# THE MECHANISM OF ACTION OF ADENOSYLCOBALAMIN

I. 3-FLUORO-1,2-PROPANEDIOL AS SUBSTRATE FOR PROPANEDIOL DEHYDRASE – MECHANISTIC IMPLICATIONS.

II. GLYCEROL AND OTHER SUBSTRATE ANALOGUES AS SUBSTRATES AND INACTIVATORS FOR PROPANEDIOL DEHYDRASE. KINETICS, STEREOSPECIFICITY AND MECHANISM.

III. THE INVOLVEMENT OF A 5'-DEOXYADENOSINE INTERMEDIATE CONTAINING THREE EQUIVALENT HYDROGENS IN THE MECHANISM OF GLYCEROL INACTIVATION OF PROPANEDIOL DEHYDRASE HOLOENZYME.

IV. HYDROGEN TRANSFER FROM ENZYME-BOUND ADENOSYLCOBALAMIN AS A PARTIAL RATE LIMITING STEP IN CATALYSIS.

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# DEDICATION

To Jean

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## PART I

3-Fluoro-1,2-propanediol has been found to be a substrate for propanediol dehydrase and has very similar binding and catalytic constants compared to the natural substrate. The only isolable products of the reaction are acrolein and inorganic fluoride; with 3-fluoro-3,3-dideuterio-1,2-propanediol as substrate, only 3,3-dideuterioacrolein is obtained. These results indicate that the primary product of the reaction is 3-fluoropropionaldehyde which spontaneously loses hydrogen fluoride to yield acrolein. The similar kinetic parameters for the fluorinated as compared to the normal substrate suggest that significant charge does not develop on the fluorinated or, by implication, the natural substrate during any rate-limiting steps of the reaction. These results support a radical, as contrasted to an ionic, pathway for reactions involving adenosylcobalamin and diol dehydrase.

## PART II

A number of vicinal diols were found to react with dioldehydrase, typically resulting in the conversion of

enzyme-bound adenosylcobalamin to cob(II)alamin and formation of aldehyde or ketone derived from substrate. Moreover all are capable of effecting the irreversible inactivation of the enzyme. The kinetics and mechanism of product formation and inactivation were investigated.

Glycerol, found to be a very good substrate for diol dehydrase as well as a potent inactivator, atypically, did not induce cob(II)alamin formation to any detectable extent. With glycerol the inactivation process was accompanied by conversion of enzyme-bound adenosylcobalamin to an alkyl or thiol cobalamin, probably by substitution of an amino acid side chain near the active site for the 5'-deoxy-5'-adenosyl ligand on the cobalamin.

The inactivation reaction with glycerol as the inactivator exhibits a deuterium isotope effect of 14, strongly implicating hydrogen transfer as an important step in the mechanism of inactivation. The isotope effect of the rate of product formation was found to be 8.0.

Experiments with isotopically substituted glycerols indicate that dioldehydrase distinguishes between "R" and "S" binding conformations, the enzyme-"R"-glycerol complex being predominately responsible for the product-forming reaction while the enzyme-"S"-glycerol complex results primarily in the inactivation reaction. Mechanistic implications are discussed.

A method for removing enzyme-bound OH-Cbl that is non-

destructive to the enzyme and a technique for measuring the binding constants of (R)-and (S)-1,2-propanediol are presented.

## PART III

The kinetics of inactivation of adenosylcobalamin dependent propanediol dehydrase by glycerol, (RS)-1,1-dideuterioglycerol, (R)-1,1-dideuterioglycerol, and perdeuterioglycerol in the presence of 1,2-propanediol and 1,1-dideuterio-1,2-propanediol were investigated. The results (lead to the conclusion) that hydrogen (or deuterium) located on C-1 of 1,2-propanediol can participate in the inactivation-reaction and contribute to the expression of an isotope effect on the inactivation rate constant. The mechanism by which this occurs must involve the cofactor as intermediate hydrogen carrier, presumably in the form of 5'-deoxyadenosine. Moreover, the results can be quantitatively accounted for by a mechanism involving transfer of hydrogen from an intermediate containing three equivalent hydrogens, such as 5'-deoxyadenosine, as the rate-determining step in the inactivation pathway.

When dioldehydrase holoenzyme is inactivated with 1-$^{3}$H-glycerol, 5'-deoxyadenosine enriched in tritium by a factor of 2.1 over that in glycerol can be isolated from the reaction mixture.

PART IV

The rate of catalysis by the adenosylcobalamin dependent enzyme diodehydrase was determined as a function of the relative amounts of 1,1-dideuterio-1,2-propanediol and 1,2-propanediol present in the reaction mixture. The rate was found to decrease sharply at low mole fractions of 1,1-dideuterio-1,2-propanediol and slowly approach the rate observed 1,1-dideuterio-1,2-propanediol at large mole fractions of 1,1-dideuterio-1,2-propanediol. We interpret this to indicate that hydrogen transfer from enzyme-bound adenosylcobalamin is at least partially rate limiting in catalysis.

# ABSTRACTS OF THE PROPOSITIONS

## PROPOSITION I

The development of an affinity absorbent for the purification of AdoCbl dependent hydrogen transfer enzymes is proposed.

## PROPOSITION II

The characterization of the alkyl or thio cobalamin species associated with glycerol inactivated diodehydrase holoenzyme is proposed.

## PROPOSITION III

A study of the galactose and ribose chemotactic receptor in S. typhimurium and E. coli is proposed.

## PROPOSITION IV

A study of the idiotypic cross reactivities of antibodies of the same specificities raised in inbred strains of mice is proposed.

## PROPOSITION V

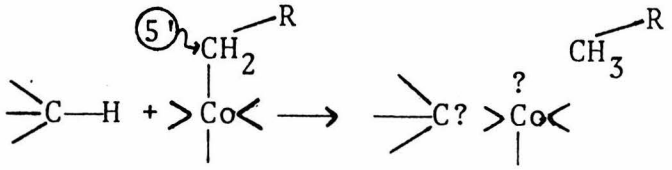
An investigation into the feasibility of applying $^{15}$NMR to the study of the ring nitrogens of porphyrins and corrins is proposed particularly in regard to the use of paramagnetic species for improvement of sensitivity.

# PART I

## 3-Fluoro-1,2-propanediol as Substrate for Propanediol Dehydrase-Mechanistic Implications

# INTRODUCTION

Most current mechanisms for rearrangement reactions involving adenosylcobalamin invoke, as one of the first steps, the cleavage of the bond between the cobalt and the 5'-methylene carbon of the deoxyadenosyl residue of the coenzyme with concomitant removal of a hydrogen atom from substrate by the 5'carbon (which becomes thereby a methyl group).



substrate coenzyme

The question of the electronic nature of this bond cleavage and hydrogen transfer (ionic, radical or concerted) remains presently somewhat unresolved, though appreciable electron spin resonance (ESR) evidence in the case of ethanolamine deaminase (Babior et al., 1972, 1973), ribonucleotide reductase (Hamilton et al., 1971, 1972; Orme-Johnson et al., 1973), and diol dehydrase (Finlay et al., 1972; Valinsky et al., 1973) supports a radical pathway.

Propanediol dehydrase, though showing relatively the same activity to both R and S forms of the normal substrate (Lee and Abeles, 1963), has almost no enzymatic activity for

other only slightly modified substances (Lee and Abeles, 1963; Toraya and Fukui, 1972). With the hope of changing the polar characteristics of potential substrates without altering significantly the stereochemical requirements, we have studied the possible role as substrates of fluorine substituted analogues of propanediol (van der Waals radii; fluorine, 1.35 Å; hydrogen, 1.2 Å). We have found that both (R)- and (S)-3-fluoro-1,2-propanediol are efficiently converted by the enzyme presumably to β-fluoropropionaldehyde which then rapidly loses HF to yield acrolein, the observed product. Other features of the reaction of these fluorinated substrates were also studied and have significance for the mechanism of the reaction catalyzed by adenosylcobalamin and diol dehydrase.

## EXPERIMENTAL SECTION

Enzyme preparation. Propanediol dehydrase ((R,S)-1,2-propanediol hydro-lyase; EC 4.2.1.28) was obtained from Klebsiella pneumoniae (ATCC 8724) by a procedure similar to that reported by Lee and Abeles (1963). Fraction E-8 was used for all determinations. Diol-free enzyme was prepared as previously reported (Frey et al., 1967).

Coenzyme $B_{12}$. Coenzyme $B_{12}$ was purchased from Sigma Chemical Company.

Assays. All assays were carried out in the dark at 37°C, and the aldehyde products were determined by a

modification of the previously reported method (Lee and Abeles, 1963). This modification increased the sensitivity of the assay approximately fivefold. In general, 2 ml of aldehyde-containing solution was assayed by adding 0.1 ml of 2 N hydrochloric acid and 0.1 ml of 2,4-dinitrophenylhydrazine (prepared by dilution of 100 mg of 2,4-dinitrophenylhydrazine plus 0.4 ml of concentrated hydrochloric acid to 25 ml with carbonyl-free methanol). After standing for 30 min, 0.5 ml of Spectroquality pyridine (Matheson Coleman and Bell) and 0.1 ml of methanolic potassium hydroxide (prepared by dilution of 10 g of potassium hydroxide dissolved in 10 ml of distilled water to 50 ml with carbonyl-free methanol) were added; and the resulting mixture was allowed to stand for 6 min and then centrifuged. Absorbance was determined at 475 mμ.

Rate determination. The relative rates of (S)-, (RS)-, and (R)-3-fluoro-1,2-propanediol and of (R)-, (RS)-, and (S)-1,2-propanediol were determined by measuring the production of the product, acrolein or propionaldehyde, at 1-min intervals. The rates were linear for at least 8 min and the slope of the least-squares line was used to determine $k_{cat}$. Reaction mixtures consisted of the following: diol dehydrase, 0.009 unit; potassium phosphate buffer, pH 8.0, 80 μmol; adenosylcobalamin, 40 μg; substrate, 100 μmol; bovine serium albumin, 0.02 mg. Total volume, 2 ml, 37°.

$K_M$ Determination. Reaction mixtures were generally the same as those described above with the amount of substrate varied. For optimum results 0.032 unit of enzyme was used. The production of acrolein was determined at 1-min intervals for each initial substrate concentration. In all cases the rate was linear through 8 min. The double reciprocal plots were also linear and the slope and intercept of the least-squares line were used to determine $K_M$.

Synthesis of Substrates.

(R)-1,2-Propanediol. (R)-1,2-Propanediol was prepared by lithium aluminum hydride reduction of D-lactic acid which was obtained by acidification of calcium D-lactate (Sigma Chemical Company) (Karrer et al., 1948). The resulting (R)-1,2-propanediol has $[\alpha]^{25}D$ -18.4° (7.5% w/w in water).[1]

(S)-1,2-Propanediol. (S)-1,2-Propanediol was prepared by lithium aluminum hydride reduction of ethyl L-lactate (Aldrich Chemical Company) (Karrer et al., 1948). The resulting (S)-1,2-propanediol had $[\alpha]^{25}D$ + 19.5° (7.5% w/w in water).[1]

Other Methods of Preparation of (R)- and (S)-1,2-Propanediol. D- and L-lactates were also prepared by deamination of the corresponding isomers of alanine (Baker and Meister, 1951) and then reduced with lithium aluminum hydride.

[1]All rotations for propanediol samples are uncorrected for water content in the preparation (Huff, 1961).

The (R)- and (S)-1,2-propanediols had $[\alpha]^{25}D$ of -19.5 and +19.5° (7.5% w/w in water),[1] respectively.

Sodium cyanoborohydride reduction of (R)-3-O-tosyl-1,2-isopropylideneglycerol (see the synthesis of (R)-3-fluoro-1,2-propanediol) by the general method of Hutchins et al. (1971), followed by acid hydrolysis of the ketal also yielded (S)-1,2-propanediol with $[\alpha]^{25}D$ + 20.8° (7.5% w/w in water).[1]

(RS)-3-Fluoro-1,2-propanediol. (RS)-3-Fluoro-1,2-propanediol was prepared by acid hydrolysis of epifluorohydrin (Aldrich Chemical Company) (Pattison and Norman, 1957).

