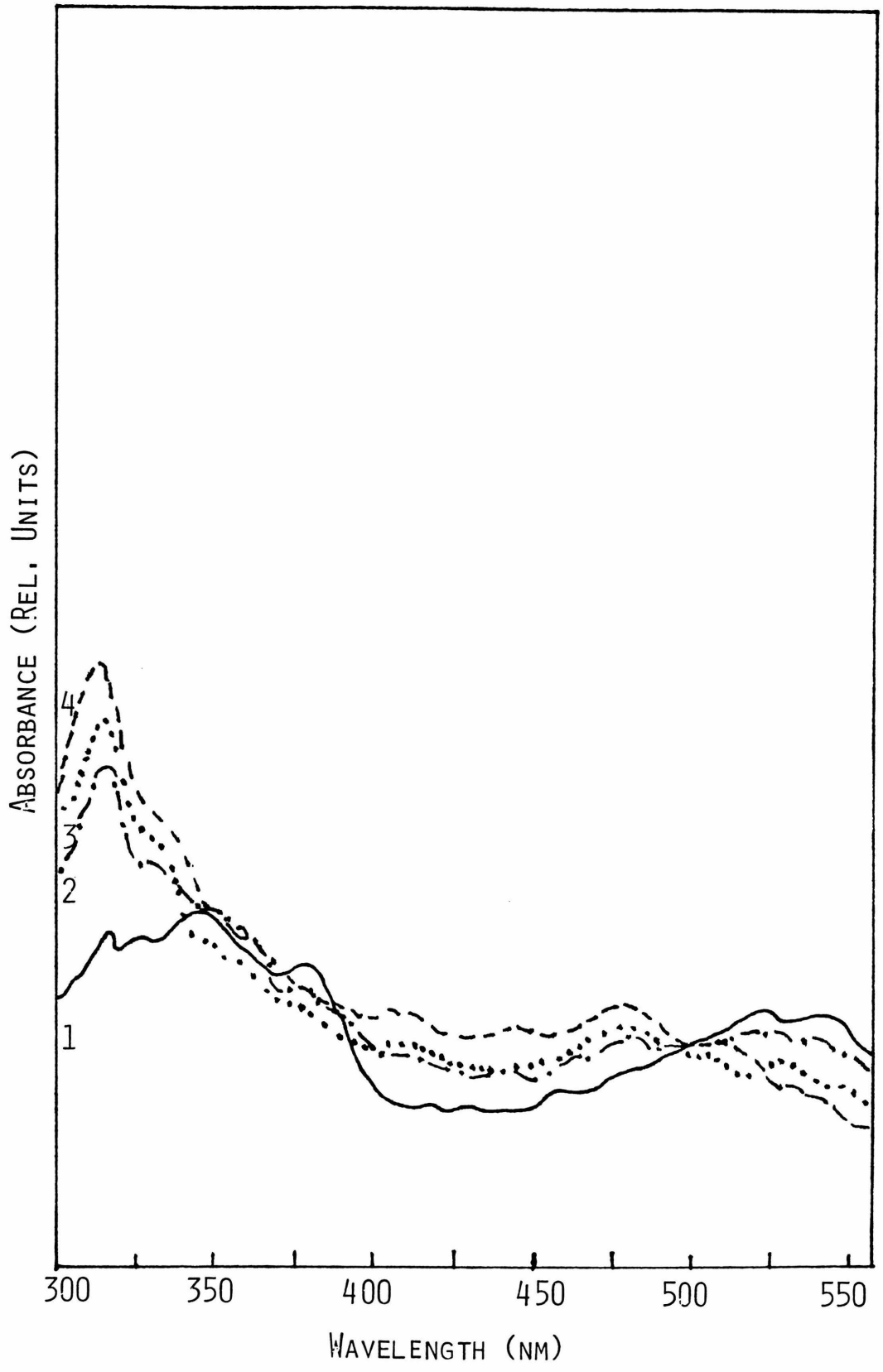

ABSORBANCE (REL. UNITS)
4
3
2
1
300
350
400
450
500
550
WAVELENGTH (NM)

Figure 7

Figure 8: A histogram describing the distribution of ^{14}C counts in an SDS-polyacrylamide gel of diol dehydratase holoenzyme that had been inactivated by [1,3-^{14}C]-glycerol, dialysed extensively as described in Experimental, and then lyophilized. After the gel was run, portions of the gel were sliced into 1 cm^2 pieces and placed in 1 mL of 0.05 M ammonium bicarbonate that contained 0.1% SDS and shaken overnight. Other parts of the slab gel were stained with Coomassie Brilliant Blue. After shaking, the gel slices were removed from the vials in which they were shaken, and 10 mL of Aquasol-2 was added to each vial and the samples counted. The approximate location of the protein bands on the gel are indicated by the labels on the bars of the histogram. When a given subunit appears on more than one bar, it indicates that the protein band overlapped into two gel slices. Gel slice 1 indicates the region ahead of the bromophenol blue dye marker, and slice 10 contains part of the stacking gel.

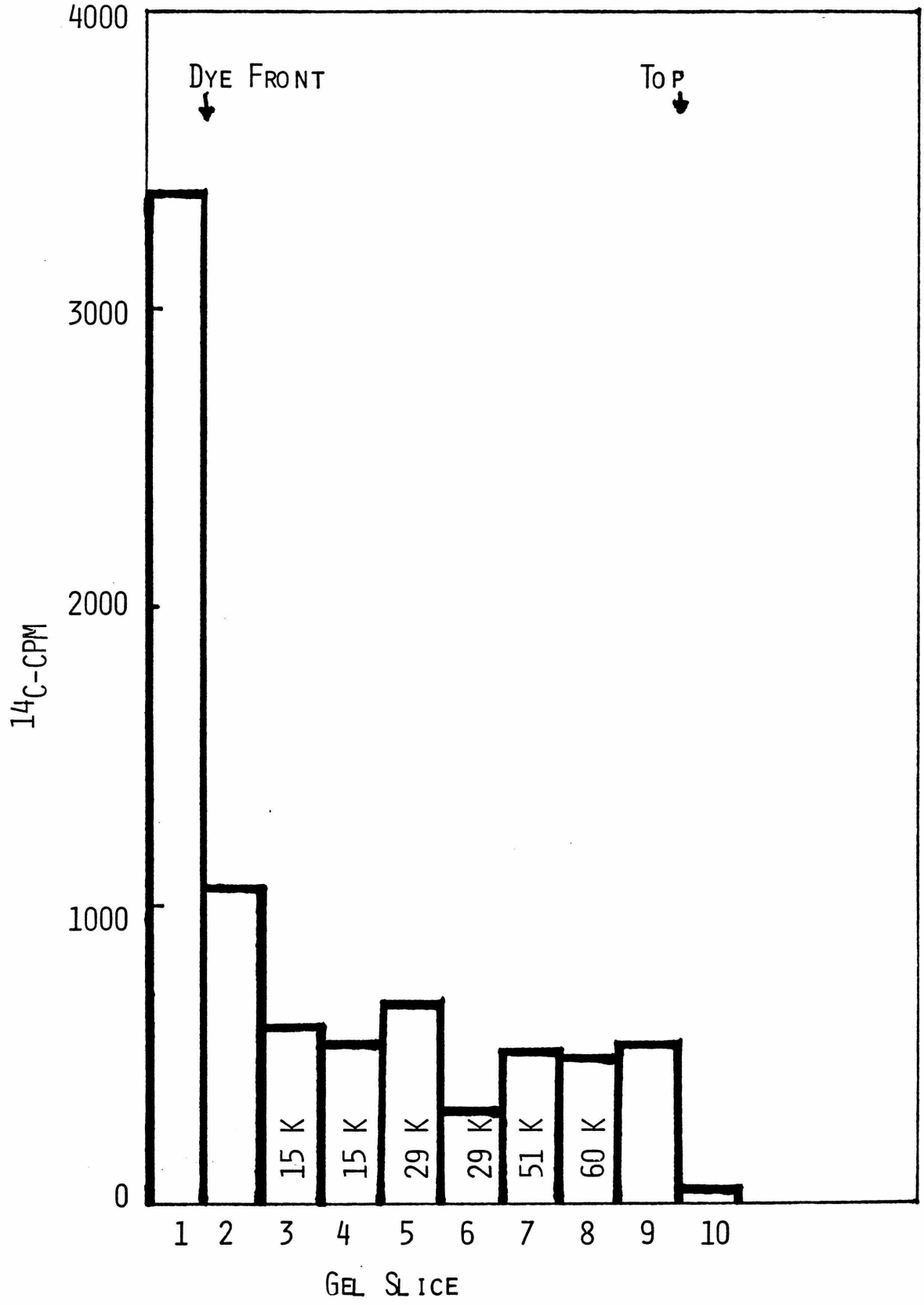

4000
3000
2000
1000
0
^{14}C-CPM
DYE FRONT
TOP
15 K
15 K
29 K
29 K
51 K
60 K
1 2 3 4 5 6 7 8 9 10
GEL SLICE

Figure 8

CHAPTER III

Tritium Isotope Effects on Catalysis by Diol Dehydratase

Introduction

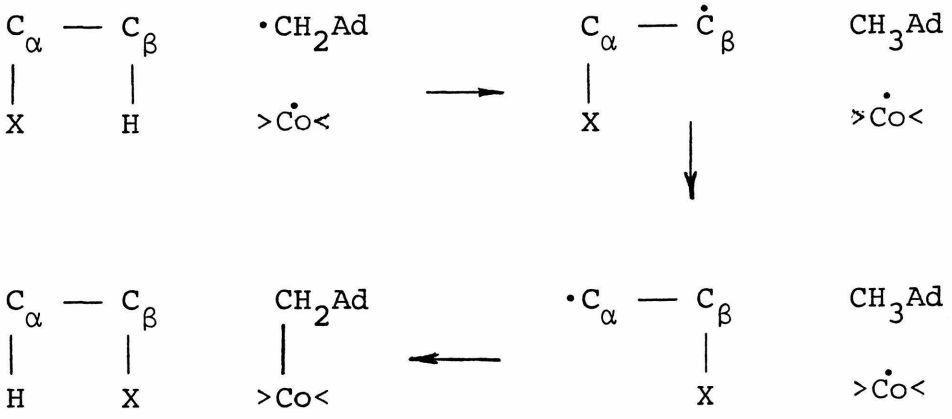
Diol dehydratase (RS-1,2-propanediol hydrolyase, E.C.4.2.1.28) is an adenosylcobalamin-dependent enzyme that catalyzes the essentially irreversible conversion of 1,2-propanediol to 1,1-propanediol which is then stereoselectively dehydrated to propionaldehyde (Zagalak *et al.*, 1966; Retey *et al.*, 1966b). In a similar fashion ethylene glycol is converted to acetaldehyde (Lee and Abeles, 1963). These reactions have many features in common with other adenosylcobalamin-dependent rearrangements (Babier, 1975) of the type shown in Scheme I, where X can be an hydroxyl,

Scheme I

alkyl, acyl, or amino group. The migration of X is known to occur intramolecularly in the reactions catalyzed by diol dehydratase (Retey *et al.*, 1966b), glutamate mutase (Barker *et al.*, 1964a,b), methylmalonyl coenzyme A mutase

(Kellermeyer and Wood, 1962; Wood *et al.*, 1964; Phares *et al.*, 1964), and ethanolamine deaminase (Babior, 1969). Moreover, the hydrogen (on C_β of the substrate) which is transferred does not exchange with water and, during the course of catalysis, apparently becomes one of three equivalent hydrogens attached to C-5' of the cofactor (Abeles and Zagalak, 1966; Retey and Arigoni, 1966; Frey *et al.*, 1967a,b; Miller and Richards, 1969; Switzer *et al*, 1969; Babior 1970; Eager *et al.*, 1972; Babior *et al.*, 1973; Moore *et al.*, 1979). These data suggest that the pathway for the migration of hydrogen involves transfer of hydrogen from C_β of substrate to C-5' of the cofactor, followed by the rearrangement of the substrate, and finally, the transfer of hydrogen from C-5' of the cofactor to C_α of the product (Scheme II). However, results of tritium isotope

Scheme II



effect experiments reported by Essenberg *et al.* (1971) were not quantitatively consistent with deuterium isotope effects studies conducted by Moore *et al.* (1979) with diol dehydratase when interpreted in light of Scheme II. A similar conflict between deuterium and tritium isotope effects was reported by Weisblat and Babior (1971) with ethanolamine deaminase.

When holoenzyme containing [C-5'-^{3}H]-adenosylcobalamin is reacted with unlabeled substrate, the rate of appearance of tritium in product seems several times too slow to be consistent with observed deuterium isotope effects. The experimentally untested explanation for the apparent discrepancy in such washout experiments between observed and predicted tritium isotope effects is that there exists an unrecognized reaction pathway (perhaps an alternate hydrogen reservoir) which does not include 5'-deoxyadenosine as an intermediate hydrogen carrier (Babior, 1979). Moreover, the observed tritium isotope effects are about three times larger than predicted (on the basis of Scheme II), suggesting that the C-5' reservoir participated in hydrogen transfer only about 33% of the time, with 67% of the reaction occurring via this alternate pathway.

Since early procedures for isolating diol dehydratase (Lee and Abeles, 1963; Poznanskaya, 1979) yielded enzyme which had been partially proteolysed (Chapter I), we decided to reinvestigate the migration of hydrogen, in the

reaction catalyzed by the pure, unproteolysed enzyme, using tritium as a trace label. First, we determined the tritium isotope effect for the transfer of hydrogen from substrate to cofactor by measuring the isotopic enrichment in unreacted [1-^{3}H]-1,2-propanediol as a function of the extent of reaction.

Next, we conducted experiments similar to those done by Frey *et al.* (1967a) where [1-^{3}H]-1,2-propanediol was reacted with diol dehydratase in the presence of relatively large amounts of unlabeled ethylene glycol. In these experiments, tritium is found at C-2 of both propionaldehyde and acetaldehyde; however, the tritium appearing in propionaldehyde must be the result of a net intramolecular transfer of tritium, for if tritium is not returned to the same three-carbon skeleton from which it was abstracted (that is, it remains in the intermediate hydrogen reservoir), the tritium will be removed from the reservoir by ethylene glycol and appear in acetaldehyde. According to Scheme II, the observed probability of net intramolecular transfer of tritium (f/n) should reflect, in part, the isotope effect for the transfer of hydrogen from cofactor to product. We found it necessary to use aldehyde trapping agents to prevent the enzyme-catalyzed exchange of tritium between acetaldehyde and propionaldehyde. This precaution was not taken in the similar experiments conducted by Frey *et al.* (1967a) which can explain why our results differ considerably from theirs.

Finally, we conducted tritium washout experiments similar to those conducted by Essenberg *et al.* (1971) where in one case, only the C-5' hydrogens of adenosylcobalamin contained tritium and, in another case, in addition to the C-5' hydrogens of adenosylcobalamin containing tritium, any other hydrogen reservoirs should have contained tritium as well. Both types of experiments yielded results which are in accord with values predicted by Moore *et al.* (1979) for the transfer of hydrogen from cofactor to product. Moreover, the results of our study suggest that the pathway containing the C-5' hydrogen reservoir is the major pathway for catalysis, although there does appear to be an alternate catalytic pathway.

Experimental

Enzyme Preparation. Diol dehydratase was isolated from *Klebsiella pneumoniae* (ATCC 8724) according to the procedure described in Chapter I and was prepared for use in the various experiments by continuous flow dialysis against appropriate buffers containing substrate or an analogue. All enzyme had a specific activity of approximately 95 units/mg (except as noted in Table II).

Substrates and Analogues. Ethylene glycol (Mallinckrodt) and 1,2-propanediol (Matheson, Coleman and Bell) were reagent grade and used without further purification.

(RS)[1-^{3}H]-1,2-Propanediol was synthesized by the

reduction of racemic ethyl lactate with [^{3}H]-$LiAlH_4$ (New England Nuclear). The distilled product had a specific activity of about 1.3 x 10^4 cpm/μmol.

[1-^{14}C]-1,2-Propanediol was purchased from Rosechem Products, Los Angeles, California.

dl-2,3-Butanediol (99%) was prepared by oxidation of cis-2-butene (61 g, 99% cis , Tridom/Fluka) which was condensed in a 2 L three-necked flask equipped with a -10°C condenser. After addition of chloroform (330 mL), a mixture of formic acid (122 g) and 30% hydrogen peroxide (135 g) was added slowly through the top of the condenser. The reaction was stirred overnight and then carefully titrated to pH 9 with 6 N sodium hydroxide. Water was removed on a rotary evaporator at 50°C and the residual sticky solid was extracted with ether in a Soxhlet apparatus. After removal of the ether, the viscous liquid was vacuum distilled giving 52.5 g (55% yield) of dl-2,3-butanediol that was approximately 99% free of the meso isomer.

dl-2,3-Butanediol (99.99%) was prepared by making the isopropylidene derivative of dl-2,3-butanediol (99%) and separating the dl- and meso-isomers by distillation with an antoannular spinning-band column (Tipson, 1948). Fractions of the distillate were analysed on a Hewlett-Packard 5880 A gas chromatograph equipped with a 30 m DB-1 column. The purified isopropylidene-dl-2,3-butanediol was 99.99% free of the meso form (the detection limit of the analyzer). The glycol was regenerated by stirring the

isopropylidene derivative with Dowex - 50W - X8 (H^+ form) in a methanolic solution at room temperature. After removal of the resin by filtration and the volatile components by rotary evaporation, the remaining viscous liquid was distilled under vacuum yielding dl-2,3-butanediol (99.99%). The diastereochemical purity of the glycol was not checked; however, formation of the meso isomer during solvolysis of the ketal should be minimal (Rubin *et al.*, 1952).

[2,8,5'-^{3}H]-5'-chloro-5'-deoxyadenosine was prepared by reacting 2.5 mCi of [2,8,5'-^{3}H]-adenosine (New England Nuclear) diluted with 2.5 mg of unlabeled adenosine in 300 µL of dry hexamethyl phosphoramide and 10 µL of thionyl chloride for 15 h (Kikugawa and Ichino, 1971). The product was purified by preparative thin layer chromatography on a silica gel plate (0.5 mm thick) impregnated with fluorescent indicators. The plate was developed with sec-butyl alcohol: water:ammonium hydroxide (100:36:14). The silica gel containing chlorinated adenosine was visualized under ultraviolet light and scraped into a beaker with a razor blade. The scrapings were washed with 4 x 100 mL of reagent acetone and the pooled washings were evaporated at reduced pressure leaving about 200 µL of a yellowish liquid (chloro-adenosine dissolved in hexamethyl phosphoramide and/or sec-butyl alcohol). The product contained about 80% of the counts initially present and no unreacted adenosine.

[2,8,5'-^{3}H]-Adenosylcobalamin was synthesized from hydroxycobalamin (Sigma) and [2,8,5'-^{3}H]-5'-chloro-5'-deoxyadenosine using a procedure similar to that of Hogenkamp and Pailes (1968). Hydroxycobalamin (25 mg) was dissolved in 6 mL of water:methanol (1:1) and deoxygenated by bubbling argon through the solution for about 30 min. Then sodium borohydride (25 mg) was quickly added to the reaction flask. Meanwhile, the yellow liquid containing the chloroadenosine was dissolved in 8 mL of water:methanol (1:1) and was deoxygenated with argon for 30 min. Once a gray-green color, characteristic of cob(I)alamin, was evident in the reaction flask, the chloroadenosine solution was added. The reaction was allowed to proceed in the dark under a continuous argon flow for 15 h. The reaction volume was then reduced to 0.5 mL at 50°C on a rotary evaporator. The crude mixture was then applied to a preparative thin layer silica gel plate (2 mm thick) which was developed in the dark at 16°C with sec-butyl alcohol:water:ammonium hydroxide (100:36:14). The band containing the tritiated coenzyme was clearly visible under red light, and was scraped from the plate. The scrapings were washed with 2 x 25 mL of water and an ultraviolet-visible spectrum of the material was obtained. The average deviation of extinction coefficients from the six absorption bands of adenosylcobalamin reported by Hogenkamp (1975) was only ±5%. The amount of material obtained was about 4 mg (2.6 μmol) which represents an overall yield of 30%. The

specific activity of the material was 1.77 x 10^8 cpm/μmol with about 20% of the tritium being at the reactive C-5' position (see Results).

Adenosylcobalamin was purchased from Sigma Chemical Co.

Assays. Diol dehydratase activity was assayed using yeast alcohol dehydrogenase (Sigma) and NADH (Sigma) to reduce propionaldehyde to 1-propanol (Bachovchin *et al.*, 1977). One unit of activity is defined as the amount of enzyme that will decompose one μmol of 1,2-propanediol per min at saturating concentrations at 37°C.

Radiochemical assays were performed by liquid scintillation counting with Aquasol-2 as the cocktail. Measurements were performed on a Beckman LS-250 scintillation spectrometer. When both carbon-14 and tritium were present in samples, corrections for channel crossover were necessary. The correction factors were determined by counting samples containing only tritium or carbon-14 radioisotopes.

Isotopic Discrimination Against Tritium in Propanediol. By measuring the increase of the $^3H/^{14}C$ ratio (Y_x) of unreacted 1,2-propanediol during catalysis by diol dehydratase one can determine the tritium isotope effect (A, equation 1). Reaction mixtures contained 100 units of diol dehydratase in 2M ethylene glycol, 0.01 M K_2HPO_4, pH 8.0, 9.08 x 10^5 cpm (70 μmol) [1-^{3}H]-1,2-propanediol, 8.33 x 10^5 cpm (9.5 μmol) [1-^{14}C]-1,2-propanediol, total volume 14 mL. The reaction mixture was incubated for 25 min at 37°C and the reaction was initiated by the addition of 0.1 mL of a 2 mg/mL solution

of adenosylcobalamin. At appropriate intervals 0.75 mL aliquots were withdrawn and added to 0.5 mL of a solution that contained 3% 2,4-dinitrophenylhydrazine, 15% sulfuric acid, 65% ethanol, and 20% water (w/v/v/v). After standing for about an hour, decolorizing charcoal (200 mg) was added to each tube. After 20 min of periodic mixing, the aliquots were centrifuged and the supernatants were filtered through glass wool plugs. Two of the aliquots were zero-reaction controls which were added to the 2,4-dinitrophenylhydrazine solution before the reaction had been initiated. Comparison of the carbon-14 counts in the time point aliquots to those in the zero controls yielded the extent of reaction. By determining the $^{3}H/^{14}C$ ratio (Y_x) for each given extent of reaction (x), the tritium isotope effect (A) was determined (Figure 1 and equation 3).

Measurement of the Probability of Net Intramolecular Transfer. Reaction mixtures contained approximately 100 units of diol dehydratase in a total volume of 14 mL of 2 M ethylene glycol, 0.01 M K_2HPO_4, pH 8.0 and [1-^{3}H]-1,2-propanediol and [1-^{14}C]-1,2-propanediol such that the propanediol to ethylene glycol molar ratio was between 1×10^{-4} and 2.5×10^{-3} and the initial $^{3}H/^{14}C$ ratio was approximately 40. After incubating the reaction mixture at 37°C for 25 min, a 1.0 mL aliquot was removed for determination of initial $^{3}H/^{14}C$ ratio (Y_o) and the amount of [1-^{14}C]-propanediol present. Catalysis was initiated by adding about 2 mg of solid adenosylcobalamin. When trapping

agents were to be employed, another 1.0 mL aliquot was transferred to a test tube in a 37°C bath 30 sec after catalysis was initiated (before the trapping agent was added); this allows one to use the 2,4-dinitrophenylhydrazine solution to remove aldehydes so that x can be determined (as described earlier). Then (if appropriate) potassium borohydride (0.18 g) or dithiothreitol (50 mg) was immediately added to the reaction mixture. The reaction was quenched by adding 200 μL of concentrated sulfuric acid to the mixture containing the trapping agent, and simultaneously the reaction in the 1.0 mL aliquot (which did not contain the trapping agent) was quenched with 250 μL of the 2,4-dinitrophenylhydrazine agent. The same amount of the 2,4-dinitrophenylhydrazine reagent was also added to the 1.0 mL zero-control. The total reaction time was varied, but about 20 min gave optimal results. Addition of concentrated acid to borohydride solutions causes severe frothing; therefore, these reactions are best done in a 250 mL or a 500 mL flask.