(R)-3-Fluoro-1,2-propanediol. (R)-3-Fluoro-1,2-propanediol was prepared from D-mannitol by the intermediate synthesis of (R)-1,2-isopropylideneglycerol, (R)-3-O-tosyl-1,2-isopropylideneglycerol, and (R)-3-fluoro-1,2-isopropylidenepropanediol as described by Ghangas and Fondy (1971). Careful distillation yielded (R)-3-fluoro-1,2-propanediol with $[\alpha]^{25}D$ - 14.9° (13% w/w in absolute ethanol) as opposed to the value $[\alpha]^{25}D$ - 7.6° (50% v/v in absolute ethanol) previously reported. 3-Fluoro-1,2-propanediol isomers with smaller [α]D values showed a considerable change in catalytic rates.

(S)-3-Fluoro-1,2-propanediol. (S)-3-Fluoro-1,2-propanediol was prepared from the R enantiomer by the intermediate synthesis of (R)-3-fluoro-1,2-di-O-tosylpropanediol and (S)-3-fluoro-1,2-di-O-benzoylpropanediol as

described by Lloyd and Harrison (1971). The (S)-3-fluoro-1,2-propanediol showed $[\alpha]^{25}D$ + 14.8° (13% w/w in absolute ethanol).

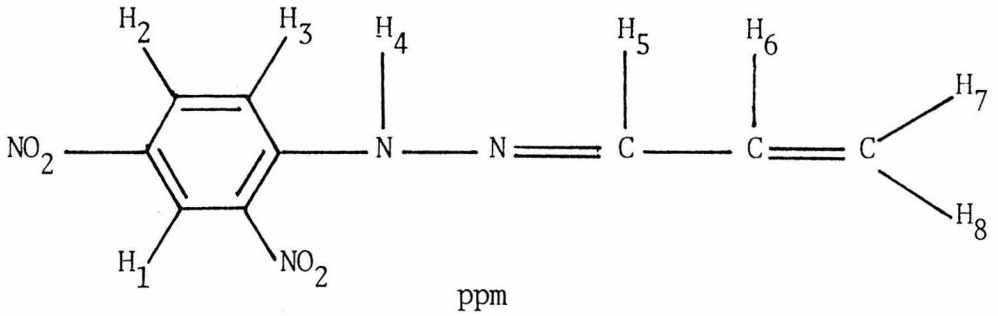
(R)-3-Fluoro-3,3-dideuterio-1,2-propanediol. Potassium (R)-1,2-isopropylideneglycerate was prepared from (R)-1,2-isopropylideneglycerol by alkaline potassium permanganate oxidation (Reichstein et al., 1935). The methyl ester was prepared directly from the postassium salt by reaction with methyl iodide in hexamethylphosphoramide (Shaw et al., 1973). Potassium (R)-1,2-isopropylideneglycerate (3.7 g, 10 mmol) was dissolved in 50 ml of hexamethylphosphoramide which contained 5.1 ml of water and potassium hydroxide (1.7 g, 30 mmol). The mixture was stirred for 30 min and methyl iodide (11.3 g, 80 mmol) was added. The reaction was allowed to proceed for 2 hr while maintaining a pH above 8 by addition of potassium hydroxide when necessary. The mixture was poured into 100 ml of water and extracted twice with 75 ml portions of ethyl ether. The combined ether extracts were washed twice with 25-ml portions of water and once with 10 ml of saturated sodium chloride solution, and dried over anhydrous sodium sulfate. Evaporation of the ether under reduced pressure yielded crude methyl (R)-1,2-isopropylideneglycerate (2.3 g, 73%) which was used without further purification. The crude ester (5 g, 31 mmol) was dissolved in 50 ml of anhydrous ethyl ether and added dropwise to a solution of lithium aluminum deuteride (1.0 g,

24 mmol) (99% $^{2}H$ from Stohler Isotope Chemicals) in 150 ml of ice cold anhydrous ether with stirring. After 3 hr, the reaction was quenched by adding 1 ml of water, 1 ml of 15% sodium hydroxide, and 3 ml of water (Fieser and Fieser, 1967). The salts were removed by filtration and evaporation of the ethereal solution yielded (R)-3,3-dideuterio-1,2-isopropylideneglycerol (4.25 g, 88%). (R)-3-Fluoro-3,3-dideuterio-1,2-propanediol, $[\alpha]^{25}D$ - 14.1° (13% w/w in absolute ethanol), was prepared from this material by a method analogous to the one described above. Deuterium content was at least 98% as determined by $^{1}H$ nuclear magnetic resonance (NMR).

(S)-3-Fluoro-3,3-dideuterio-1,2-propanediol. (S)-3-Fluoro-3,3-dideuterio-1,2-propanediol, $[\alpha]^{25}D$ + 14.8° (5% w/w in absolute ethanol), was prepared from the R enantiomer as described above. Deuterium content was at least 98% as determined by $^{1}H$ NMR.

3-Fluoro-1,1-dideuterio-1,2-propanediol. 3-Fluoro-1,1-dideuterio-1,2-propanediol was prepared from ethyl 3-fluorolactate (Gottwald and Kun, 1965) by lithium aluminum deuteride reduction in a manner similar to that previously described. Deuterium content was at least 98% as determined by $^{1}H$ NMR.

Acrolein 2,4-Dinitrophenylhydrazone. Acrolein 2,4-dinitrophenylhydrazone was prepared as described previously ( Shriner et al., 1964). The $^{1}H$ 100-MHz NMR spectrum was




| H | ppm from $Me_4Si$ | |
|---|---|---|
| 1 | 9.12 | $J_{1,2}$ = 2.5 Hz |
| 2 | 8.32 | $J_{2,3}$ = 9.5 Hz |
| 3 | 7.96 | $J_{5,6}$ = 9.1 Hz |
| 4 | 11.12 | $J_{6,7}$ = 10.5 Hz |
| 5 | 7.79 | $J_{6,8}$ = 16.7 Hz |
| 6 | 6.63 | $J_{7,8}$ = -0.2 Hz |
| 7 | 5.78 | |
| 8 | 5.75 | |

Figure 1: NMR data for acrolein 2,4-dinitrophenylhydrazone. Only a single resonance is observed for the $H_5$ proton. Presumably due to steric hindrance only the anti (vinyl) isomer is formed as is the case for a number of aldehyde 2,4-dinitrophenylhydrazones (Curtin et al., 1959).

determined in deuterated chloroform on a Varian XL-100 spectrometer and was matched with a computer-simulated spectrum with the following chemical shifts relative to tetramethylsilane and coupling constants (Figure 1). The acrolein 2,4-dinitrophenylhydrazone showed $R_f$ 0.52 on silica gel thin-layer chromatography using chloroform as an eluting solvent.

Determination of Reaction Products from 3-Fluoro-3,3-dideuterio-1,2-propanediol. The dinitrophenylhydrazones of acrolein from the enzymatic rearrangement of (R)- and (S)-3-fluoro-3,3-dideuterio-1,2-propanediol were isolated by preparative thin-layer chromatography. Their $^1H$ NMR spectra were determined on a Varian XL-100 spectrometer using the Fourier transform technique. The acrolein from both isomers showed >98% deuterium on C-3 of the acrolein. No other deuterium was detected.

## RESULTS

Nature of the Reaction. The rate of conversion of both (R)- and (S)-3-fluoro-1,2-propanediol to aldehyde by diol dehydrase is linear for at least 30 min. The only products observed are acrolein (identified by the acrolein-specific assay of Circle et al. (1945), and by the NMR of the 2,4-dinitrophenylhydrazine (Dnp) adduct) and inorganic fluoride. Following the reaction by $^{19}F$ magnetic resonance showed only substrate and fluoride anion; if β-fluoropropionaldehyde is

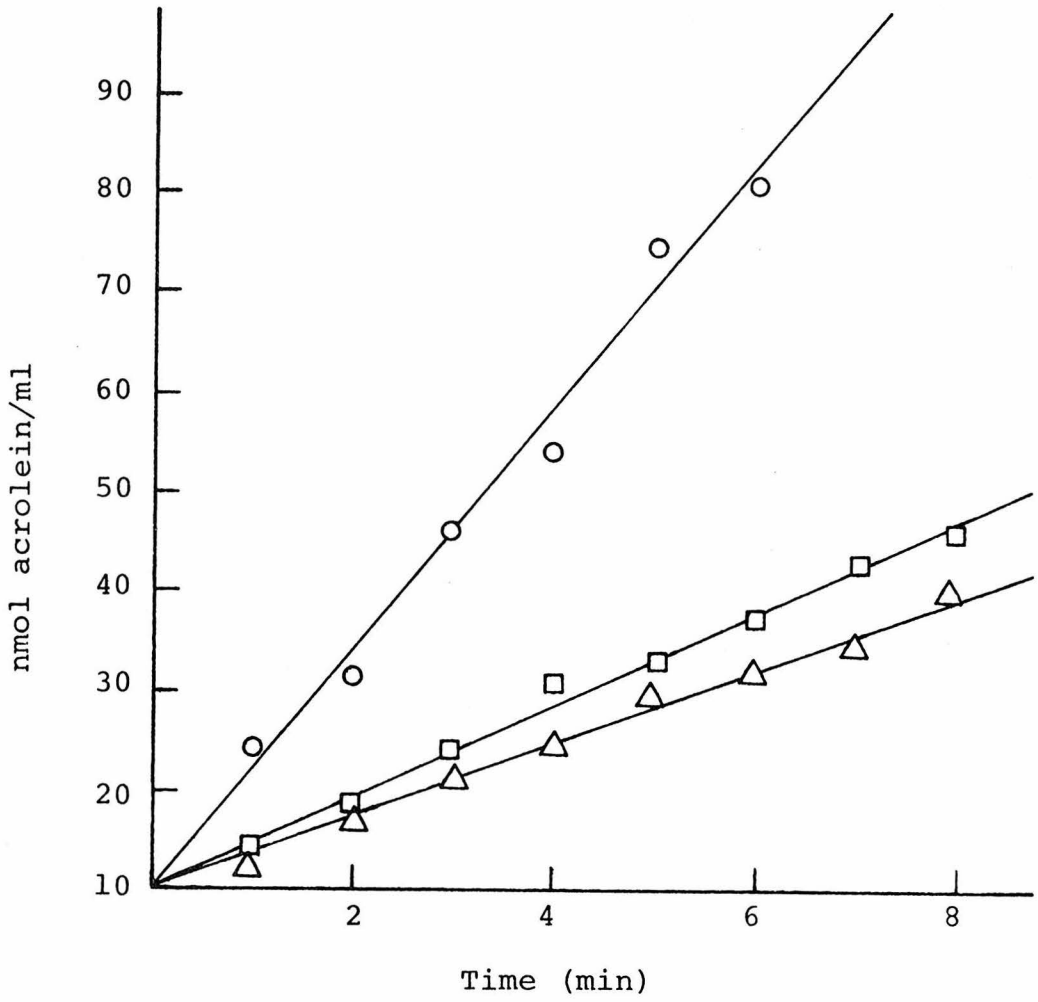



Fig. 2. Relative rates of (S)-, (RS)-, and (R)-3-fluoro-1,2-propanediol. Reaction mixtures consisted of the following: diol-dehydrase, .009 units; potassium phosphate buffer, pH 8.0, 80 μmoles; DBC coenzyme, 40γ; substrate, 100μmoles; BSA, .02 mg. Total volume, 2 ml, 37°. ○, (S)-3-fluoro-1,2-propanediol; □, (RS)-3-fluoro-1,2-propanediol; △, (R)-3-fluoro-1,2-propanediol.