The [1-^{3}H]-1,2-propanediol was not stereospecifically labeled, hence only one half of the tritium was in a reactive position. To remove the unreactive tritium, the product-aldehyde (or alcohol) was oxidized under acidic conditions with potassium dichromate. After adding carrier propionic acid, the aqueous solution was extracted four times with one volume of diethyl ether. The pooled ethereal extracts were then dried over anhydrous magnesium sulfate and the ether was removed by rotary evaporation at a

temperature of 20-25°C. The remaining acids were chromatographed on silica gel (Varner, 1957) with chloroform as the eluting solvent. This system provided quantitative separation of propionic acid from acetic acid. The fractions from the column were neutralized by adding 1 mL of water containing phenolpthalein to each fraction and titrating with 0.2 N sodium hydroxide. The aqueous phases containing the acid salts were then transferred to separate tubes which were placed in an 80°C oven until the fractions had evaporated to dryness (this insures complete removal of chloroform which is a scintillation quencher). Finally, 1.0 mL of water and 10 mL of Aquasol-2 were added to each fraction and counted to an error of $\leq$5%. A weighted average for all the fractions containing propionic acid yielded the $^{3}H/^{14}C$ ratio of derivatized product (P_x) for a given extent of reaction. With A, Y_o, x and P_x determined, one can calculate the probability of net intramolecular transfer (f/n) according to equation 5.

Tritium Washout Rate from "Tritiated Holoenzyme". In order to tritiate all possible hydrogen reservoirs of diol dehydratase, approximately 100 units (4 nmol) of enzyme in 50 mM dl-2,3-butanediol (99%), 0.02 M K_2HPO_4, pH 8.0 were incubated with approximately 2 x 10^6 cpm (150 µmol) of [1-^{3}H]-1,2-propanediol under argon. Then adenosylcobalamin (3.8 mmol) was added in the dark. After three minutes, potassium borohydride (10 mg) was added to prevent exchange of product aldehyde with the hydrogen reservoir(s). Potassium

borohydride does not affect the removal of tritium from the intermediate reservoir (Essenberg *et al.*, 1971). A large excess of borohydride should be avoided, because evolution of hydrogen causes difficulty in running the subsequent column. After reacting for 1 h, the mixture (about 5 mL) was applied to a 2.6 x 20 cm Bio-Gel P-4 column that was pre-equilibrated with 50 mM dl-2,3-butanediol (99.99%), 0.01 M K_2HPO_4, pH 8.0. The high stereochemical purity of the butanediol is necessary to prevent tritium from being washed out of the hydrogen reservoir(s) by reaction with meso-2,3-butanediol (Moore and Richards, 1979). Approximately 1 mL fractions were collected in the dark and the absorbance at 280 nm was used to determine the location of the protein. The column was sufficiently long to separate the tritiated holoenzyme complex from most of the small molecules; however, some tritiated propanediol and/or propanol adhered to diol dehydratase in its native conformation. Upon denaturation, these small molecules were released into the solution yielding a high, but constant background. By using extreme care in pipetting solutions and counting samples to an error of 0.7%, reliable data were obtained. The protein-containing fractions from the column were pooled together and added to a vial containing 5 mg of alcohol dehydrogenase and 5 mg NADH. This system serves as both an aldehyde trap and as a means of assaying the catalysis rate. Approximately 60-80% of the original holoenzyme activity remained at this point. The loss of activity can be attributed to separation of

coenzyme from the protein during chromatography and/or reaction of the holoenzyme with small amounts of meso-2,3-butanediol; hence, the specific activity of the remaining active holoenzyme is unchanged. Since the appearance of tritium in the product from the tritiated holoenzyme is a first-order process, knowledge of the precise number of active sites is not critical to determine k_{3_H} (see Results).

To initiate the reaction by which tritium is removed from the hydrogen reservoir(s), 182 µL of a solution which contained 35.0 mM 1,2-propanediol, 5.56 M dl-2,3-butanediol (99.99%) was added to 900 µL aliquots of the holoenzyme solutions. Both the propanediol/butanediol and holoenzyme solutions were incubated separately at 37°C for at least 5 min before the reaction was initiated; the mixture was stirred by a teflon-coated stirring bead. Catalysis was quenched at appropriate times by the addition of 10 µL of concentrated sulfuric acid. A zero-time control was made by adding the sulfuric acid before adding the propanediol/butanediol solution.

To remove tritiated coenzyme from the solutions, activated charcoal (100 mg) was added to each time-point aliquot and the solutions vortexed, allowed to stand 10 min, vortexed again, and centrifuged. The supernatants were filtered through a glass wool plug and equi-volume aliquots were counted. This method of separating coenzyme from tritiated product is similar to the method employed by Weisblat and Babior (1971).

To determine k_{cat} under identical conditions, 140 units of diol dehydratase (5.7 mmol) apoenzyme was coupled with 3.8 nmol of adenosylcobalamin in 50 mM dl-2,3-butanediol (99.99%), 0.01 M K_2HPO_4, pH 8.0 for 20 minutes under argon. The solution was added to a vial containing 5 mg of NADH and 5 mg of alcohol dehydrogenase. The total volume was recorded and 1.80 mL aliquots were removed and incubated at 37°C for at least 5 min. By adding 364 µL of the same propanediol/butanediol mixture mentioned earlier, the rate of propionaldehyde production could be determined spectrophotometrically. Over the time interval pertinent to the washout of tritium from the holoenzyme, the catalysis rate was nearly linear. If one divides the production rate of propionaldehyde (mol/sec) by the amount of holoenzyme present (mol), one obtains k_{cat} (sec^{-1}). The holoenzyme complex was stable for at least 90 min at room temperature under these conditions; hence, no time-dependent correction factor for catalytic activity was necessary.

Tritium Washout Rate from "Tritiated Coenzyme". Diol dehydratase (5.3 nmol) was incubated under argon in 1 M dl-2,3-butanediol (99.99%), 0.1 M K_2HPO_4, pH 8.0 for 30 min at which time 1.0 nmol of unlabeled adenosylcobalamin and 1 mg of potassium borohydride were added. The mixture was then allowed to incubate for an additional 30 min at room temperature in the dark. This procedure removes most of the 1,2-propanediol or meso-2,3-butanediol that might be initially present in the solutions. If these substrates

are not removed prior to the addition of tritiated coenzyme, they could react with holoenzyme containing tritium, thus removing tritium from the C-5' reservoir prematurely. This premature removal of tritium from the reservoir is undesirable, for it would increase the background level of radiation in the solution, making it more difficult to obtain good data.

Next, [2,8,5'-^{3}H]-adenosylcobalamin (3.3 mmol) were added, and after 5 min, the solution was transferred to a vial containing NADH (6 mg) and alcohol dehydrogenase (6 mg) and the volume recorded. After an additional 5 min at 37°C, 0.182 mL of 25.0 or 50.0 mM 1,2-propanediol was added to several 0.900 mL aliquots of the holoenzyme solution. The reactions were quenched with 10 μL of concentrated sulfuric acid at appropriate times. The aliquots were then treated as in the "tritiated holoenzyme" experiment.

To determine k_{cat}, 364 μL of the appropriate propanediol solution was added to 1.8 mL of the same diol dehydratase holoenzyme solution containing alcohol dehydrogenase and NADH and the rate of propionaldehyde production was monitored spectrophotometrically (an absorbance filter was placed in the reference beam to bring the absorbance at 340 nm down to about 1.5). Comparison of values obtained for k_{cat} at the beginning and end of each experiment showed that the catalytic activity did not diminish over the time period of the experiment. After several minutes, these reactions were quenched with acid and treated as the other

time aliquots. The isotope effects thus measured are referred to as "tritiated coenzyme" isotope effects.

Results

Figure 1 shows the results of measuring the $^{3}H/^{14}C$ ratio of unreacted propanediol (Y_x) at various extents of reaction (x). Because the tritiated propanediol was not stereospecifically labeled, only one half of the tritium was in a reactive position. The equation describing such a situation is given by Melander (1960) as

$$Y_x = Y_o [0.5(1-x)^{(\frac{1}{A} - 1)} + 0.5] \quad (1)$$

which can be algebraically manipulated to yield

$$\ln [2(Y_x/Y_o)-1] = -(1-\frac{1}{A}) \ln (1-x) \quad (2)$$

When $\ln [2(Y_x/Y_o)-1]$ is plotted versus $-\ln(1-x)$ (Figure 1), A can be calculated from the slope of the line according to equation 3.

$$\text{Slope} = 1 - \frac{1}{A} \quad (3)$$

The results (for A) obtained in the presence of 2 M ethylene glycol (as described in Experimental) were nearly identical to those obtained by Moore (1979), for propanediol alone,

demonstrating that the high glycol concentrations employed in some experiments do not significantly alter the kinetic process.

The $^{3}H/^{14}C$ ratio of product (P_x), which has been oxidized to remove unreactive tritium from C-1, will vary relative to the initial $^{3}H/^{14}C$ ratio of propanediol (Y_o) according to the relation (Moore *et al.*, 1979) when only

$$P_x = 0.5\ Y_o\ (1/x)\ [1 - (1-x)^{1/A}] \qquad (4)$$

propanediol is being acted upon. However, if one carries out the reaction under conditions where there is a large amount of ethylene glycol relative to the radioactive propanediol, tritium appearing at C-2 of propionaldehyde (which is subsequently oxidized in the workup to propionate) will result only from the net intramolecular transfer of tritium (Frey *et al.*, 1967a). Hence, the appropriate equation for describing the $^{3}H/^{14}C$ ratio of the oxidized product when ethylene glycol is present as a function of the extent of reaction is given by

$$P_x = 0.5\ Y_o\ (1/x)[1-(1-x)^{1/A}]\ (f/n) \qquad (5)$$

where f/n represents the probability that a tritium abstracted from C-1 of propanediol will be returned to C-2

of the same carbon skeleton. Table I shows, if aldehyde trapping agents are not employed, that f/n, when calculated according to equation 5, appears to decrease as the extent of reaction increases. This apparent decrease can be explained by the enzyme-catalyzed exchange between [2-^{3}H]-propionaldehyde and acetaldehyde (Frey *et al.*, 1967a). Because the specific activity of propionaldehyde is higher than that of acetaldehyde, there should be a net tritium flux towards acetaldehyde, thus reducing the amount of tritium observed at C-2 of propionaldehyde. This exchange can explain the low value of f/n (approximately 0.03) observed by Frey *et al.* (1967a) who allowed the reaction of [1-^{3}H]-1,2-propanediol and ethylene glycol with diol dehydratase to go to completion in the absence of any trapping agents. With trapping agents present, the enzyme-catalyzed exchange cannot occur and f/n for tritium appears to be 0.33 $\pm$ 0.02 (Table I).

A control experiment was carried out to see if any loss of tritium could have occurred at C-2 during the oxidation process or subsequent workup used in the experiment just described. In the control experiment, propanol was oxidized in DCl/D_2O by potassium dichromate and worked up according to the same procedure, described earlier, to isolate the propionic acid. The acid was then titrated in D_2O with NaOD and evaporated to dryness in a hot oven. The sodium propionate was then taken up in D_2O and proton

magnetic resonance showed that there was no measurable incorporation (±5%) of deuterium at C-2 of propionate.

The equation describing the rate of appearance of tritium as it is removed from holoenzyme containing tritiated adenosylcobalamin by reacting with unlabeled substrate is given by

$$-dT_c/d\tau = dPT/d\tau = (CH_2T)k_{3_H} \quad (6)$$

where T_c represents the amount of tritium at C-5' of adenosylcobalamin, PT is the amount of tritiated product being produced, (CH_2T) represents the amount of holoenzyme in the solution which contains tritium at C-5', and k_{3_H} is the observed first-order rate constant. Since the transfer of hydrogen from cofactor to product is the rate-limiting step of catalysis (Moore *et al.*, 1979)

$$k_{3_H} = \frac{1}{3} k_{HT} \quad (7)$$

where k_{HT} is a rate constant describing the pathway where hydrogen is abstracted from substrate, but, due to randomization of the hydrogens in the intermediate reservoir, tritium is transferred to product. The factor of 1/3 appears because only one of three possible positions in the C-5' reservoir can be occupied by tritium (since tritium is a trace label). Moreover, T_c will equal (CH_2T),

thus after making the appropriate substitutions into equation 6, integration yields

$$T_c = T_{c,o} \exp\left(-\frac{1}{3} k_{HT}\tau\right) \quad (8)$$

When put in terms of the tritium appearance in product as a function of time (τ), the semi-log relationship becomes

$$-\ln\left(1 - \frac{T}{T_\infty}\right) = \frac{1}{3} k_{HT}\tau \quad (9)$$

where T represents the amount of tritium in product and T_∞ represents the total amount of tritium that can be washed from the coenzyme reservoir into product. Consequently, one must divide the slope obtained from a plot, such as in Figure 2, by 1/3 in order to obtain k_{HT} for a given experiment. Comparison of k_{HT} to k_{HH} (k_{cat} for protiated substrate) should yield the isotope effect for the transfer of hydrogen from cofactor to product.

Abeles (1972) showed that only the C-5' hydrogens of deoxyadenosine participate in catalysis; thus, when [2,8,5'-^{3}H]-adenosylcobalamin was added to diol dehydratase and reacted with 1,2-propanediol, only about 20% of the total radioactivity initially in the coenzyme appears in product. It is possible for hydrogen at C-8 to exchange with water under certain conditions (Bullock and Jardetzky, 1964; Bradbury and Chapman, 1972); therefore, a control

experiment was done to see if exchanged tritium from C-2 or C-8 could affect the observed appearance rate of tritium in solution during a washout experiment. By looking for protium-deuterium exchange in adenosine dissolved in D_2O at the acidic and alkaline pH's pertinent to the washout experiments, we determined that exchange at C-2 or C-8 does not occur at a rate that would affect the results. Therefore, in the "tritiated coenzyme" experiments, we observe the appearance of tritium from only the C-5' reservoir.

Figure 2 shows results for the washout of tritium from "tritiated holoenzyme" with data from the other washout experiments summarized in Table II. One can see that both "tritiated holoenzyme" and "tritiated coenzyme" experiments yielded similar results when native enzyme was used; however, when proteolysed enzyme was used in the "tritiated holoenzyme" experiments, nearly a two-fold increase in the observed tritium isotope effect was obtained. Although the magnitude of the observed isotope effect was not as large as that observed by Essenberg *et al.* (1971) in "tritiated coenzyme" experiments, the change was in the direction of their result. Hence, the large isotope effects that they observed were not necessarily due to any experimental error on their part (although the methods used to measure the isotope effects were somewhat different), but were perhaps due to some change in the mechanism of catalysis when the enzyme is partially proteolysed. The nature of the change in mechanism will be considered in the Discussion.

Discussion

The Tritium Isotope Effect on the Transfer of Hydrogen from Substrate to Cofactor. The first set of experiments involves the measurement of the increase in the $^{3}H/^{14}C$ ratio (Y_x) in unreacted 1,2-propanediol as a function of the extent of reaction. In one case, 1,2-propanediol was the only substrate present; in the other case, 2 M ethylene glycol was present as well as a much lower concentration of 1,2-propanediol. As shown in Figure 1, identical results were obtained in both cases, suggesting that the high glycol concentrations employed in some experiments do not significantly alter the kinetic process. The observed isotope effect of A = 6.1 $\pm$ 0.5 for the conversion of [1-^{3}H]-1,2-propanediol to [2-^{3}H]-propionaldehyde by diol dehydratase is comparable to the value of 5.8 observed by Weisblat and Babior (1971) for the conversion of [1-^{3}H]-ethanolamine to [2-^{3}H]-acetaldehyde by ethanolamine deaminase.

To determine the meaning of A, one needs to consider Scheme III, shown below, which is based on Scheme II, given

Scheme III

$$SH + E \xrightarrow{k_{1H}} (H_3) \xrightarrow{k_{2H}} E + PH$$

$$ST + E \xrightarrow{k_{1T}} (H_2T) \xrightarrow{\frac{2}{3}k_{2H}} E + PH$$

$$(H_2T) \xrightarrow{\frac{1}{3}k_{2T}} E + PT$$

in the introduction of this chapter. In Scheme III, E represents free enzyme, ST and SH represent substrate with and without tritium, (H_3) and (H_2T) represent the composition of the intermediate hydrogen reservoir, and PT and PH represent product with and without tritium. The rate constants (which are not necessarily microscopic) describe the first and second hydrogen transfers, with the isotope that is transferred being denoted in the subscript by H or T. The first hydrogen transfer is essentially irreversible, for tritium initially in the C-5' reservoir has never been observed in free substrate with diol dehydratase. The second hydrogen transfer is somewhat reversible, because Frey *et al.* (1967a) observed that propionaldehyde could remove tritium from the C-5' reservoir. By reducing product-aldehyde to an alcohol, the tritium exchange between product and the C-5' reservoir can be stopped.

Scheme III assumes that there is no significant isotope effect on the Michaelis constant of propanediol upon isotopic substitution at C-1. Moore (1979) observed that 1,1-d_2-1,2-propanediol had the same Michaelis constant as propanediol, so this is a reasonable assumption.

At steady state, the following equations apply to Scheme III:

$$\frac{dPH}{dt} = k_{2H} (H_3) = X_{SH} k_{1H} (E) \qquad (10)$$

$$\frac{dPT}{dt} = \frac{1}{3} k_{2T} (H_2T) = X_{ST} k_{1T} (E) \qquad (11)$$

Since ST is stoichiometrically insignificant in relation to SH, the amount of PH produced by reaction of the holoenzyme with ST was ignored in writing equation 10. The parameters X_{SH} and X_{ST} represent the mole fractions of SH and ST, respectively. Since X_{SH} is proportional to the ^{14}C counts in unreacted propanediol and X_{ST} is proportional to the ^{3}H counts in unreacted propanediol;

$$\frac{dPT}{dPH} = Y_x \frac{k_{1T}}{k_{1H}} , \tag{12}$$

therefore, A pertains only to the first hydrogen transfer and is given by

$$A = \frac{k_{1H}}{k_{1T}} . \tag{13}$$

The Probability of Net Intramolecular Transfer of Tritium. To determine f/n (from equation 5), [1-^{3}H]-1,2-propanediol was reacted with diol dehydratase in the presence of relatively large amounts of unlabeled ethylene glycol. Under these conditions, tritium appearing at C-2 of propionaldehyde should result from tritium being transferred from C-1 of substrate into the intermediate hydrogen reservoir followed by transfer of tritium from the reservoir back to C-2 of the molecule from which it was initially abstracted (Frey *et al.*, 1967a). If tritium is not transferred back

to the same three-carbon skeleton from which it originated, the tritium will be removed from the reservoir in subsequent turnovers by ethylene glycol and the tritium will appear in acetaldehyde.

Frey *et al.* (1967a) observed a value of f/n which was about 0.027 when the reaction with [1-^{3}H]-1,2-propanediol and unlabeled ethylene glycol was allowed to go to completion in the absence of aldehyde-trapping agents. Exchange of tritium between propionaldehyde and acetaldehyde could lower the observed value of f/n. As shown in Table I, the apparent values of f/n do decrease as the extent of reaction increases when trapping agents, which would prevent this exchange, are absent from the reaction mixture. When trapping agents are present, f/n appears to be 0.33 $\pm$ 0.02. Scheme III predicts that

$$f/n = \frac{k_{2T}}{k_{2T} + 2k_{2H}} \qquad (14)$$

which suggests that, according to equation 14, $k_{2H} = k_{2T}$ when f/n = 0.33. However, Moore *et al.* (1979) determined that the second hydrogen transfer was the rate-limiting step of catalysis and calculated the isotope effect for this transfer to be 10.8 for deuterium. According to the Swain relationship (Swain *et al.*, 1958) k_{2H}/k_{2T} should be about $(10.8)^{1.44}$ or 31; therefore, f/n should equal about 0.016. Consequently, Scheme III (as well as Scheme II) appears to

be inadequate in describing the mechanism of action. Before consideration of an alternate kinetic scheme, it will be useful to examine the results of the washout experiments.

Tritium Washout Experiments. To test the hypothesis that there exists an unrecognized pool of enzyme-bound hydrogens (Babior, 1979) which could function in a manner similar to the C-5' hydrogens of adenosylcobalamin, "tritiated holoenzyme" (see Experimental) was prepared by reacting holodiol dehydratase with (RS)-[1-^{3}H]-1,2-propanediol. Then, the appearance rate of tritium in product was measured relative to propionaldehyde production when the intact "tritiated holoenzyme" complex was allowed to react with unlabeled propanediol. This experiment should yield a tritium isotope effect for all potential hydrogen reservoirs. These results were then compared to those of a similar experiment where the only source of tritium is adenosylcobalamin which has been synthetically (or enzymatically, as was done by Essenberg *et al.*, 1971) prepared to contain tritium at C-5'. This "tritiated coenzyme" experiment should yield a tritium isotope effect for the C-5' reservoir only. If another hydrogen reservoir exists, the observed tritium isotope effect for "tritiated holoenzyme" should be significantly smaller than that observed in the "tritiated coenzyme" experiments, because all of the reservoirs will contain tritium which could participate in catalysis, as opposed to just the single C-5' reservoir. Moreover, the magnitude of the isotope effect for "tritiated holoenzyme" should agree

with the predicted value (k_{HH}/k_{HT} = 31) of Moore *et al.* (1979). In contrast, if the C-5' hydrogen reservoir is the only reservoir, both experiments should give the same isotope effect.

Table II shows that for native enzyme the same tritium isotope effect is observed in both "tritiated holoenzyme" and "tritiated coenzyme" experiments. Moreover, when the data are interpreted according to equation 9, one obtains a value of k_{HH}/k_{HT} (29 $\pm$ 2) which is in excellent agreement with the predicted value (31) based on Scheme II. Therefore, it appears that the C-5' hydrogens of adenosylcobalamin are the only holoenzyme-hydrogens which participate directly in catalysis in the native enzyme.

The values for $k_{cat}/k_{^3H}$ reported in Table II for native enzyme are nearly threefold less than the values reported by Essenberg *et al.* (1971) for proteolysed enzyme and nearly twofold less than our measurements carried out with proteolysed enzyme. Since Scheme II cannot account for this apparent increase in the isotope effect, it appears that there may be an alternate kinetic pathway which does not directly involve the C-5' hydrogen reservoir as an intermediate in catalysis. Moreover, it appears that proteolysis of the enzyme can cause the alternate pathway to make a more significant contribution to catalysis.

Mechanism of Action. In formulating a possible mechanism of action, we need to take account of the phenomena

to be explained. The first is the large value of f/n (0.33 as opposed to the predicted value of 0.016) observed when tritiated propanediol is reacted with the holoenzyme in the presence of large amounts of ethylene glycol. The second is the extraordinarily large isotope effects observed in the washout experiments with proteolysed enzyme. The third is the close agreement of observed isotope effects, in "tritiated holoenzyme" and "tritiated coenzyme" washout experiments with native enzyme, to those predicted by Scheme II. Moreover, the modified mechanism of action should be consistent with previous work.

The kinetic scheme shown in Figure 3 seems to adequately account for all of the phenomena observed with diol dehydratase. As drawn, the scheme describes catalysis when tritiated propanediol is the substrate. The alternate pathway, described by steps 1-4 in Figure 3, deviates from the normal catalytic pathway, described by Scheme II, when the C-5' radical, generated by homolysis of the carbon-cobalt bond in adenosylcobalamin, abstracts hydrogen from some group RH (perhaps a free sulfhydryl group) on the protein instead of the tritium on C-1 of substrate. The R radical then abstracts tritium from C-1 of substrate generating a substrate radical which undergoes the usual rearrangement to a product-like radical. Finally, the same tritium that was initially abstracted from C-1 is always returned to its original three-carbon skeleton at C-2 since the R reservoir only contains one hydrogen (or tritium). This mechanism assumes

that hydrogen can be returned to product only from the reservoir which originally received hydrogen from C-1 of substrate. It is also assumes that the reverse of step 1 of the alternate pathway is slow relative to k'_{1T}.

Once hydrogen has been returned to product (step 4 in Figure 3), it is unclear whether the R radical abstracts hydrogen from C-5' (essentially step 5) after the product is released, or whether it can remain until another substrate molecule binds. If the R radical can remain after product is released, several catalytic cycles through the alternate pathway could be obtained from the formation of a single R radical, thus amplifying the events where hydrogen is abstracted from RH by the C-5' radical.

The kinetic scheme in Figure 3 can account for the observed value for f/n of 0.33 if, when tritiated propanediol is the substrate in the presence of large amounts of ethylene glycol, catalysis follows the alternate pathway one third of the time. If this is the case, one third of the reactive tritium initially present at C-1 of propanediol will be found at C-2 of propionaldehyde, for the alternate pathway must always return the tritium that it abstracts from substrate to product. The remainder, two thirds, of the reactive tritium in substrate will then follow the pathway described by Scheme III; as predicted by equation 14, only about 1/60th of this tritium will be detected in product from this pathway. Therefore, the observed tritium at C-2

of propionaldehyde will be, for the most part, a result of catalysis via the alternate pathway.