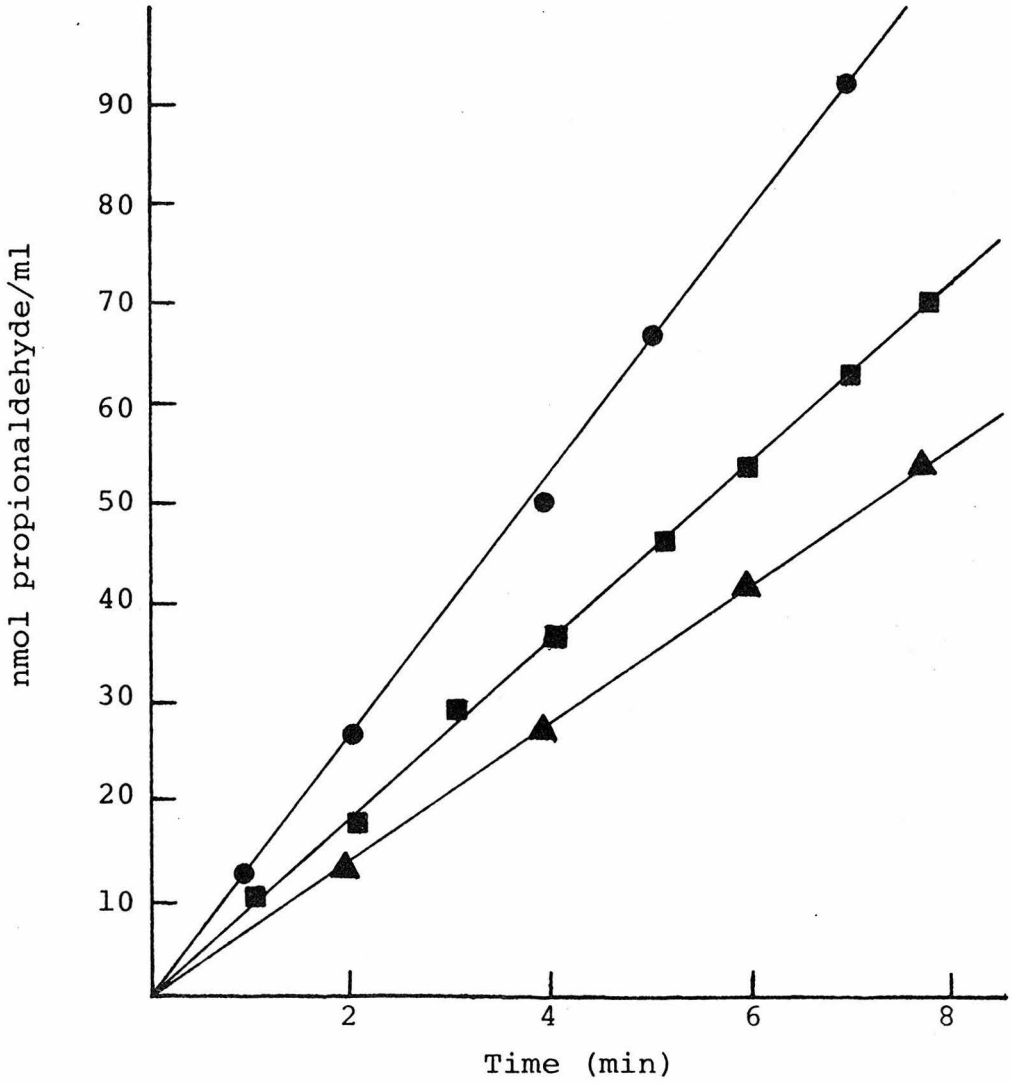



Fig. 3. Relative rates of (S)-, (RS)-, and (R)-1,2-propanediol. Reaction mixtures consisted of the following: diol-dehydrase, 0.009 units; potassium phosphate buffer, pH 8.0, 80 μmoles; DBC coenzyme, 40 γ; substrate, 100 μmoles; BSA, 0.02 mg. Total volume, 2 ml, 37°. ●, (R)-1,2-propanediol; ■, (RS)-1,2-propanediol; ▲, (S)-1,2-propanediol.

formed its lifetime must be very short at 35°C (NMR probe temperature). The possibility that reaction of 3-fluoro-1,2-propanediol proceeded by primary abstraction of hydrogen from C-3 (the carbon to which the fluorine is bound) was eliminated by studying the enzymatic conversion of both (R)- and (S)-3, fluoro-3,3-dideuterio-1,2-propanediol; in both cases, the only product was 3,3-dideuterioacrolein (as indicated by $^1H$ magnetic resonance spectra of the Dnp adducts) and fluoride anion.

In contrast to the behavior of 3-fluoro-1,2-propanediol as essentially normal substrate, neither 1-fluoro-2-propanol nor 1,3-difluoro-2-propanol shows detectable reactivity (less than 1% of that for 1,2-propanediol). Moreover, neither exhibits any inhibition of enzymatic conversion of 1,2-propanediol when present in 100-fold excess over substrate.

Kinetic Aspects. Comparative Rates. Table 1 summarizes the values of $k_{cat}$ obtained for (R)-, (S)-, and (RS)-3-fluoro-1,2-propanediol and for (R)-, (S)-, and (RS)-1,2-propanediol. (A note about formal stereochemical notation may avoid some confusion. The conventions are such that, for example, (S)-3-fluoro-1,2-propanediol is, in fact, the 3-fluoro analogue of (R)-1,2-propanediol.) The rate of the RS mixture (more pronounced with 3-fluoro-1,2-propanediol than with 1,2-propanediol) more nearly equals that of the slower isomer than the average of the rates for the two isomers. This suggests

Table 1. Kinetic Parameters for (R)-, (S)-, and (RS)-1, 2-Propanediol and (R)-, (S)-, and (RS)-3-Fluoro-1,2-Propanediol

| Substrate[a] | $V_{max}$ (nmol/min) | $k_{cat}$[b] ($sec^{-1}$) | $K_m \times 10^4$ (M) |
|---|---|---|---|
| (S)-F-diol | 12.10 ± 0.66 | 340 ± 18 | 13.2 ± 0.30 |
| (RS)-F-diol | 4.56 ± 0.25 | 128 ± 7 | 3.07 ± 0.10 |
| (R)-F-diol | 3.70 ± 0.20 | 104 ± 5 | 1.47 ± 0.05 |
| (R)-H-diol | 13.09 ± 0.60 | 368 ± 16 | 0.381 ± 0.028 |
| (RS)-H-diol | 8.90 ± 0.34 | 250 ± 10 | 0.212 ± 0.023 |
| (S)-H-diol | 6.82 ± 0.17 | 191 ± 5 | 0.123 ± 0.07 |

[a] F-diol = 3-fluoro-1,2-propanediol; H-diol = 1,2-propanediol.

[b] Based on a molecular weight of 250000 and a specific activity of 60 units/mg (Essenberg et al., 1971).

that the Michaelis values for the R and S isomers may be unequal as discussed subsequently.

Determination of Michaelis Constants.

Fluorodiols. The color from the Dnp derivative of acrolein is 2.75 times as intense as that from the Dnp derivative of propionaldehyde. This fact, together with the increased sensitivity of the modified Dnp assay, enables one to measure accurate initial velocities for 3-fluoro-1,2-propanediol at initial concentrations as low as $5 \times 10^{-5}$ M. Accordingly, $K_M$ values for those substrates could be and were determined. They are summarized along with the $K_{cat}$ values in Table 1.

1,2-Propanediol. The modified Dnp assay for propionaldehyde alone allowed meaningful initial velocities to be obtained at initial concentration only as low as $1 \times 10^{-4}$ M. Near saturation behavior is obtained at this concentration for (R)-, (S)-, and (RS)-1,2-propanediol. Michaelis constants for these natural substrates were determined by another method and are listed in Table I for comparison (W. W. Bachovchin, R. G. Eagar, and J. H. Richards, manuscript in preparation). The ratio of $K_M$ values for (R)- and (S)-1,2-propanediol determined by this method was 3.81/1.23 which agrees well with that of 3.2 previously reported by Jensen et al. (1975).

Kinetic Isotope Effects. The kinetic isotope effect $k_H/k_D$ for (RS)-1,1-dideuterio-3-fluoro-1,2-propanediol was

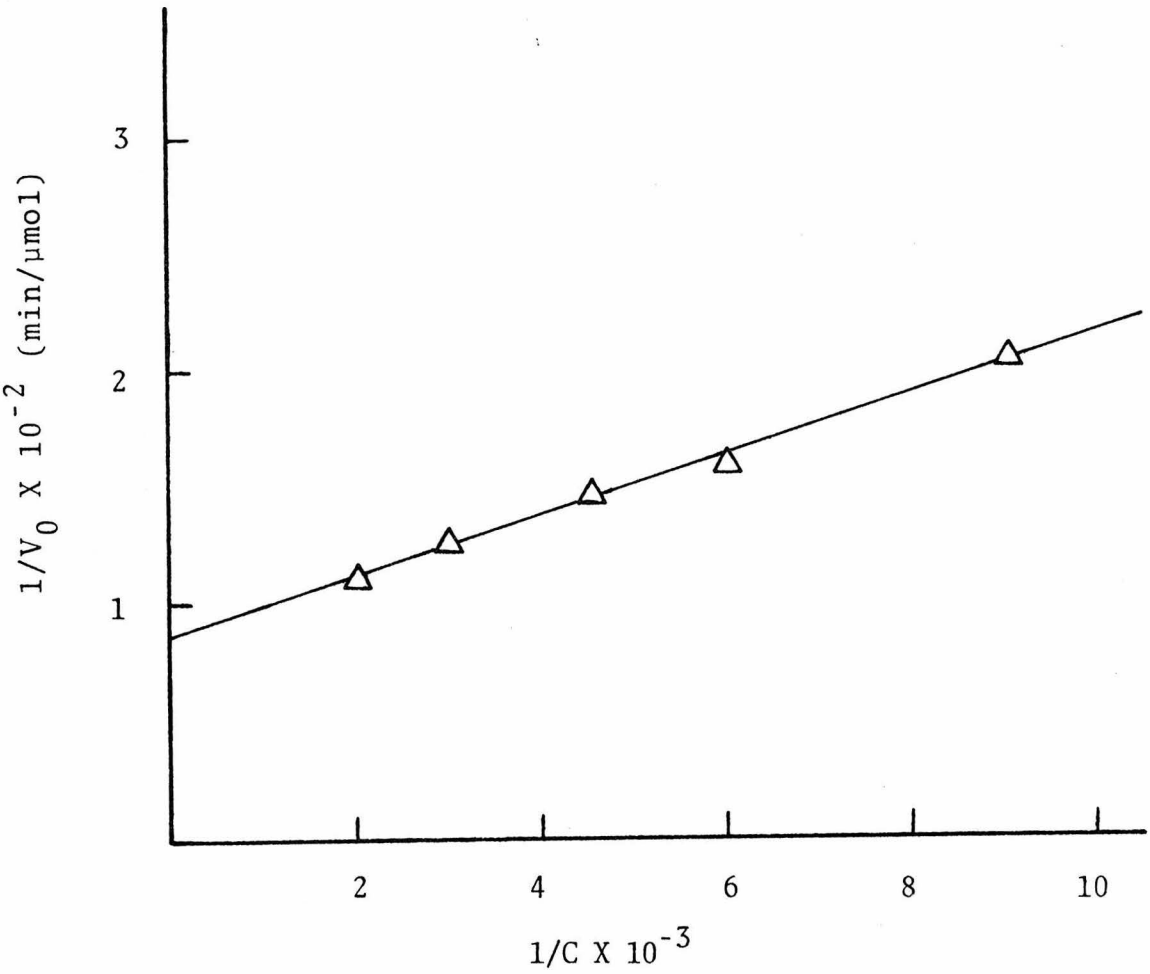



Fig. 4. The Lineweaver-Burk plot for (R)-3-fluoro-1,2-propanediol. Reaction mixtures consisted of the following; apoenzyme, 0.028 units; potassium phosphate buffer, pH 8.0, 80 μmoles; DBC coenzyme, 40 γ; BSA, 0.02 mg; varying amounts of substrate. Total volume, 2 ml, 37°. The production of acrolein was determined at one minute intervals for each initial substrate concentration. In all cases the rate was linear through eight minutes. Each initial velocity plotted above represents the slope of the least squares line.