When tritiated propanediol is the substrate, step 1 of the alternate pathway is a hydrogen abstraction from RH of the protein and this competes with an abstraction of tritium from C-1 of the substrate in the normal pathway. Since the tritium isotope effect on the first hydrogen transfer is about 6.1 (this assumes that k_{1T} is about the same as k'_{1T}), the presence of tritium at C-1 of substrate will enhance the relative amount of the R radical formed by at least sixfold over the amount formed when protium is at C-1 of substrate. Since roughly one third of the turnovers occur via the alternate pathway when tritiated propanediol is being acted upon, this suggests that the alternate pathway contributes only about 33/6.1 = 5% to catalysis when protiated substrate is being acted upon by native enzyme. In other words, about 95% of catalysis occurs via Scheme II when diol dehydratase acts on unlabeled substrate.

Since 95% of catalysis occurs via Scheme II when unlabeled propanediol is the substrate, the agreement of the results obtained from washout experiments, with native enzyme, with the results predicted on the basis of Scheme II is understandable. Moreover, if proteolysis of the enzyme caused the formation of the R radical to be more favorable, this could account for the larger isotope effects observed in washout experiments where proteolysed enzyme was used.

Presumably, proteolysis could affect the protein conformation so that k_H (which describes step 1 of the alternate pathway) increases relative to k_{1T} (or k_{1H} in the normal catalytic pathway), thus increasing the fraction of catalytic events that would not directly involve the C-5' reservoir.

Previous work which remains to be discussed in light of the newly proposed mechanism of action include experiments where deuterated substrate is being acted upon and inactivation studies with glycol analogues of propanediol. When deuterium is at C-1 of propanediol, initially there will be some increase in the contribution of the alternate pathway to catalysis over its contribution when protium is at C-1. Moore *et al.* (1979) calculated that the deuterium isotope effect on the first hydrogen transfer is about 2.2 which suggests that initially the alternate pathway will contribute 33/2.2 = 12% to catalysis. However, once steady state has been reached, the C-5' reservoir will become fully deuterated, suggesting that eventually the R radical will have to abstract deuterium from the C-5' (in step 5) reservoir, thus deuterating the alternate hydrogen carrier of the protein. When this occurs, the competing steps between the alternate and normal pathways will be described by k_D and k_{1D}, respectively. Assuming the deuterium isotope effects are about the same, one would expect to see the same contribution of the alternate pathway to catalysis at steady state when deuterated propanediol is being acted upon as when protiated propanediol is being acted upon. However, if the deuterium isotope effect

on step 1 of the alternate pathway was larger than the isotope effect on the transfer of hydrogen from substrate to cofactor via the normal pathway, one might observe less contribution of the alternate pathway to catalysis than when protiated substrate is acted upon.

For example, Essenberg *et al.* (1971) observed a value of k_{cat}/k_{3_H} of 250 when tritium was washed from the C-5' reservoir by reaction of proteolysed holoenzyme with unlabeled substrate and a k^D_{cat}/k_{3_H} of 14 when tritium was removed from the reservoir by deuterated substrate. The value of 250 with protiated substrate is about 2.8 times larger than predicted from Scheme II, but the value of 14 with deuterated substrate is only about 1.6 times larger than predicted ($\frac{93}{10.8} = 8.6$) from Scheme II. Assuming that a larger deviation from the predicted isotope effects suggests a larger contribution of the alternate pathway to catalysis, it appears that less of a contribution to catalysis is made by the alternate pathway when deuterated substrate is being acted upon. This suggests that $k_H/k_D \geqslant k_{1H}/k_{1D}$ for the mechanism shown in Figure 3. This being the case, when deuterated substrate is acted upon by enzyme, catalysis proceeds essentially by the normal catalytic pathway. Therefore, observed deuterium isotope effects with native enzyme pertain to the normal catalytic pathway.

As discussed in Chapter II, analogues of propanediol act as inactivators of diol dehydratase. Although it was not

necessary to invoke an alternate catalytic pathway to account for the observed isotope effects and other data which pertain to the inactivation reactions, the presence of a minor alternate catalytic pathway, whose contribution to catalysis might be affected by protein conformation or possibly substrate structure, suggests that this alternate pathway might somehow be related to the inactivation reactions.

Future Studies. It might be possible to gain more information about the alternate kinetic pathway by doing experiments similar to those conducted by Miller and Richards (1969) with methylmalonyl coenzyme A mutase or Eager *et al.* (1972) with glutamate mutase. These experiments would involve reacting 1,1-dideuterio-2-^{13}C-1,2-propanediol with diol dehydratase in the presence of relatively large amounts of unlabeled 1,2-propanediol, followed by analysis of the products by mass spectrometry. From such experiments, it should be possible to determine f/n for deuterium. The kinetic scheme in Figure 3 would predict that f/n for deuterium would be less than 0.33, since the isotope effect on the abstraction of deuterium from C-1 of substrate will be smaller than the isotope effect for tritium.

Another useful experiment would be to measure the value of f/n for tritium when a small amount of [1-^{3}H]-1,2-propanediol is reacted with diol dehydratase in the presence

of large amounts of d_4-ethylene glycol. The deuterated ethylene glycol should eventually deuterate the R group of the protein which is proposed to be an intermediate in the alternate pathway. Therefore, the contribution of the alternate pathway should be governed by the ratio of k_D (step 1) to k_{1T}, which predicts that f/n will be significantly smaller in this experiment (the magnitude of f/n will depend on k_H/k_D (step 1) relative to k_{1H}/k_{1T} for the normal pathway).

Theoretically, one could tritiate the R group of the protein by reacting the enzyme with labeled substrate (no ethylene glycol or other unlabeled substrate being present). In practice, it could be very difficult to incorporate measurable amounts of tritium into the protein since one can never really fully tritiate the C-5' reservoir (from which the R radical would have to abstract tritium). Moreover, spurious labeling of the protein by product-aldehyde (described in Chapter II) would complicate matters further.

It would also be interesting to determine f/n for tritium for a number of substrate analogues. As discussed in Chapter II, the number of catalytic events per inactivation event with substrate analogues is usually very high. Thus, reacting substrate analogues which contain tritium at C-1 in the presence of large amounts of 1,2-propanediol might yield information about the effects on substrate structure on the contribution of the putative alternate pathway to catalysis.

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Table I. Determination of the Probability of Net Intramolecular Transfer[a]

Propanediol / ethylene glycol	x[b]	f/n[c]	Trapping Agent
2.5×10^{-3}	0.76	0.24	None
"	0.87	0.23	None
"	0.98	0.12	"
"	0.994	0.09	"
"	0.70	0.31	DTT[d]
"	0.55	0.32	DTT
1.0×10^{-4}	0.65	0.35	KBH_4

[a]Determinations made as described in Methods. [b]The extent of reaction at which catalysis was quenched. [c]The apparent probability of net intramolecular transfer when calculated according to equation 5. [d]Dithiothreitol seems to alkylate aldehydes under the conditions of the experiment.

Table II. Rates of Tritium Transfer and Product Formation for Diol Dehydratase[a]

Propanediol (mM)	Reservoirs[b]	Enzyme[c]	k_{cat} (s^{-1})	k_{3_H} (s^{-1})	$\frac{k_{cat}}{k_{3_H}}$	$\frac{k_{HH}}{k_{HT}}$
5.9	All	Proteo.	2.76	0.0156	177	--[d]
"	"	"	2.76	0.0160	173	--
"	"	Native	2.74	0.0290	94.5	31.5
"	"	"	2.99	0.0333	89.8	29.9
"	"	"	2.95	0.0315	93.7	31.2
4.1	C-5'	"	2.03	0.0259	78.3	26.1
4.1	"	"	1.99	0.0236	84.3	28.1
8.2	"	"	3.96	0.0467	84.9	28.3

[a]All measurements were made at 37°C in the presence of 1 M dl-2,3-butanediol (99.99%). [b]"All" denotes experiments using "tritiated holoenzyme" and "C-5'" denotes those using "tritiated coenzyme" (see Methods). [c]"Native" means that pure, unproteolysed enzyme was used. "Proteo." denotes the use of a nonhomogeneous mixture of protein containing diol dehydratase which elutes from DEAE-cellulose at high salt concentrations during the purification of diol dehydratase (McGee and Richards, 1981). [d]Since the appropriate statistical factor for this preparation is unknown k_{HH}/k_{HT} is not given.

Figure 1. The $^{3}H/^{14}C$ ratio of nonstereospecifically labeled [1-^{3}H]-1,2-propanediol (Y_x) was measured at various extents of reaction (x) and plotted according to equation 2. The circles denote measurements made in the presence of 2 M ethylene glycol as described in Experimental. Triangles denote measurements made by Moore (1979) where propanediol was the only substrate present in the reaction mixture. In the latter case, the extent of reaction was determined by the colorimetric assay of propionaldehyde 2,4-dinitrophenylhydrazone by the method of Eager *et al.* (1975).

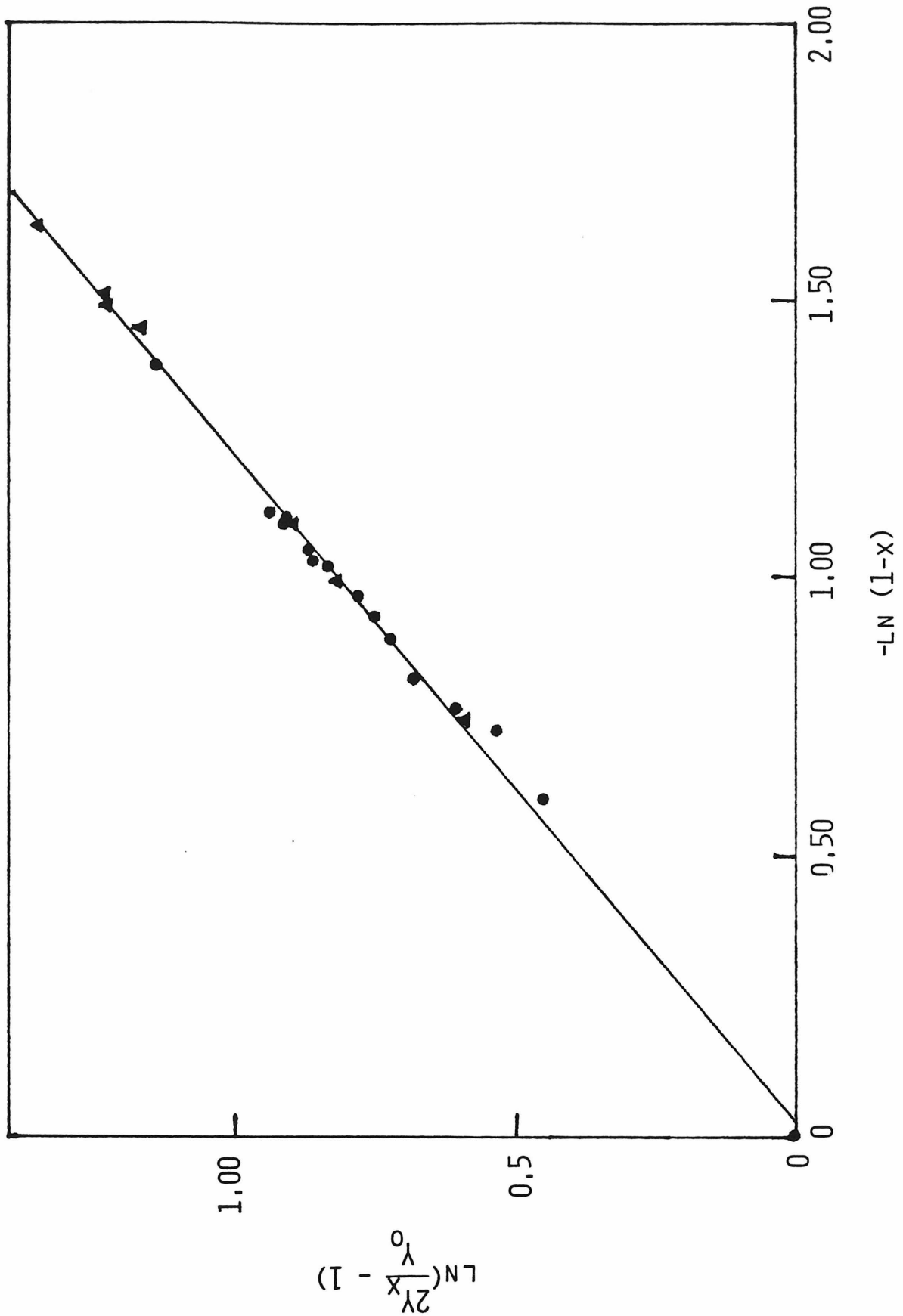


Figure 1

Figure 2. Shown are the results for a "tritiated holoenzyme" washout experiment. The points represent the data plotted according to equation 9 with the error bars denoting the possible effects of counting error on the value of the points.

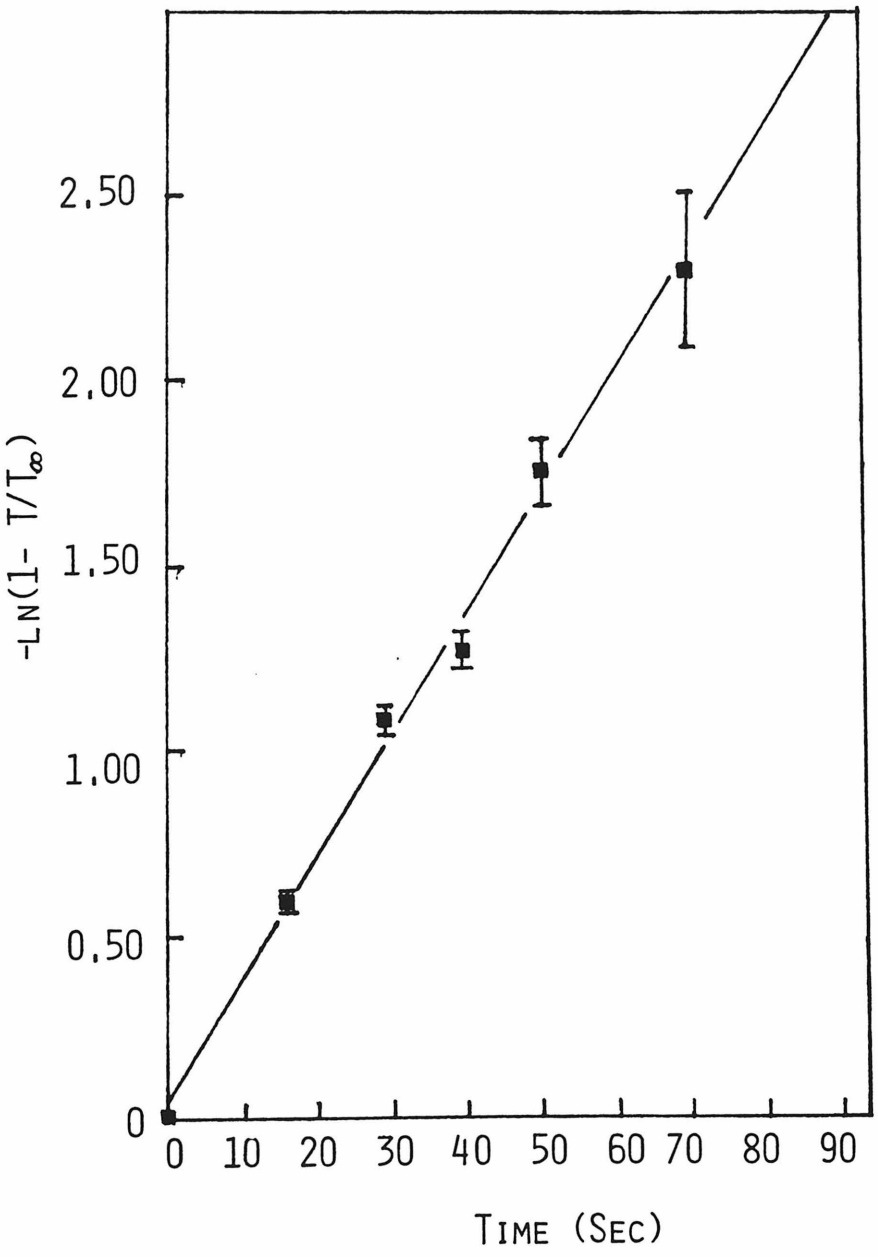


Figure 2

Figure 3. This figure shows the modified kinetic scheme proposed to account for the observed data in this study. As shown, diol dehydratase acts on tritiated substrate (ST) to produce either tritiated product (PT) or unlabeled product (PH). The active site of the holoenzyme is drawn to contain adenosylcobalamin and some group on the protein (RH) which can also function as an intermediate hydrogen carrier in the alternate kinetic pathway (given by steps 1-4). The constants k_{on} and k_{off} describe the binding and release of the substrate, respectively. Once substrate is bound to the enzyme, it can either follow the normal catalytic pathway, previously described by Schemes II and III, where tritium is abstracted from substrate by the C-5' radical according to the rate constant k_{1T}, or it can follow the alternate kinetic pathway, described by steps 1-4 in the figure, by the C-5' radical abstracting protium from RH of the protein according to the rate constant k_H in step 1. Once formed, the R radical then abstracts tritium from substrate (step 2) followed by the usual rearrangement of the substrate (step 3). Finally, the product radical obtains tritium from the protein (RT) in step 4. This mechanism assumes that hydrogen can be transferred to product only from the intermediate hydrogen carrier (RT or C-5') which intially abstracted the hydrogen from substrate. Once PT is formed, it can be released from the enzyme (not shown).

Step 5 depicts the abstraction of hydrogen (or perhaps tritium in some cases) from the C-5' reservoir by R· which

provides a pathway whereby catalysis can return to its normal process. If step 5 is slow, which would suggest that step 1 is not readily reversible, it may be possible for PT to be released from the enzyme and still have R• present when another substrate molecule binds to the enzyme. Such a case could allow several cycles to proceed through the alternate pathway for each R• formed in step 1.

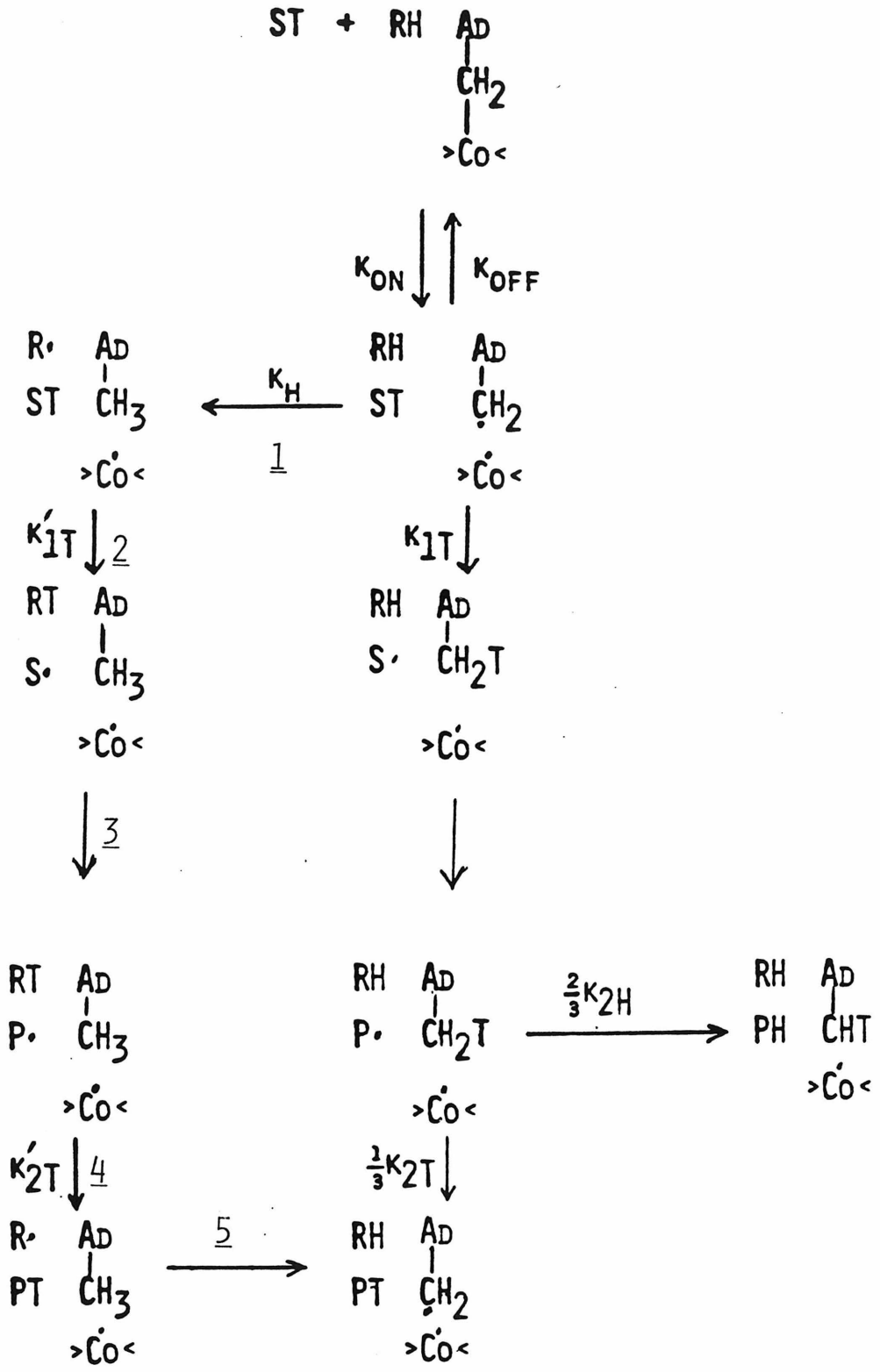

ST + RH AD
CH2
>Co<
KON
KOFF
R· AD
ST CH3
>Co<
KH
1
RH AD
ST CH2
>Co<
K'1T
2
K1T
RT AD
S· CH3
>Co<
RH AD
S· CH2T
>Co<
3
RT AD
P· CH3
>Co<
RH AD
P· CH2T
>Co<
2/3K2H
RH AD
PH CHT
>Co<
K'2T
4
1/3K2T
R· AD
PT CH3
>Co<
5
RH AD
PT CH2
>Co<

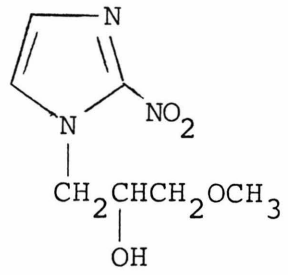
Figure 3

PROPOSITION I

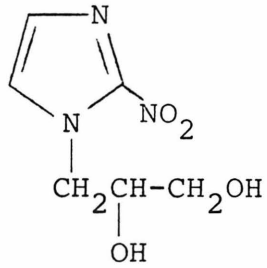
In cancer radiotherapy, the relative radioresistance of hypoxic cells present in some tumors (Thomlinson, 1960; Hewitt *et al.*, 1967) presents a serious limitation in attempts to maximize the therapeutic ratio between tumor control and normal tissue damage. Accordingly, many chemical compounds have been tested to determine their effectiveness in sensitizing tumor cells to the lethal effects of ionizing radiation (Adams, 1973). A great majority of these sensitizers are effective against only hypoxic cells, with their mode of action resembling that of oxygen. The relative efficiencies of these sensitizers have been shown to relate directly to the electron affinities of these compounds (Adams and Dewey, 1963; Adams and Cooke, 1969; Raleigh *et al.*, 1972). These observations have been the basis for in vivo and in vitro studies of nitroiomidazoles (some are shown in Figure 1) as a class of radiation sensitizers of potential clinical interest (Denekamp and Harris, 1974, 1976; Brown, 1975; Sheldon *et al.*, 1976; Asquith *et al.*, 1974; Petterson, 1978; Griffin *et al.*, 1979; Dische *et al.*, 1980; Dische *et al.*, 1981b).

Both misonidazole (Thomlinson *et al.*, 1976) and desmethylmisonidazole (Dische *et al.*, 1981a,b) (shown in Figure 1) have been tested clinically, and these drugs do

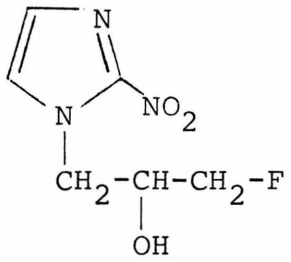
Figure 1: Shown are some nitroimidazoles that are being clinically tested as radiation sensitizers. The labels - Ro-07-0582, Ro-05-9963, and Ro-07-0741 - are designations used by Hoffmann-La Roche, Inc. who produces these compounds.