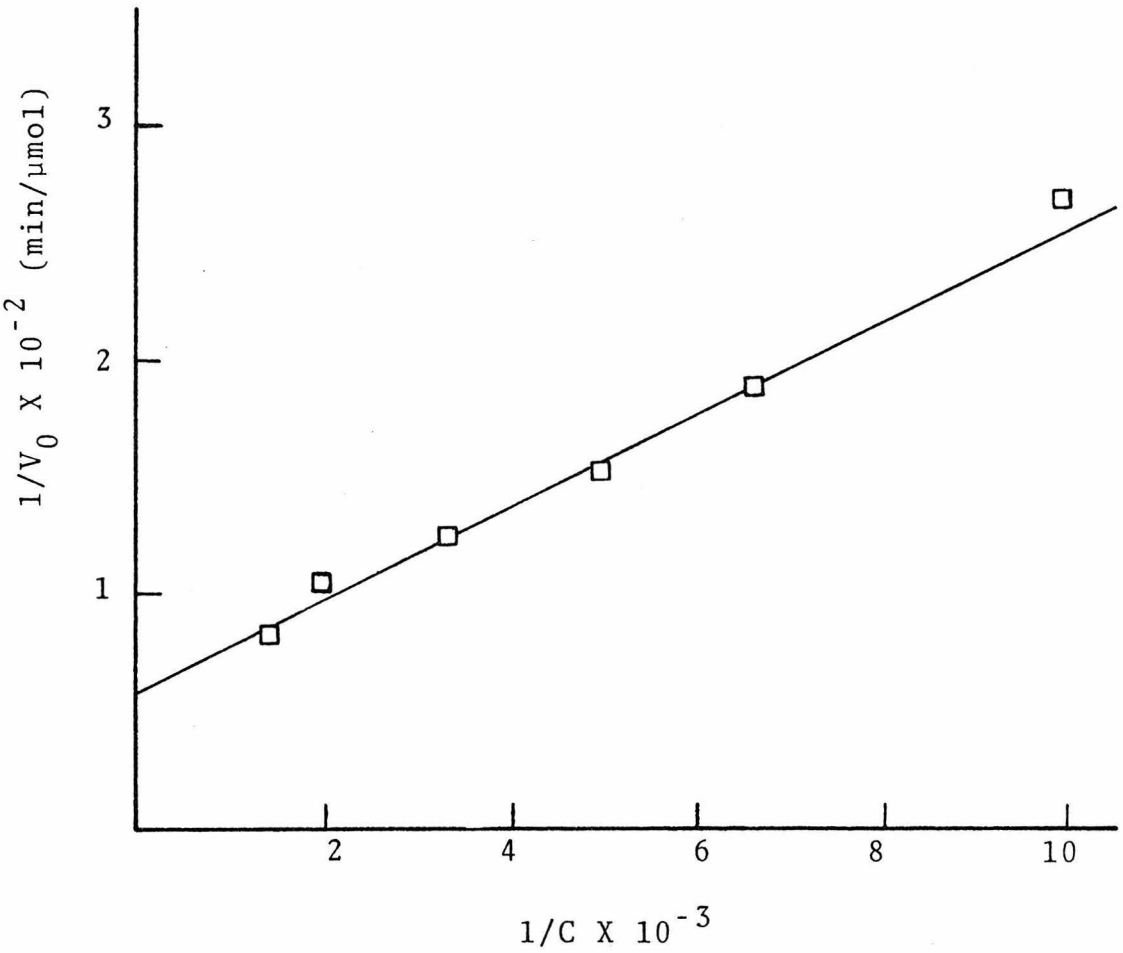



Fig. 5. The Lineweaver-Burk plot for (RS)-3-fluoro-1,2-propanediol. Reaction mixtures consisted of the following; apoenzyme, 0.032 units; potassium phosphate buffer, pH 8.0, 80 µmoles: DBC coenzyme, 40 γ; BSA, 0.02 mg; varying amounts of susstrate. Total volume, 2 ml, 37°. The production of acrolein was determined at one minute intervals for each substrate concentration. In all cases the rate was linear through eight minutes. Each initial velocity plotted above represents the slope of the least squares line.

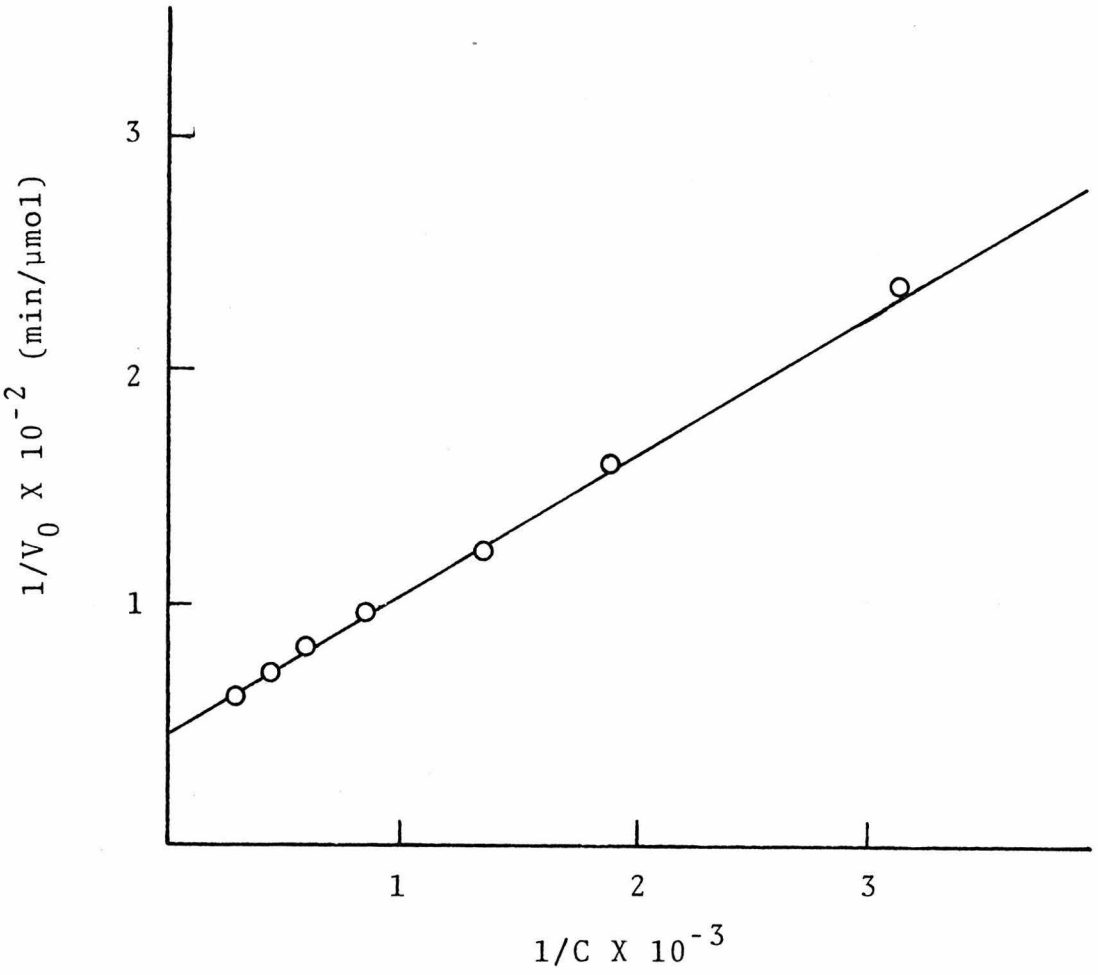



Fig. 6. The Lineweaver-Burk plot for (S)-3-fluoro-1,2-propanediol. Reaction mixtures consisted of the following: apoenzyme, 0.016 units; potassium phosphate buffer, pH 8.0, 80 μmoles; DBC coenzyme, 40 γ; BSA, 0.02 mg; varying amounts of substrate. Total volume, 2 ml, 37°. The production of acrolein was determined at one minute intervals for each substrate concentration. In all cases the rate was linear through eight minutes. Each initial velocity plotted above represents the slope of the least squares line.

determined to be 12.9; there was no concomitant change in the value of $K_M$. This kinetic isotope effect is the same as the value of 10-13 reported for 1,1-dideuterio-1,2-propanediol (Abeles, 1972; Frey et al., 1965; Lee and Abeles, 1962). Substitution of deuterium at C-3 of 3-fluoro-1,2-propanediol had no observable effect on the rate or binding behavior.

Spectral Observation of Intermediates. The visible spectrum of a reaction mixture of enzyme, coenzyme, and 3-fluoro-1,2-propanediol as substrate was identical with that of a similar solution with 1,2-propanediol itself as substrate (Wagner et al., 1966).

## DISCUSSION

Competition for One Site. That the R and S isomers compete for the active site can be demonstrated from the experimentally observed values of $K_M$ and $k_{cat}$ in Table I.

For two substrates competing for the same site the relevant equations are:

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

$$E + R \underset{k_5}{\overset{k_4}{\rightleftharpoons}} ER \xrightarrow{k_6} E + P$$

For a mixture of R and S isomers the overall reaction rate ($V_{RS}$) is given by:

$$V_{(RS)} = k_{cat}([ES] + [ER]) = k_3[ES] + k_6[ER]$$

where $k_{cat}$ is the value actually observed for the RS mixture and

$$V_{(RS)} = k_{cat}[[E_0]/(1 + \overline{K}_S[S] + \overline{K}_R[R])][\overline{K}_S[S] + \overline{K}_R[R]] =$$

$$[[E_0]/(1 + \overline{K}_S[S] + \overline{K}_R[R][k_3\overline{K}_S[S] + k_6\overline{K}_R[R]]$$

In a racemic mixture [R] = [S] = ½[RS] and the equation becomes:

$$V_{(RS)} = k_{cat}[[E_0][RS]/(2 + \overline{K}_S[RS] + \overline{K}_R[RS]][\overline{K}_S + \overline{K}_R] =$$

$$[[E_0][RS]/(2 + \overline{K}_S[RS] + \overline{K}_R[RS])[k_3\overline{K}_S + k_6\overline{K}_R]$$

whence

$$k_{cat} = (k_3\overline{K}_S + k_6\overline{K}_R)/(\overline{K}_S + \overline{K}_R)$$

The individually determined values of $k_3$, $k_6$, $K_S$, and $K_R$ in Table I lead to predicted values of "$k_{cat}$" for (RS)-3-fluoro-1,2-propanediol of 128 $sec^{-1}$ (compared to the observed value of 128 $sec^{-1}$) and for (RS)-1,2-propanediol of 235 $sec^{-1}$ (compared to the observed value of 250 $sec^{-1}$). We accordingly conclude that both the R and S isomers of these substrates exhibit simple competitive behavior for the same active site on the enzyme.

Binding. The observation that substitution of deuterium for hydrogen at C-1 of 3-fluoro-1,2-propanediol as substrate reduces the value of $k_{cat}$ by a factor of 13 while the value of $K_M$ remains unchanged demonstrates that the observed

Michaelis constant reflects enzyme-substrate dissociation relatively unperturbed by subsequent catalytic events (i.e., $K_M \approx K_S$).

The question of whether the increased values of $K_M$ observed for the fluorodiols relative to the normal substrates might arise from the nonproductive binding of the fluorodiols in which the 3-fluoro substituent occupies the site normally reserved for the 1-hydroxyl group was probed by studying the possible substrate and/or inhibitor role of 1-fluoro-2-propanol and 1,3-difluoro-2-propanol. Neither substance showed any detectable reactivity (less than 1% that of 3-fluoro-1,2-propanediol); nor did either inhibit the enzyme in concentrations up to 100 times that of propanediol. Accordingly, we conclude that the fluorine substituent does not mimic a hydroxyl group and that 3-fluoro-1,2-propanediol binds to the enzyme in a manner essentially analogous to 1,2-propanediol itself, with the fluoromethyl group occupying the methyl site.

Transition State Energies. If one accepts the point made above, that $K_M \approx K_S$, one can use the kinetic and binding data to estimate the free energies of the Michaelis complex relative to free enzyme and substrate and to estimate the activation energy from the E-S complex to the rate-determining transition state. The resulting values for these free energies collected in Table II demonstrate that there is an approximate, though not exact, quantitative compensation between binding affinity and catalysis such that the

Table II. Comparison of the Energetics of Binding and Catalysis.

| | $\Delta G$ (kcal/mol) Binding | $\Delta G^{\ddagger}$ (kcal/mol) Catalysis |
|---|---|---|
| (R)-H-diol[a] | 6.27 | 14.53 |
| (S)-H-diol | 6.97 | 14.93 |
| (R)-F-diol | 5.44 | 15.31 |
| (S)-F-diol | 4.08 | 14.58 |

[a]H-diol = 1,2-propanediol; F-diol = 3-fluoro-1,2-propanediol.

comparative overall reactivities of the R and S isomers of both 1,2-propanediol and 3-fluoro-1,2-propanediol are remarkably similar.