Misonidazole
Ro-07-0582



Desmethylmisonidazole
Ro-05-9963



Ro-07-0741

Figure 1

indeed increase response of various tumors to radiation. However, there is a neurological disorder which developes as a side effect of the use of these drugs, thus compromising their effectiveness (Dische *et al.*, 1977; Urtrasun *et al.*, 1978).

Histological studies of the pathological symptoms observed with several nitroimidazoles have shown that there is a good deal of similarity between nitroimidazole-induced neuropathy and thiamine defficiency (Sharer, 1972; Parkes, 1976; Rogulja *et al.*, 1973; Griffin, 1979). Since thiamine is poorly absorbed into the central nervous system (Cohen, 1962) and poorly by the gastro-intestinal tract (Thomson, 1966; Thomasula and Kates, 1968), it must be actively transported. Interestingly, a class of thiamine antagonists which contain an imidazole ring, instead of the normal thiazole ring of thiamine, have been shown to hinder thiamine uptake in microorganisms (Kurata *et al.*, 1967).

Structural similarity between the nitroimidazoles and thiamine and the pathological symptoms associated with the drug use implicate the interference of the drugs with thiamine metabolism. What is not clear is whether the drugs require the thiamine transport system to be effective as radiation sensitizers or if the associated neuropathy is merely a side effect which could be overcome by administration of large doses of thiamine.

To resolve this question, I propose studying the effects of misonidazole and desmethylmisonidazole in

addition to 2,5-dinitroimidazole, 3-nitro-2-methylthiopyrrole and 1-methyl-2-nitromimidazole (Rupp *et al.*, 1978) on the transport of thiamine as carried out by Cohen (1962) or Thomson *et al.* (1970). Concurrently, the effectiveness of the durgs as radiation sensitizers in tumored mice would be studied. In E. coli, Rupp *et al.* (1978) found that 2,5-dinitroimidazole, 3-nitro-2-methylthiopyrrole and 1-methyl-2-nitroimidazole were all more effective as radiation sensitizers than is misonidazole, with 2,5-dinitroimidazole showing much lower toxicity and mutagenicity. A correlation between inhibition of thiamine transport and effectiveness as a sensitizer would suggest that the drugs were being actively transported by the thiamine system. If large amounts of thiamine did not significantly alter the effectiveness of the drug as a sensitizer, this would suggest that the drug was being transported passively or by some other system as well. Such studies could also suggest structural modifications which could be made on the drugs to enhance their effectiveness and minimize unwanted side effects.

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Raleigh, J.A., Chapman, J.D., Borsa, J., Kremers, W. and Ruever, A.P. (1972). Int. J. Radiat. Biol. *23*, 377.

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PROPOSITION II

All adenosylcobalamin-dependent enzymes appear to contain more than one subunit (Babior, 1975); however, the roles for the individual subunits are not understood. It would be of particular interest to know with certainty which subunit(s) bind the coenzyme and/or pariticpate directly in catalysis. There is considerable evidence, obtained through studies employing analogues of the adenosylcobamides, that these enzyme bind both the adenosyl group and the corrin ring with considerable avidity (Smiley and Sobolov, 1962; Lee and Abeles, 1963; Pawelkiewicz and Zagalak, 1964; Kellermeyer *et al.*, 1964; Cannata *et al.*, 1965; Blakely, 1966; Tomao *et al.*, 1967; Morely *et al.*, 1968; Babior *et al.*, 1969; Carty *et al.*, 1971; Yamada *et al.*, 1971; Hamilton *et al.*, 1971; Uchida *et al.*, 1973; Hull *et al.*, 1975; Toraya *et al.*, 1977; Rosendahl *et al.*, 1982). Therefore, by preparing photoaffinity labels which are analogues of adenosine or cobalamin, it may be possible to covalently link these labels to specific subunits of adenosylcobalamin-dependent enzymes and eventually determine individual amino acids involved in enzyme-cofactor interactions. This use of photoaffinity labels is being developed in many different systems (Vaughan and Westheimer, 1969; Converse and Richards, 1969; Katzenellenbogen *et al.*, 1974; Macfarlane *et al.*, 1982).

Before preparing the radiolabeled photoaffinity labels, an initial set of screening experiments should be carried out to determine the binding affinities of the analogues to the enzyme. These could be determined by measuring the inhibition-effect of the analogues on the rate of formation of the holoenzyme complex using the procedure of Toraya *et al.* (1977). The most promising analogues may prevent the holoenzyme complex from forming altogether when preincubated with the apoenzyme before the addition of the coenzyme (Toraya *et al.*, 1977).

Two analogues of adenosine which could be of interest are 8-azidoadenosine and 2-azidoadenosine. Both nucleotides have been used as photoaffinity labels for different proteins (Jovin *et al.*, 1969a,b; Moses *et al.*, 1970; Knippers, 1970; Kornberg and Kornberg, 1974; Macfarlane *et al.*, 1982). Since 8-azidoadenosine is readily reduced to 8-aminoadenosine by dithiothreitol and other dithiols (Cartwright *et al.*, 1976), care should be taken to remove dithiols commonly included in buffers for the adenosylcobalamin-dependent enzymes which, typically, are sensitive to oxidation (Babior, 1979); if necessary, 2-mercaptoethanol, a monothiol, could be used in place of dithiothreitol. A potential complication with 2-azido-adenosine stems from the tendency of 2-azidopurines to undergo rearrangement to tetrazole forms (Temple *et al.*, 1966); however, Macfarlane *et al.* (1982) have studied this rearrangement in detail and managed to overcome related complications.

The sensitivity of the azido group to reduction precludes the possibility of alkylating cob(I)alamin with a 2- or 8-azidoadenosine derivative directly, for these reactions are carried out in the presence of borohydride (Johnson *et al.*, 1963). It might be possible to introduce the azido functionality into the adenosine ring by reacting 8-bromoadenosylcobalamin with sodium azide in dimethyl sulfoxide. Similar conditions were employed by Houstek and Smrt (1979) to prepare 8-azidoadenosine from 8-bromoadenosine. In other solvents, tri-n-octylamine azide (Haley and Hoffman, 1974) or tetramethylguanidinium azide (Cartwright *et al.*, 1976) have been used in place of sodium azide.

The 8-bromoadenosylcobalamin could be prepared by chlorinating or brominating 8-bromoadenosine at the 5' position by the method of Kikugawa and Ichino (1971), and then alkylating cob(I)alamin as described by Hogenkamp (1975). Yields might be lower since cob(I)alamin might displace the halogen from the adenine ring instead of attacking at the 5'-position of the ribose moiety (Babior, 1979). It should, however, be fairly easy to separate the products by chromatography on silica gel.

It might also be possible to prepare analogues of cobalamin which contain a photoaffinity label. For example, derivatives of 5,6-dimethylbenzimidazole (the normal β ligand of cobalt in adenosylcobalamin) might be used as the β ligand of cobalt in cobamide. In adenosylcobalamin the base is reversibly ligated to the cobalt, with binding being

just a matter of having the appropriate pH (Babior, 1979), hence it should be fairly easy to attach an appropriate 5,6-dimethylbenzimidazole analogue to the cobalt in cobamide (the corrin ring of adenosylcobalamin). For additional binding affinity, the α ligand of the cobalt could be 5'-deoxyadenosine. Similar adenosylcobamides have been used in studying ribonucleotide reductase (Blakeley, 1965; Vitols *et al.*, 1967; Morely *et al.*, 1968; Blakeley, 1966).

The 8-azido derivatives of 1-β-D-ribofuranosylbenzimidazole and 1-β-ribofuranosyl-5,6-dimethylbenzimidazole (Hogenkamp, 1974) could be of interest. These benzimidazole derivatives probably could be brominated at the 8-position in the same manner as was adenosine (Houstek and Smrt, 1979), followed by treatment with sodium azide as mentioned earlier. These studies should permit the identification of the subunit(s) involved in binding the cofactor in adenosylcobalamin-dependent enzymes.

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PROPOSITION III

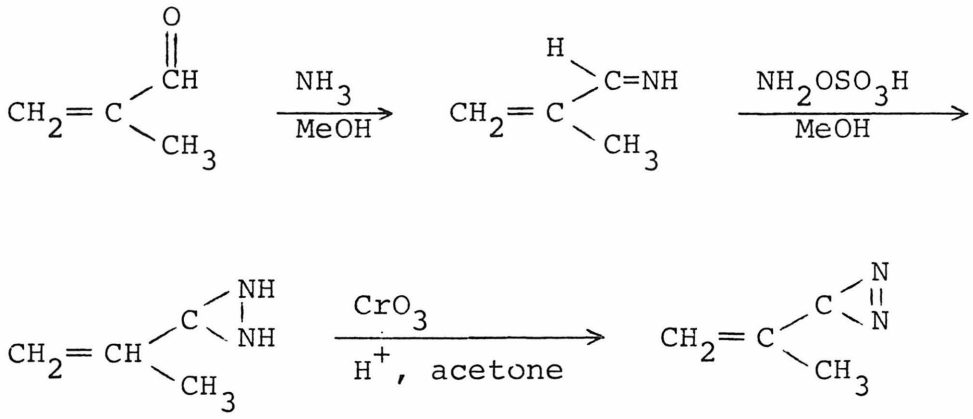
In recent years, the development of polymers, composites, and adhesives for dental applications has been significant. Although mechanical strength and modulus may be tailored to specific needs, adhesion, especially to dentine, has been one of the most challenging problem areas (National Research Council, 1981). The development of improved interfacial bonding polymers, such as covalently reactive acrylics, will lead to simpler dental procedures (with less etching and grinding) and inhibition of subsequent caries.

Dentine is the region of the tooth that is directly below the surface enamel. It is composed of an organic matrix within which the inorganic crystallites are laid down. The organic matrix of dentine is almost entirely collagen (Stack, 1951). Nakabayashi *et al.* (1977) have shown that it is possible to modify the polypeptide chains of the matrix so as to allow covalent coupling between the collagen and the prosthetic polymer. They achieved this by irradiating an emulsion which contained p-azidobenzoyloxyethyl methacrylate with ultraviolet light on the surface of an acid-etched tooth. The nitrene generated by photolysis of the azido group inserted itself into the polypeptide chain, and the methacrylate later became incorporated into the synthetic polymer.

Another photosensitive functionality which may be of use is the diazirine group. The synthetic scheme shown below employs reactions used by Bayley and Knowles (1980) to introduce

the diazirine group into adamantanone. Since the diazirine group can function in aliphatic systems, there is a great deal of flexibility in the type of molecules that can be made. For example, Scheme I shows a hypothetical synthesis of 3,3'-diazirine-2-methylpropene. Other starting aldehydes

Scheme I



or ketones could be prepared from the appropriate methylacrylate esters (shown below) by oxidation of the compounds

using dicyclohexylcarbodiimide in dimethyl sulfoxide (Pfitzner and Moffatt, 1965). This flexibility could lead to the development of useful photo-activated adhesives.

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PROPOSITION IV

In addition to adenosylcobalamin, diol dehydratase requires a monovalent cation for activity (Lee and Abeles, 1963; Manners *et al.*, 1970; Toraya *et al.*, 1971). The ion usually employed is K^+, but other cations, including Na^+, NH_4^+, Rb^+, Cs^+ and Ti^+, are also suitable, with Li^+ being inactive. Toraya *et al.* (1971) correlated the observed effectiveness of a given ion as a cofactor with its ionic radius by determining k_{cat} with the various cofactor ions. Highest activities were obtained with ions whose radii are in the vicinity of 1.4 Å (K^+, Rb^+, Tl^+ and NH_4^+) with significantly lower activities being observed with Na^+ and Cs^+.

The presence of the monovalent ion stabilizes the enzyme-coenzyme complex, with the degree of stabilization correlating with the effectiveness of the ion as a replacement for K^+ in the catalytic reaction (Toraya *et al.*, 1970, 1971; Toraya and Fukui, 1972). However, the change in k_{cat} as a function of the ionic radius of the cofactor ion cannot be attributed soley to the increased dissociation constant of the coenzyme, because the kinetic assays are done in the presence of saturating coenzyme (about 100 times K_M) and substrate. Moreover, the presence of substrate stabilizes the enzyme-coenzyme complex so that dissociation is practically undetectable (Toraya *et al.*, 1971; Essenberg *et al.*, 1971). Hence, one or more steps in catalysis may be affected by the nature of the monovalent ion(s) bound to the protein.