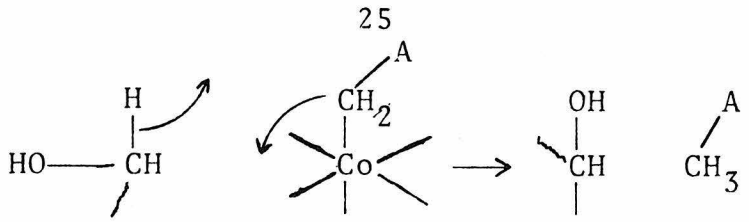
Reaction Mechanism. The observation of a kinetic isotope effect of about 13 observed when deuterium is substituted for hydrogen at C-1 of 3-fluoro-1,2-propanediol indicates that hydrogen removal from C-1 is one of the major rate-determining steps in the enzymatic reaction of the fluorodiol, which is analogous to the situation for 1,2-propanediol itself. More significantly we interpret the great similarity between the activation energies for 3-fluoro-1,2-propanediol and 1,2-propanediol as substrates for diol dehydrase to support the proposal that the hydrogen abstraction occurs without the development of significant charge on the substrate molecule and most likely involves, therefore, a radical (or concerted), as distinct from an ionic, pathway.

The effect of fluorine as a substituent has been determined in many types of simple organic reactions. The effects are large for ionic processes and can be significant or very small for radical pathways. For example, in reactions involving primary carbonium ions, a fluorine on the cationic carbon can stabilize the intermediate by 29.5 kcal/mol. When on the adjacent carbon, fluorine destabilizes the carbonium ion by 9.9 kcal/mol (Clark and Lilley, 1970; Martin et al., 1966). In anionic situations fluorine generally exerts a stabilizing influence. For example, the rate of base

catalyzed hydrogen exchange at C-9 of fluorene is accelerated by a factor of $10^5$ by the substitution of a trifluoromethyl group for one of the hydrogens (Streitwieser and Mares, 1968). Further, the substitution of a fluorine for one of the hydrogens of acetic acid results in a decrease in $pK_a$ from 4.76 (acetic acid) to 2.77 (fluoroacetic acid).

The effect of a fluorine substituent on a radical process depends significantly on the polarity of the transition state. For attack by highly reactive, strongly electronegative radicals (such as Cl· or F·) the transition states tend to have significant polar character, while less reactive radicals generate transition states with much less polar character. Studies of free radical halogenation of 1-halobutanes showed that, compared to butane itself the reactivity at C-1 of 1-fluorobutane was reduced to 1/3 if attack was by F· and increased tenfold if attack was by Br··. In either case, the effect of a fluorine on hydrogen abstraction from C-3 was small (a relative rate of 1.3 for butane vs. 1.0 for 1-fluorobutane for attack by F· and of 80-82 for butane vs. 82-90 for 1-fluorobutane for attack by Br·) (Fredericks and Tedder, 1960; Galiba et al., 1966).

The possibility that removal of hydrogen from C-1 of propanediol is concerted with formation of a new covalent carbon-cobalt bond through a four-center transition state should also be considered. Recent model studies suggest that substances with a covalent carbon-cobalt bond can lead



to rearrangements such as those observed with adenosylcobalamin - enzyme complexes. (The observed rearrangement was an analogue of that of methylitaconate $\rightleftharpoons$ ∝-methylglutarate: Dowd et al., 1975.) The detailed mechanism of such rearrangements remains unresolved though cobalt-$\pi$ type intermediates have been suggested in other model studies (Silverman et al., 1972; Silverman and Dolphin, 1973). The presence of fluorine compared to hydrogen at C-3 of propanediol should have a negligible effect on the energy of such a transition state though quantitative precedents for this assertion are lacking.

The central points from these remarks are (i) the observed kinetic isotope effect indicates that removal of hydrogen from C-1 of propanediol is the rate-determining step and (ii) this removal of hydrogen from C-1 is unlikely to involve cationic or anionic species because substitution of fluorine at C-3 has so little effect on the observed rate. Such a small effect of fluorine at C-3 is, however, consistent with a radical or a concerted pathway for removal of the C-1 hydrogen.

Although one has traditionally regarded the active site of enzymes as providing environments which stabilize ionic situations, there are many amino acid side chain residues (for

example, phenyl, indolyl) which nature might find useful in constructing environments which could effectively stabilize radical type intermediates. This could be accomplished either by rendering the environment very hydrophobic or, more positively, by providing possibilities for stabilization of single electrons of the type seen, for example, in the interaction between halogen radicals and aromatic substances (Huyser, 1965).

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# PART II

## Glycerol and Other Substrates Analogues as Substrates and Inactivators for Propanediol Dehydrase.

## Kinetics, Stereospecificity and Mechanism

# INTRODUCTION

Dioldehydrase ((RS)-1,2-propanediol hydrolase E.C. 4.2.1. 28) from Klebsiella pneumoniae, ATCC 8724) is one of nine known enzymes which utilize adenosylcobalamin as a cofactor to catalyze 1,2 rearrangements of the type

$$\begin{matrix} C_\alpha \\ | \\ X \end{matrix} - \begin{matrix} C_\beta \\ | \\ H \end{matrix} \longrightarrow \begin{matrix} C_\alpha \\ | \\ H \end{matrix} - \begin{matrix} C_\beta \\ | \\ X \end{matrix}$$

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

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

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

# EXPERIMENTAL

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

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

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

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

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

Alcohol Dehydrogenase-β-Nicotinamide Adenine Dinucleotide Assay. Yeast alcohol dehydrogenase (ADH, Sigma Chemical Co. and β-nicotinamide adenine dinucleotide (reduced form, NADH, Sigma Chemical Co.) reduce propionaldehyde to 1-propanol. The maximum velocity of this reaction is 30% of that for acetaldehyde reduction (Bruemmer and Roe, 1971). The

production of propionaldehyde of dioldehydrase can be measured by monitoring the decrease of absorbance at 340 nm, due to the oxidation of NADH to NAD in the presence of excess ADH.

Determination of Inactivation Rates: Coupled Enzyme Assay. Reaction mixtures consisted of: ADH, 15 units; NADH, 0.20 mM, dioldehydrase, 0.1 unit; bovine serum albumin, 0.06 mg; potassium phosphate buffer, pH 8.0, 30mM; AdoCbl, 0.02mM; and the desired amount of 1,2-propanediol and/or inactivator. Total volume 2.50 ml. The reaction mixture was incubated at $37^0$ with stirring. The inactivation reaction was started by the addition of AdoCbl and stopped by addition of 50 µl of a 6.0 M 1,2-propanediol solution. Enzyme activity (measured by the rate of production of propionaldehyde) was determined at a minimum of four different inactivation times; the slope of the corresponding semilog plot represents the observed inactivation rate constant ($k_{i,obs}$) for the given concentration of inactivator and 1,2-propanediol.

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

Determination of Inactivation Rates: DNP Method. Reaction mixtures consisted of the following: dioldehydrase ≃0.06 units; potassium phosphate buffer pH 8.0, 30 mM, AdoCbl 0.013 mM; bovine serum albumin 0.4 mg and the desired amount of substrate and/or inactivator. Total volume, 2 ml 37°. The reaction was started by addition of AdoCbl and the inactivation was quenched with 50µl of 6 M 1,2-propanediol. The amount of enzyme remaining active was a function of time after the addition of AdoCbl was determined by measuring the production of propionaldehyde at 1.0 minute intervals for eight minutes after the addition of 1,2-propanediol. The rate of propionaldehyde production was linear for at least 8 min. after the addition of 1,2-propanediol.

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

β-Hydroxypropionaldehyde can also be determined satisfactorily using the DNP assay as described for propionaldehyde

The reaction mixtures used were the same as above with 0.6 unit of dioldehydrase giving the best results. Absorbance was measured at 475 mμ. The extinction coefficient at 475 mμ from the dinitrophenylhydrazone of β-hydroxypropionaldehyde is somewhat smaller than that from the dinitrophenylhydrazone of propionaldehyde.

$^{14}$C-glycerol Labeling of Dioldehydrase. Reaction mixtures consisted of the following: dioldehydrase, 70 units; $^{14}$C-glycerol (1.33 x $10^9$ cpm/mmole), 0.02 mmoles; potassium-phosphate buffer pH 8.0, 30mM, AdoCbl, 3.2 μM. Total volume 2.0 ml. Two controls were performed, one in which AdoCbl was omitted from the reaction mixture and the other in which OH-Cbl was substituted for AdoCbl. The reaction mixtures were incubated at 37°C for 20 min. Two basic procedures were used for removing glycerol, TCA precipitation of the proteim or dialysis against guanidine·HCl.

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

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

Substrates. Perdeuterioglycerol ($D_5$-glycerol) was purchased from Merk, Sharp, and Dohme, Ltd., and used without further purification.

1-$^{14}$C-glycerol was purchased from Amersham.

(R)-1,1-Dideuterioglycerol (R-$D_2$-glycerol) was synthesized from D-mannitol via the intermediate preparation of the 1,2,5,6-diisopropylidene derivative (Baer, 1952), and of D-2,3-isopropylidene glycerol (Ghangas and Fondy, 1971). The experimental details of the following steps are given by Eagar et al. (1975). Potassium D-2,3-isopropylidene glycerate was prepared by alkaline potassium permanganate oxidation of D-2,3-isopropylidene glycerol and was esterified with methyl-iodide in hexamethylphosphoramide. The methyl ester was then reduced with lithium aluminum deuteride (99% $^2$H, Stohler Isotope Chemicals). The ketal was hydrolyzed in ethanol-sulfuric acid and the product distilled to give (R)-1,1-dideuterioglycerol with $[\alpha]_D^{25}$ + 0.014°[1]. Deuterium content was at least 98% as determined by proton NMR.

(RS)-1,1-Dideuterioglycerol (RS-$D_2$-glycerol) was prepared from (RS)-1,2-isopropylidene glycerol (Ghangas and Fondy, 1971) by a method analogous to that described above. Deuterium content was at least 98% as determined by proton NMR.

(RS)-1,1-Dideuterio-1,2-propanediol was prepared by lithium aluminum deuteride reduction of dl-ethyl lactate

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

(Fieser and Fieser, 1967). Deuterium content was at least 98% as determined by proton NMR. The isotope effect on catalysis was 13, somewhat greater than the value of 10-12 reported by Abeles (1972). The uv-visible spectrum of the holoenzyme-dideuteriopropanediol complex was identical to those reported for 1,2-propanediol (Wagner et al., 1966) and 3-fluoro-1,2-propanediol (Eagar et al., 1975).

3,3,3-Trifluoro-1,2-propanediol was prepared from 3,3,3-trifluoro-1-bromacetone by lithium aluminum hydride reduction to the corresponding alcohol (McBee and Burton, 1952), which was collected by fractional distillation at 124-124.5°. The alcohol (12.8 g, .0674 mole) was then added to a solution of 4 g NaOH in 10 ml water, and 3,3,3-trifluoro-1,2-epoxypropane distilled out of the reaction mixture in 95% yield. The epoxide was refluxed overnight in 1% $H_2SO_4$, the solution was extracted five times with ethyl ether, and the ether evaporated to give the diol.

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

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

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

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

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

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

NMR spectra were obtained at 60 MHz in the ourier transform mode on a Varian T-60 spectrometer in deuteriochloroform or $^{2}H_{2}O$.

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

# RESULTS

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

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

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

where $E_a$ is the amount of active enzyme remaining at time t, $E_o$ is the initial amount of enzyme present, and $k_{i,obs}$

is the observed first-order rate constant for inactivation. Thus a plot of $\ln[E_a]$ versus time is linear and the slope gives a value of $k_{i,obs}$ for a given glycerol concentration. A plot of $k_{i,obs}$ versus glycerol concentration (Fig. 1) shows Michaelis-Menten type saturation behavior. The inactivation process can, accordingly, be described in terms of reversible association between enzyme and glycerol followed by irreversible inactivation

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

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

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

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

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

where E is active enzyme free in solution, with

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

the rate of inactivation (eq 3) becomes

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

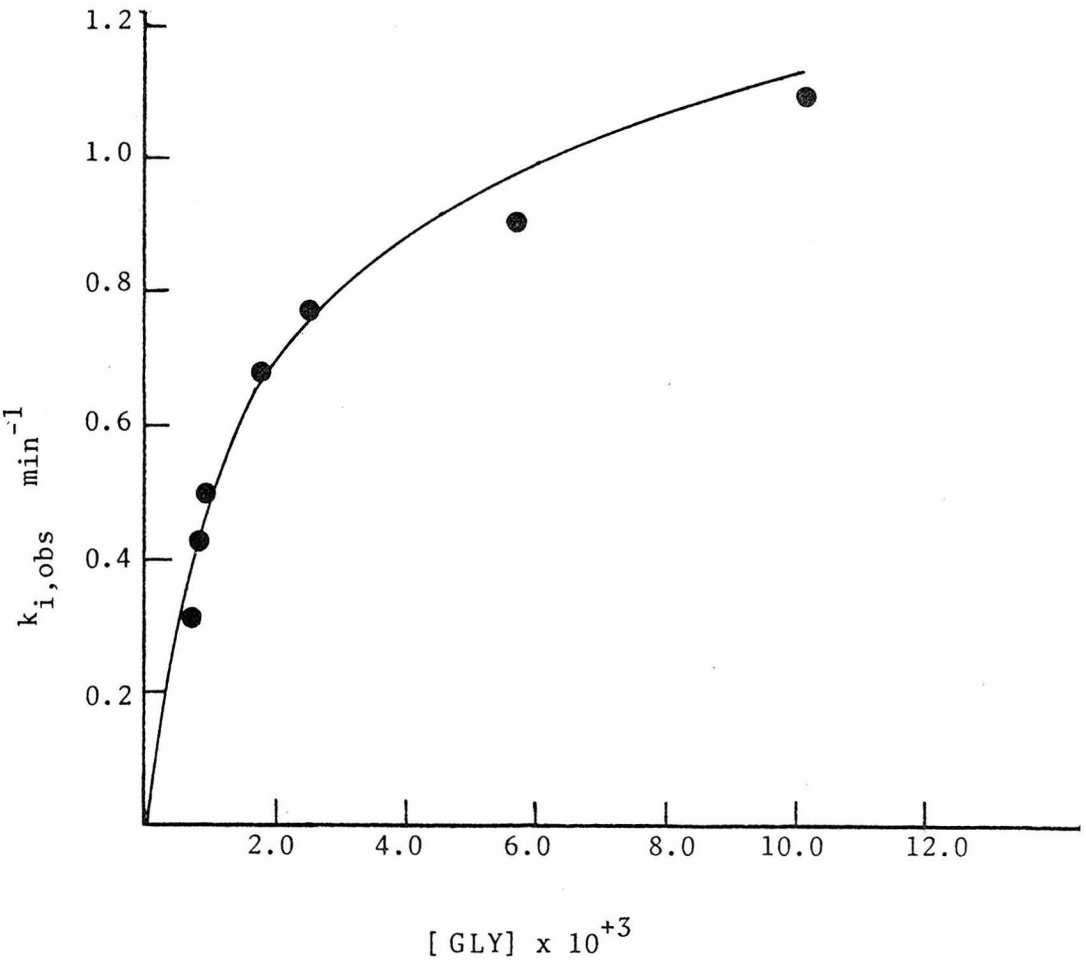



Figure 1: Dependence of the observed rate of inactivation, $k_{i,obs}$, on glycerol concentration under anaerobic conditions and in the absence of 1,2-propanediol. Values of $k_{i,obs}$ were obtained from the least squares line of the linear plot of ln% enzyme activity remaining vs. inactivation time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

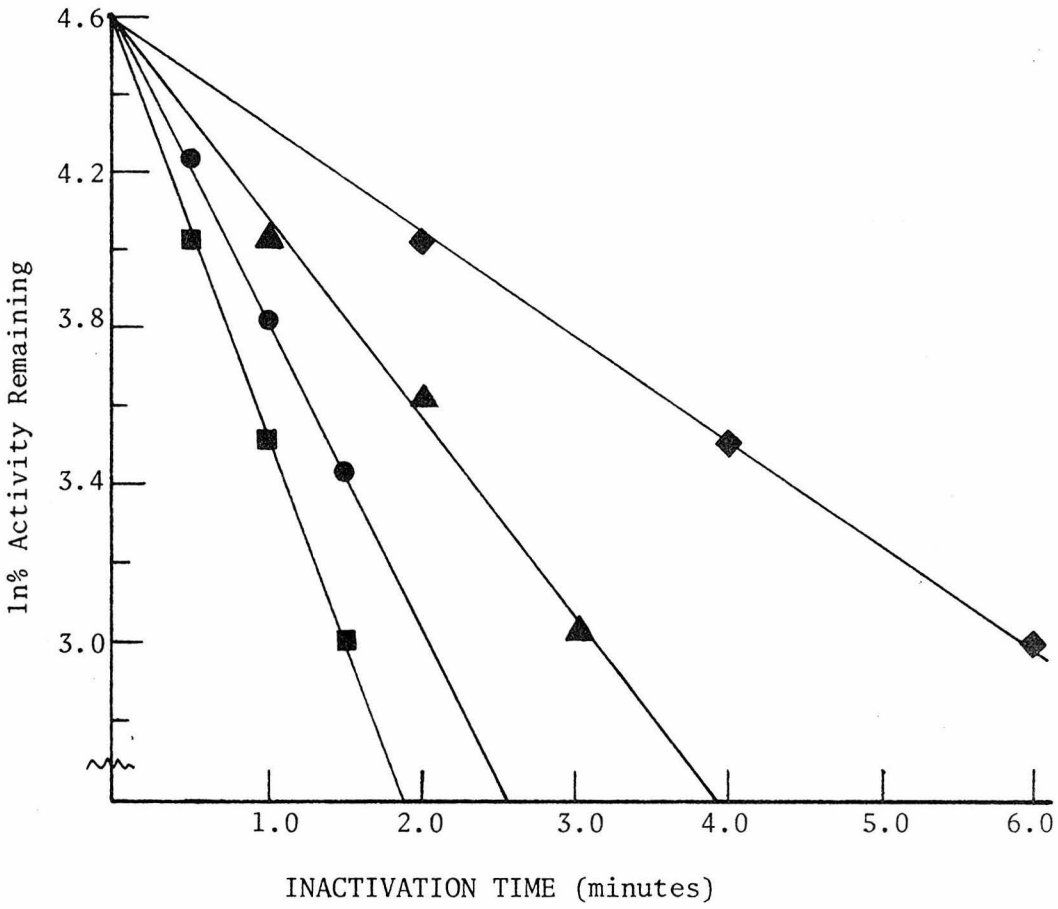



FIGURE 1B: Plot of ln% activity remaining versus inactivation time. ■, 1.04 x $15^{-2}$ M glycerol; ●, 2.5 x $10^{-3}$ M glycerol; ▲, 9.0 x $10^{-4}$ M glycerol; ◆, 5.9 x $10^{-4}$ M glycerol. Values of $k_{i,obs}$ were obtained for each glycerol concentration from slope of the above least squares lines.

Integration gives

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

where

$$k_{i,obs} = \frac{k_i}{1 + \frac{K_G}{[G]}} . \tag{6}$$

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

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

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

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

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

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

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

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

where

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

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

Thus, the above equation allows what we believe to be the first reliable determination of the Michaelis constant for 1,2-propanediol and dioldehydrase (Table I). The (S)-isomer of 1,2-propanediol is more effective at inhibiting

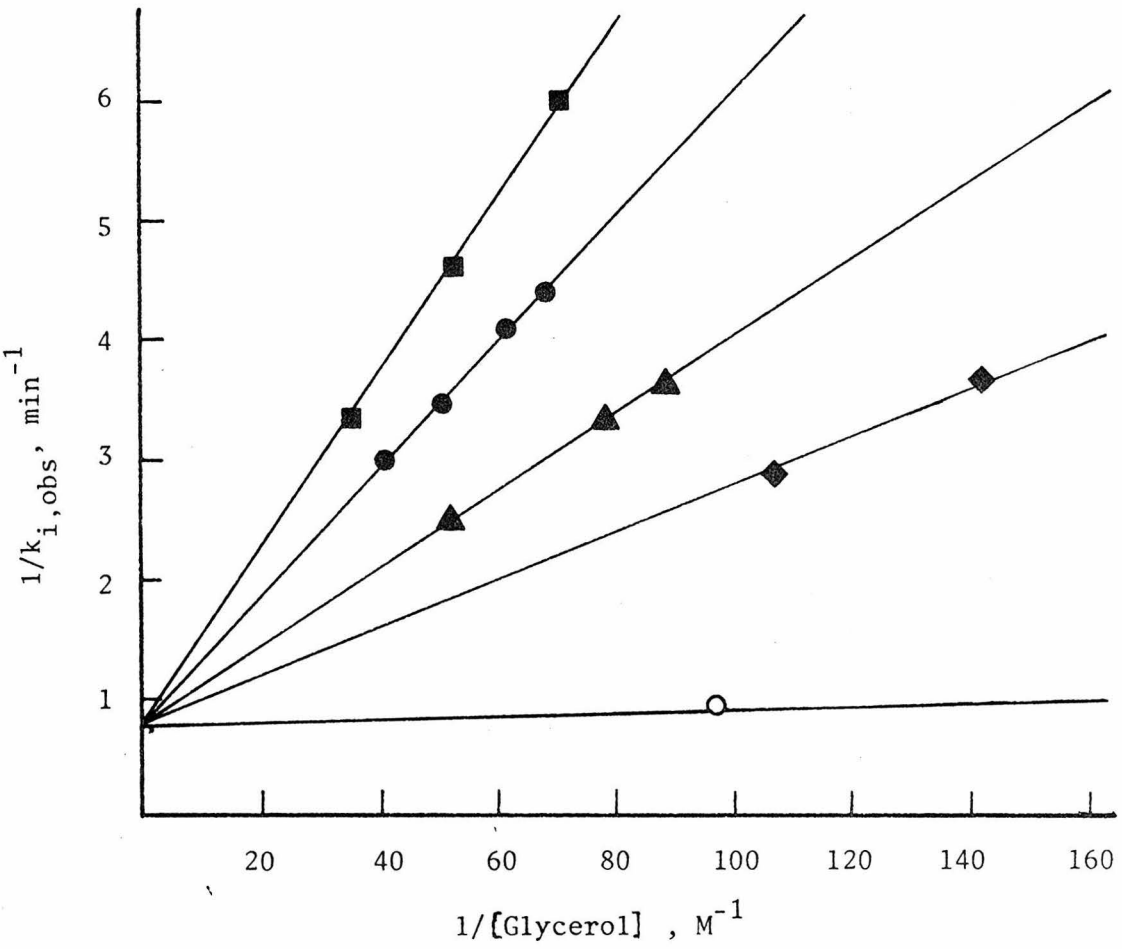



Figure 2: Plot of the reciprocal of the observed inactivation rate constant vs the reciprocal of glycerol concentration in the presence of the following concentrations of (RS)-1,2-propanediol: ■, $1.9 \times 10^{-3}$ M; ●, $1.34 \times 10^{-3}$ M; ▲, $8.6 \times 10^{-4}$ M; ◆, $4.75 \times 10^{-4}$ M;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