By measuring kinetic isotope effects of various deuterated and tritiated 1,2-propanediols when diol dehydratase has cofactor ions other than K^+, one might gain further insight into the role of the monovalent ion in the holoenzyme complex as well as the mechanism for catalysis. A kinetic study, such as that conducted by Moore *et al.* (1979) with 1,2-propanediol containing deuterium at C-1, could help establish the rate-limiting step when the various monovalent ions act as cofactor. Also, the fractionation factor (Chapter III) may vary with the cofactor ion used; therefore, studies to determine f/n with tritiated substrate and coenzyme, similar to those conducted in Chapter III, could be carried out to determine if the proposed alternate pathway becomes more significant with an alternate cofactor ion bound to the protein.

An absence of any effect on the fractionation factor would suggest that the cofactor ion does not have a significant role in determining the pathway for the transfer of hydrogen from product into the intermediate reservoir, but rather a later step in catalysis. This later step may become rate influencing, especially when Cs^+ or Na^+ are used. If this is the case, secondary isotope effects, similar to those observed in Chapter II, might be measurable when 2-deuterio-1,2-propanediol or 3,3,3-trideuterio-1,2-propanediol are substrates.

By reducing hydroxyacetone with $NaBD_4$, one could prepare 2-deuterio-1,2-propanediol (Aasen, 1970) for studying α-deuterium isotope effects. By using the procedures outlined by Kollonitsch (1966a,b), one could prepare 3,3,3-trideuterio-1,2-propanediol from CD_3I and ethyl oxalylchloride for studying β-deuterium isotope effects.

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PROPOSITION V

Thermally induced [3.3] sigmatropic rearrangements have found widespread applications in synthetic organic chemistry (Ziegler, 1977). In order for such a rearrangement to occur, for example, in the Cope rearrangement shown below, the two terminal methylene groups are required to be

aligned in the transition state so as to allow formation of the new bond. By analogy with the known conformations of cyclohexane (Eliel, 1962), possible transition states include boat and chair geometries.

Doering and Roth (1962) established that the chair-like transition state has the lowest energy, and in a majority of instances, the stereochemistry of [3.3] sigmatropic rearrangements of substituted 1,5-dienes are best understood in terms of this transition state (Rhoads and Paulino, 1975). Hence, independent study of transition states of higher energy, such as the boat geometry in the Cope rearrangement, has been accomplished only recently (Goldstein and Benzon, 1972; Shea and Phillips, 1978, 1980).

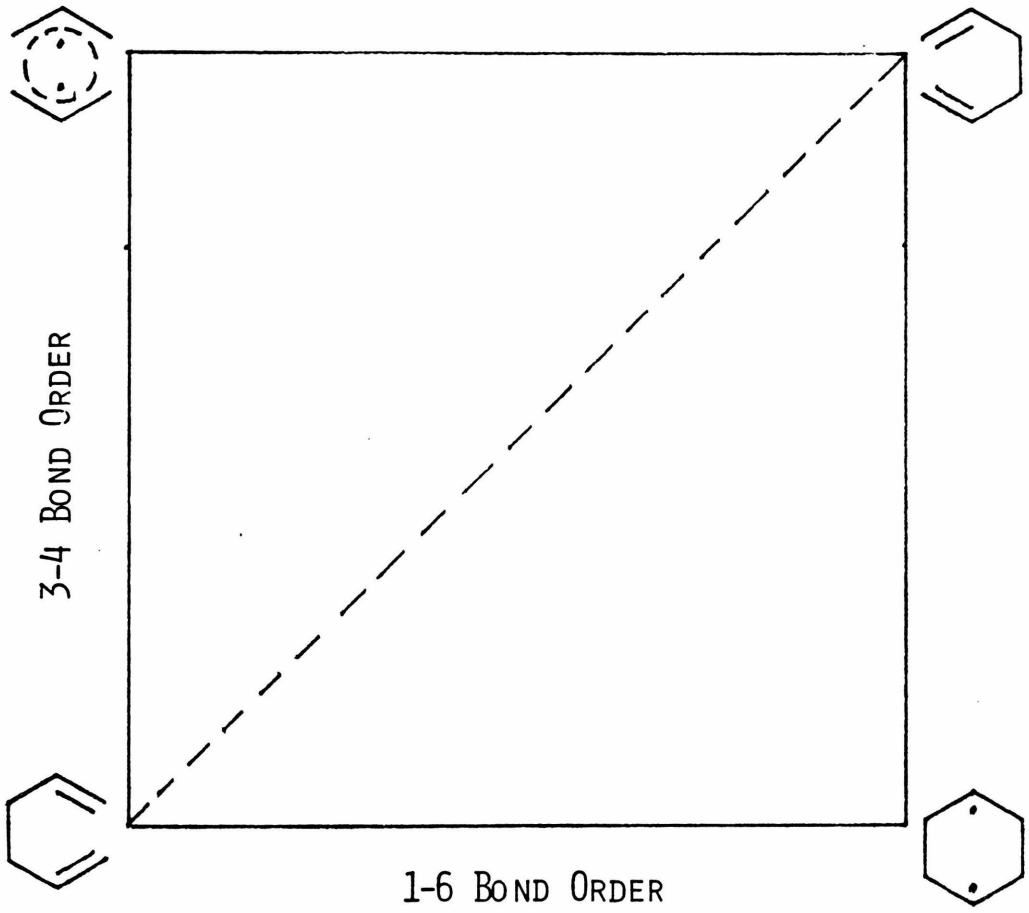
Shea and Phillips (1978, 1980) found that the dl and meso isomers of 2,2'-bismethylene cycloalkanes undergo Cope

rearrangements exclusively, due to steric restrictions, via the chair and boat transition states, respectively. By using appropriate deuterated 2,2'-bismethylene cycloalkanes, experiments similar to those done by Gajewski and Conrad (1979) could be used to determine the effects of radical-stabilizing groups on the boat and chair transition states.

Dewar and Wade (1973, 1977) observed that 2-phenyl and 2,5-diphenyl-1,5-hexadiene rearrange 69 and 4900 times faster, respectively, than the parent compound, 1,5-hexadiene. By measuring bond-breaking and bond-making α-deuterium isotope effects and equilibrium isotope effects for those compounds, Gajewski and Conrad (1979) showed that 2,5-diphenyl-1,5-hexadiene had a transition state that had much more cyclohexane-1,4-diyl character than the parent compound (indicating that bond making has gone farther than bond breaking in the transition state), with the 2-phenyl compound having a transition state intermediate in 1,4-diyl character.

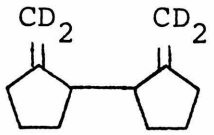
To depict the variation of transition state structures, a More O'Ferral-Jencks diagram (More O'Ferrall, 1970; Jencks, 1972) is used (Figure 1). Rather than plotting bond distances or bond orders, the ratios of the α-secondary kinetic to thermodynamic isotope effects at the reacting sites are used as structural coordinates. Gajewski and Conrad (1979) reasoned that, since neither bond distances nor bond orders in transition states have been measured, and since kinetic isotope effects result only from bonding changes

Figure 1. A More O'Ferrall-Jencks coordinate system is shown. The 3-4 bond order is given by $(1-\frac{BBKIE-1}{EIE-1})$ and the 1-6 bond order is given by $(\frac{BMKIE-1}{EIE-1})$ where BBKIE is the bond-breaking kinetic isotope effect, BMKIE is the bond-making kinetic isotope effect, and EIE is the equilibrium isotope effect. The dotted line predicts the coordinates for compounds which have a concerted transition state (i.e., bond making has proceeded to the same extent as bond breaking).

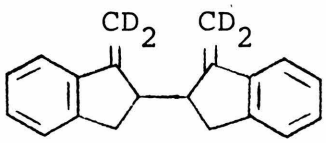

3-4 Bond Order
1-6 Bond Order

at the reacting center, including some fractional change represented by the equilibrium isotope effect, these structural coordinates should be both meaningful and accessible.

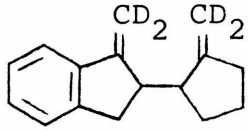
The following compounds would be useful in determining bond-making kinetic isotope effects (BMKIEs). The dl and meso isomers



I

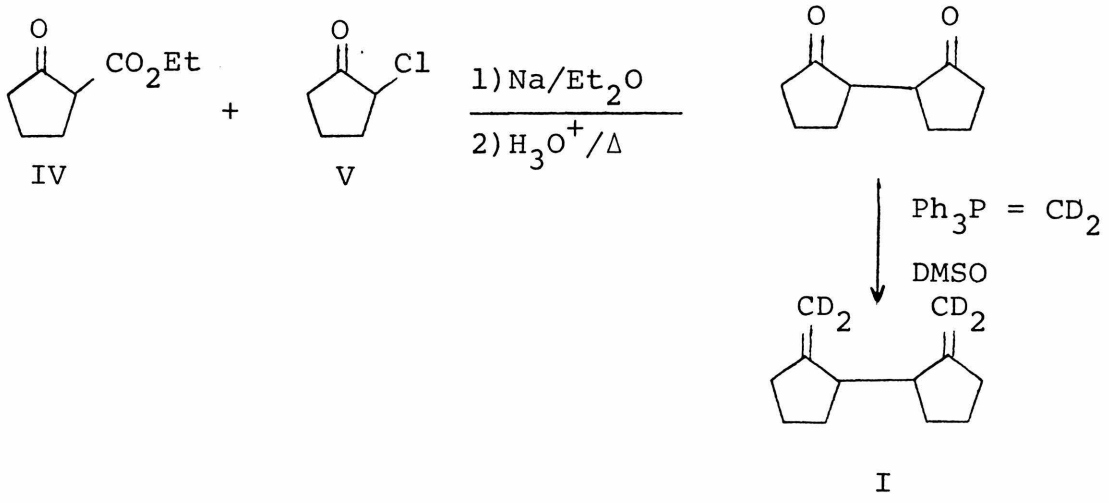


II

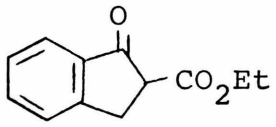


III

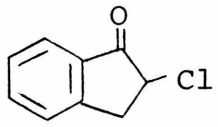
can be resolved by vapor phase chromatography (Shea and Phillips, 1980). Compounds I-III can be synthesized according to Scheme I (Shea and Phillips, 1980). The appropriate



starting materials (VI, VII) to make II and III have been synthesized previously (Dieckman, 1922; Banerjee *et al.*, 1965).

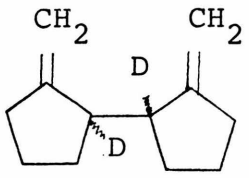


VI

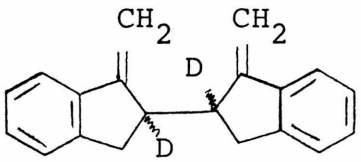


VII

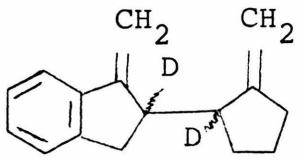
The deuterated methylenetriphenylphosphorane could be prepared from CD_3Br and triphenylphosphine (March, 1977). The following compounds would be useful for determining bond-breaking kinetic isotope effects (BBKIEs). Again, the compounds could be resolved into dl and meso isomers as mentioned earlier.



VIII

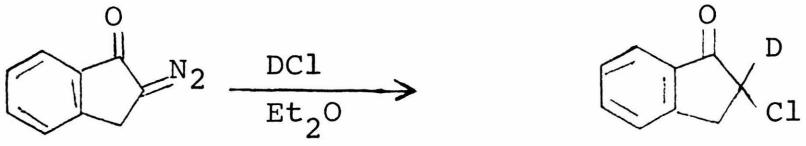


IX

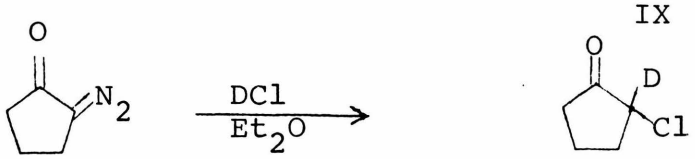


X

Only minor changes in Scheme I need be employed in making VIII-X. The deuterated analogues of V and VII could be prepared by the method of Banerjee *et al.* (1965) shown below:



IX



XII

The chlorinated ketones (XI and XII) could then be reacted with IV or VI according to Scheme I. The ester hydrolysis and decarboxylation could then be carried out in the presence of DCl and D_2O to yield VII-X.

The aromatic rings in II, III, IX and X will permit comparisons of substituent effects on the boat and chair transitions states of the Cope rearrangement.

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