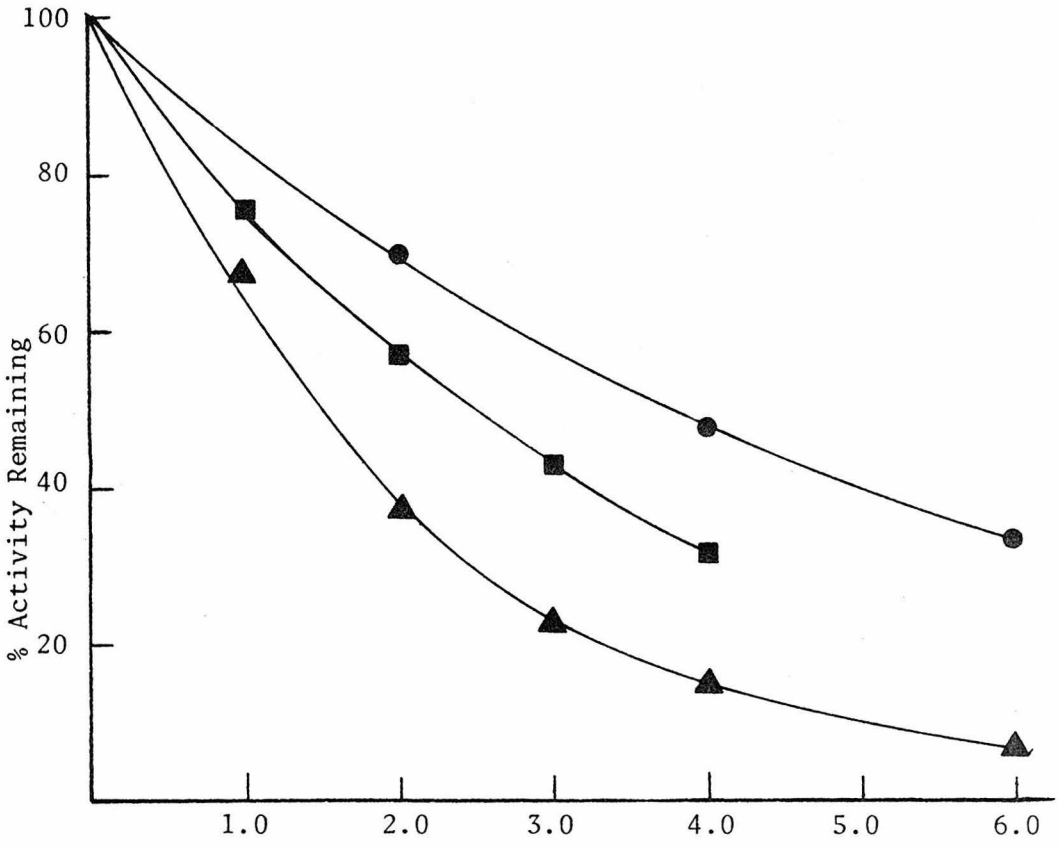



Figure 2B: Rate of inactivation at $1.4 \times 10^{-2}$ M glycerol in the presence of $9.5 \times 10^{-4}$ M 1,2-propanediol. ▲, R-1,2-propanediol; ■, RS-1,2-propanediol; ●, S-1,2-propanediol.

inactivation than is the (RS)-mixture, while (R)-1,2-propanediol is a much less effective inhibitor than the (RS)-mixture That the (R)- and (S)-isomers differ in their ability to protect dioldehydrase from glycerol inactivation reflects a difference in their respective values of $K_M$ (Table I). The values of $K_M$ obtained in this way are really $K_i$'s. However, the ratio of $K_i$ values for (R)- and (S)-1,2-propanediol determined by this method agrees well with the value of 3.2 reported by Jensen et al. (1975) and suggests that in this case $K_i = K_M$.

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

Deuterium Isotope Effects on the Rate of Inactivation by Glycerol. The rates of enzyme inactivation at saturating concentrations of R-$D_2$-glycerol, RS-$D_2$-glycerol, and $D_5$-glycerol were measured by the method just described and were considerably slower than for the undeuterated substrate. Substitution of deuterium in glycerol has little or no measurable effect on $K_M$ (manuscript in preparation) and the ratio of the rate of inactivation observed for glycerol to that

Table I. Kinetic Parameters of Substrates and Inactivators of Dioldehydrase.

| Substrate | $K_M \times 10^{+4}$ | $k_p$ $sec^{-1}$ [f] | $k_i$ $min^{-1}$ |
|---|---|---|---|
| (S)-1,2-propanediol | 0.186[a] | 191 | ~0 |
| (RS)-1,2-propanediol | 0.30[a] | 250 | ~0 |
| (R)-1,2-propanediol | 0.60[a] | 340 | ~0 |
| glycerol | 16.0[b] | 412 | 1.30 |
| 1-chloro-2,3-propanediol | 4.1 | >0[c] | 0.36 |
| trifluoropropanediol | 1.24 | >0[c] | 0.016 |
| 2,3-butanediol (meso + D) | 2.0 | 4.0[d] | 0.068 |
| 1,2-butanediol | 5.9 | >0[c] | 0.054 |
| isobutylene glycol | 8.8 | 18 | 0.47 |
| ethylene glycol | 6.6 | 150[d,e] | 0.098 |
| thioglycerol | 0.1 | ~0 | 1.74 |

a. Values of $K_M$ for the natural substrates were determined in competition with glycerol with the natural substrate serving as "inhibitors" of inactivation as described in the results. Preliminary values of $K_M$ for the 1,2-propanediols obtained by this method were previously published (Eagar et al., 1975). The values given here are slightly different and represent a refinement in technique.

b. A publication has recently appeared (Toraya et al., 1976) also reporting glycerol to be a substrate and inactivator of dioldehydrase. A value of $K_M$ for glycerol was given that agrees with the value we have determined.

c. Product formation does occur but rate was not quantitated.

d. Determined from amount of product formed before complete inactivation.

e. Direct comparison of initial rate to that of 1,2-propanediol.

f. Based on a molecular weight of 250,000 and specific activity of 60 units/mg, with 1,2-propanediol as substrate.

observed for the same concentration of one of the deuterated glycerols accordingly reflects the isotope effect on $k_i$. Thus, when measurements are made at large concentrations of glycerol and its deuterated derivatives,

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

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

The $k_{i,obs}$ values determined in these experiments should be only slightly smaller than the true $k_i$ values (because at 0.04 M inactivator with $K_M = 1.6 \times 10^{-3}$ M, $K_M/[G] \sim 4 \times 10^{-2} << 1$, see eq 6) and can therefore represent experimentally determined values of $k_i$ for each deuterated glycerol.

With $D_5$-glycerol we observe a large isotope effect (14) on the rate of inactivation. We observe smaller, nearly equal values for R-$D_2$-glycerol (1.8) and RS-$D_2$-glycerol (1.9).

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

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

| Substrate | Competitive[a] turnover number T = 60 min | $k_p$, $sec^{-1}$[b] | $(\frac{k_H}{k_D})_p$ | $k_{i,obs}$ $min^{-1}$ 0.04 M inactivator | $(\frac{k_H}{k_D})_i$ |
|---|---|---|---|---|---|
| Glycerol | 19,000 | 412 | 1 | 1.25 | 1 |
| $D_5$-Glycerol | 33,000 | 52 | 8.0 | 0.0893 | 14 |
| RS-$D_2$-Glycerol | 16,340 | 190 | 2.2 | 0.694 | 1.9 |
| R-$D_2$-Glycerol | 5,320 | 61 | 6.8 | 0.658 | 1.8 |

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

[b] Calculated from equation 9 using $k_i$ and $P_\infty/E_o$.

Turnover Number. The total amount of β-hydroxypropionaldehyde formed relative to the amount of enzyme initially present was determined for glycerol, R-$D_2$-glycerol, RS-$D_2$-glycerol and $D_5$-glycerol as substrates. A known amount of dioldehydrase was allowed to react for 60 min with saturating amounts of each of the above glycerols at 37°C. No further β-hydroxypropionaldehyde was produced during an additional 60 min period indicating that complete inactivation of the enzyme had occurred during the initial 60 min period. The total amount of β-hydroxypropionaldehyde formed was determined by the acrolein specific assay and the results are presented in Table II as "competitive turnover numbers," the average number of molecules of product formed by each molecule of holoenzyme before its inactivation.

Although both R-$D_2$-glycerol and RS-$D_2$-glycerol yield less product than glycerol itself before inactivating the enzyme, $D_5$-glycerol forms nearly twice as much product as glycerol.

Rate of Formation of β-Hydroxypropionaldehyde. The production of β-hydroxypropionaldehyde was followed for several minutes with glycerol, $D_5$-glycerol and R-$D_2$-glycerol each serving as substrate. The results in Fig. 3 show that although the initial rate of product formation is much greater for glycerol than $D_5$-glycerol, the rate at which glycerol inactivates the enzyme relative to inactivation by

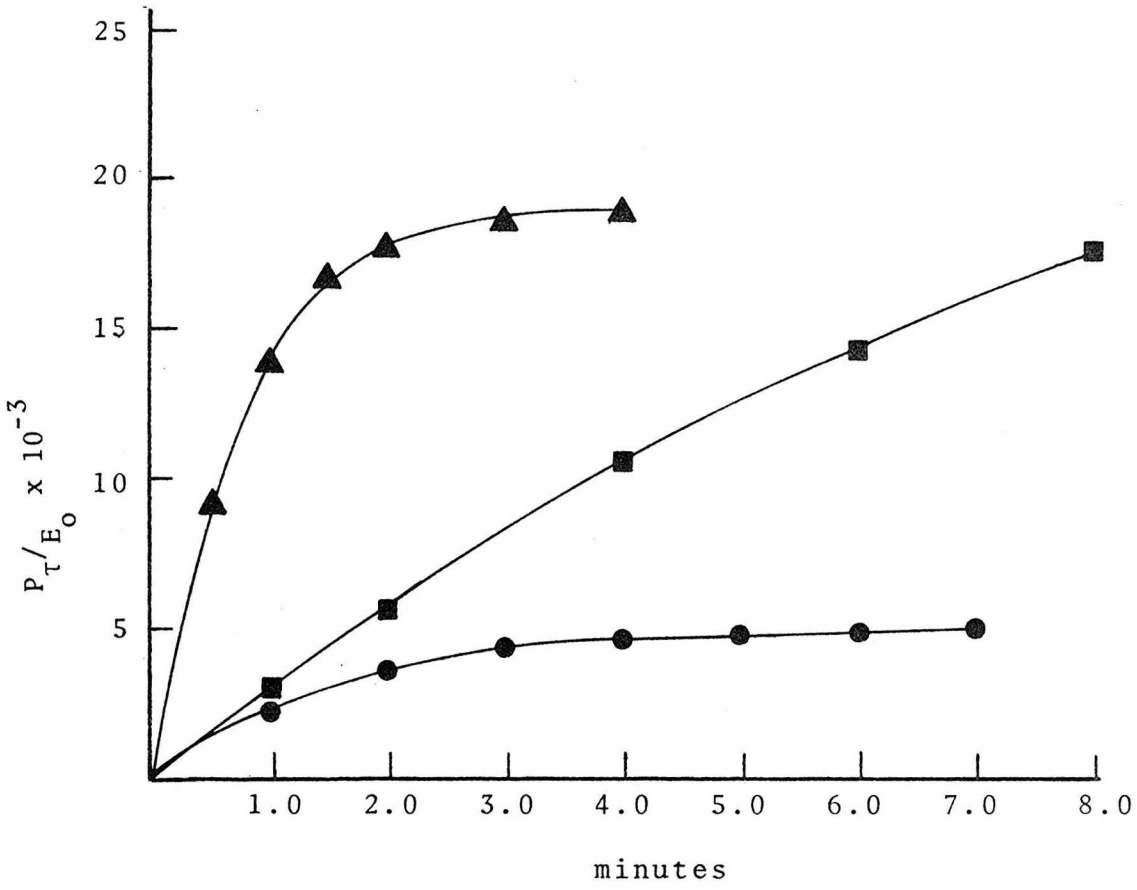



Figure 3: Production of β-hydroxypropionaldehyde as a function of time for: ▲ , glycerol; ■ , $D_5$-glycerol; ● ,R-$D_2$-glycerol. β-hydroxypropionaldehyde was determined by the DNP assay as described in the experimental and is depicted here as the competitive turnover number, $P_\tau /E_o$, for uniformity. [$E_o$] based on 60 units/mg and a molecular weight of 250,000 g/mole.

$D_5$-glycerol is greater by an even larger factor, thus resulting in a net enhancement of product formation from $D_5$-glycerol.

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

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

$$[EG] = [E_a].$$

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

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

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

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

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

$$\frac{[P_\infty]}{[E_o]} = \frac{k_p}{k_i}$$

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

The values of $\frac{[P_\infty]}{[E_o]}$ have been determined as have those of $k_i$ for glycerol and each of its deuterated derivatives (Table II) so that values of $k_p$ can be calculated; they are also listed in Table II. Indeed one can plot $[P_\tau]/[E_o]$ as a function of time using the values of $k_i$ and $k_p$ in Table II and accurately reproduce the experimental graphs of Fig. 3.

Deuterium Distribution in Products. The 2,4-dinitrophenylhydrazones of β-hydroxypropionaldehydes formed from the variously deuterated glycerols were purified by thin layer chromatography on silica gel with chloroform as the eluting solvent. The $^1H$ nmr spectra of these derivatives showed that R-$D_2$-glycerol gives product with essentially no deuterium at C-3, while RS-$D_2$-glycerol gives product with approximately 60% deuterium at C-3.

Product Formation-Other Substrates. With the exception of 1,2-butanediol and thioglycerol, all of the substrate analogues listed in Table I gave easily detectable amounts of product. Products from isobutylene glycol and 2,3-butanediol

were identified as isobutyraldehyde and 2-butanone, respectively, by thin-layer chromatography of the 2,4-dinitrophenylhydrazones on silica gel with chloroform as the eluting solvent. Aldehydes from 3,3,3-trifluoro-1,2-propanediol and 3-chloro-1,2-propanediol were detected by the DNP assay but were not rigorously characterized.

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

Even though 1,1-dideuterio-1,2-propanediol reacts more slowly by a factor of 13 than 1,2-propanediol itself, use

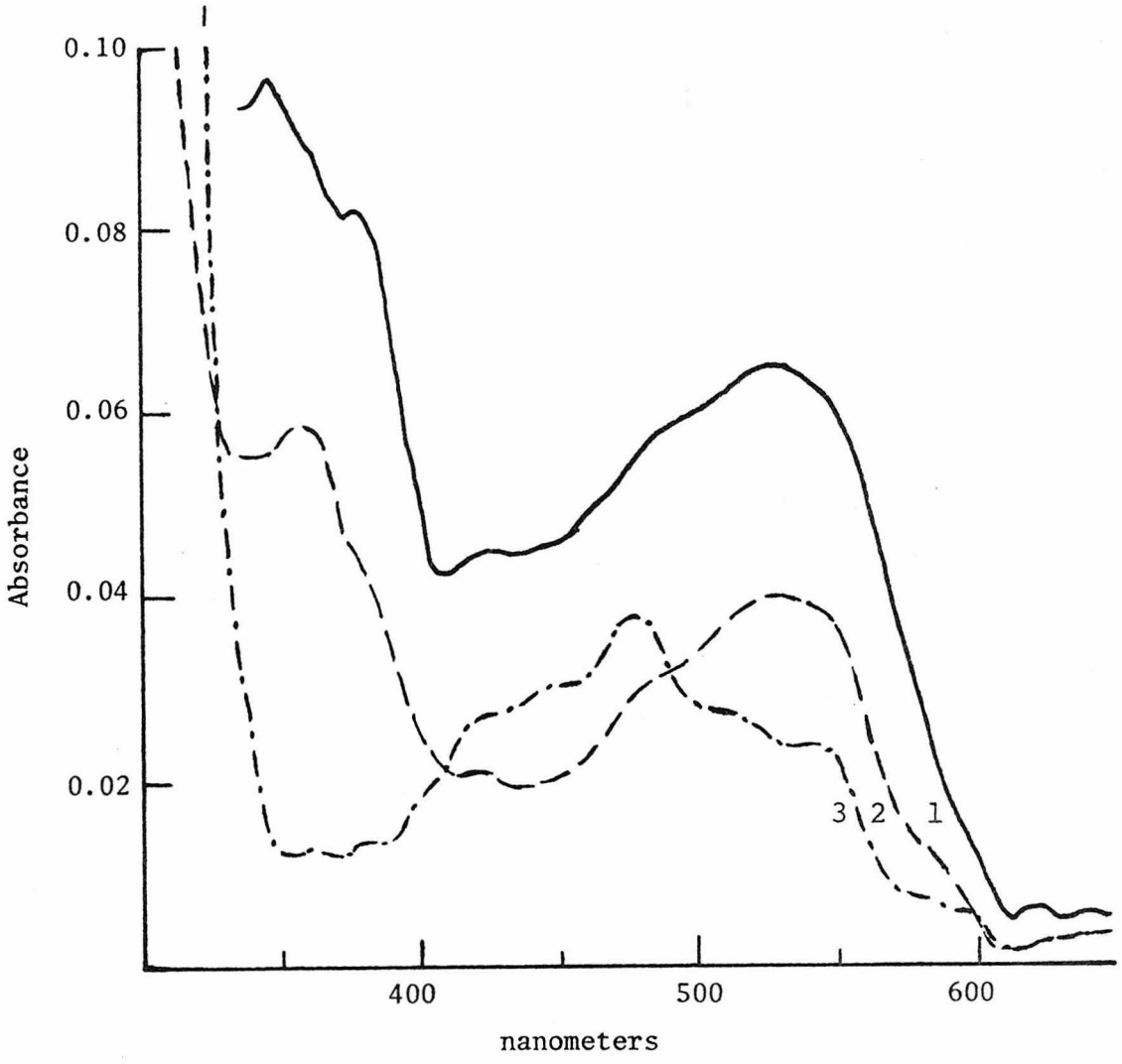



Figure 4 : Comparison of the effect of glycerol to the effect of 1,2-propanediol on the spectrum of enzyme-adenosylcobalamin. (1) diolde-hydrase, 37 units; AdoCbl, , $2.3 \times 10^{-3}$ μmoles; $K_2HPO_4$, 3 μmoles in 0.3 ml. The reference cell was identical to sample but lacked AdoCbl. (2) Glycerol, 0.06 mmoles added in 0.03 ml to both sample and reference of (1). Total volume 0.33 ml. (3) 1,2-propane-diol, 18 μmoles added to sample and reference of (1). Total volume 0.33 ml. 27°C.

of 1,1-dideuterio-1,2-propanediol in place of 1,2-propanediol does not measurably affect the amount of enzyme-bound AdoCbl observable as $Cbl^{II}$. Because $D_5$-glycerol is a reasonably good "substrate" but inactivates much more slowly than glycerol, we looked for spectral changes when $D_5$-glycerol instead of glycerol was added to holoenzyme; none were observed. Thus with either form of glycerol, we could see no evidence for $Cbl^{II}$.

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

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

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

Spectral Observation of Enzyme-Bound OH-Cbl. Although the general character of OH-Cbl remains essentially unchanged on binding to dioldehydrase, small bathochromic shifts of the bands above 300 nm do occur and serve to distinguish OH-Cbl

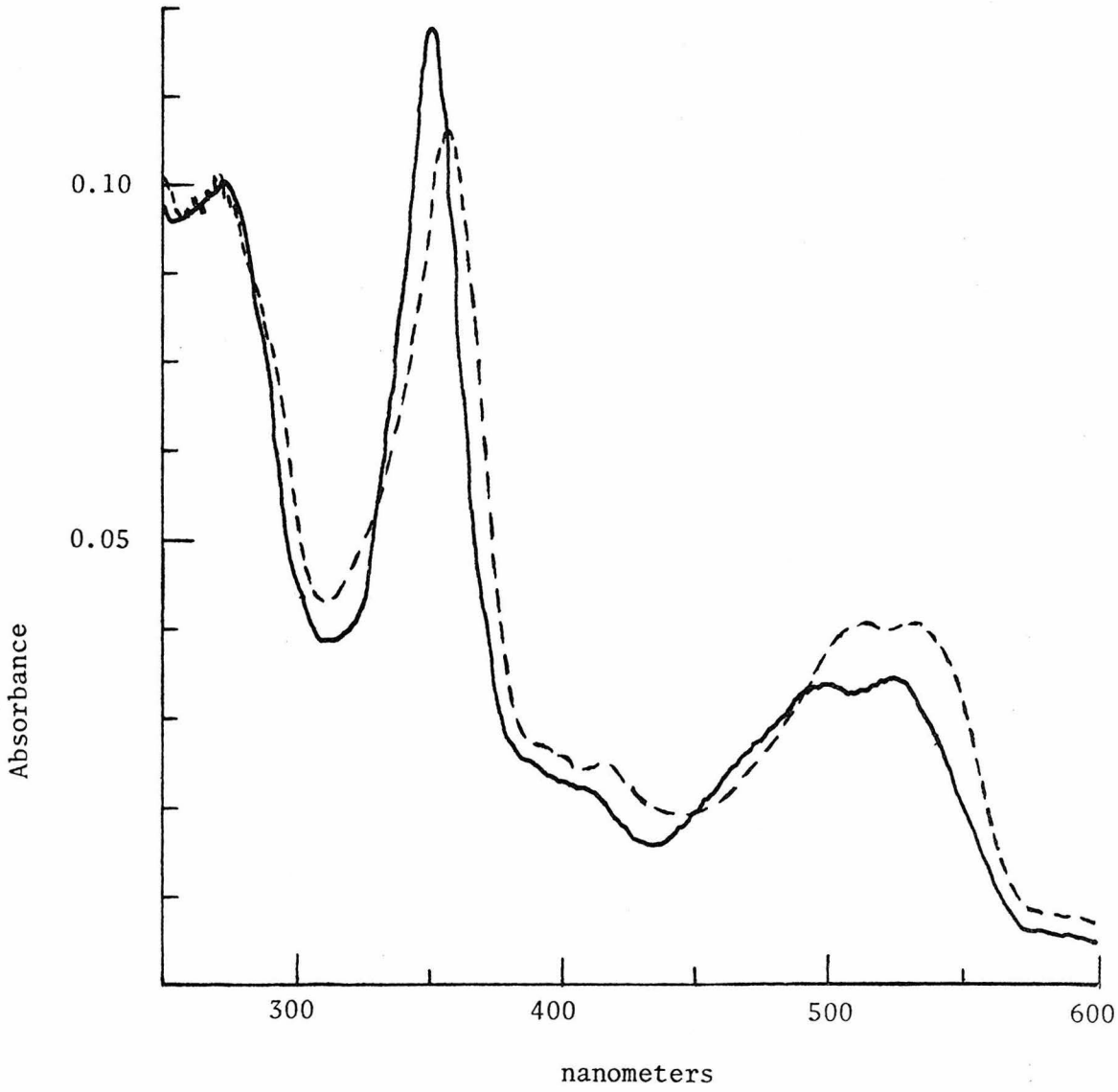



Figure 5: Comparison of the absorption spectrum of OH-Cbl free in solution with that bound to dioldehydrase. (—), OH-Cbl, $1.4 \times 10^{-3}$ µmoles; $K_2HPO_4$ buffer pH 8.0, 0.3 µmoles. Total volume 0.32 ml. (--), enzyme, 30 units; OH-Cbl, $1.4 \times 10^{-3}$ µmoles; and $K_2HPO_4$ in 0.32 ml. The reference cell was identical to the sample cell but lacked OH-Cbl, $27^{o}$ C.

free in solution from that bound to dioldehydrase (Fig. 5). OH-Cbl also binds to BSA, for example, but the resulting electronic spectrum is identical to that of free OH-Cbl. For OH-Cbl in 0.01 M $K_2HPO_4$ buffer at pH 8.0 we observe $\lambda$ max at 525, 510 and 352 nm. For OH-Cbl bound to dioldehydrase in $K_2HPO_4$ buffer at pH 8.0 the corresponding $\lambda$ max are 540, 515 and 362 respectively.

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

Recovery of Active Apoenzyme from Inactive Apoenzyme-OH-Cbl Complexes. Dialysis of an enzyme-OH-Cbl complex against a solution containing $Mg^{2+}$ and $SO_3^{2-}$ leads to recovery of the OH-Cbl in the dialysate. Subsequent dialysis to remove the $SO_3^{2-}$ (which appears to be a potent inhibitor

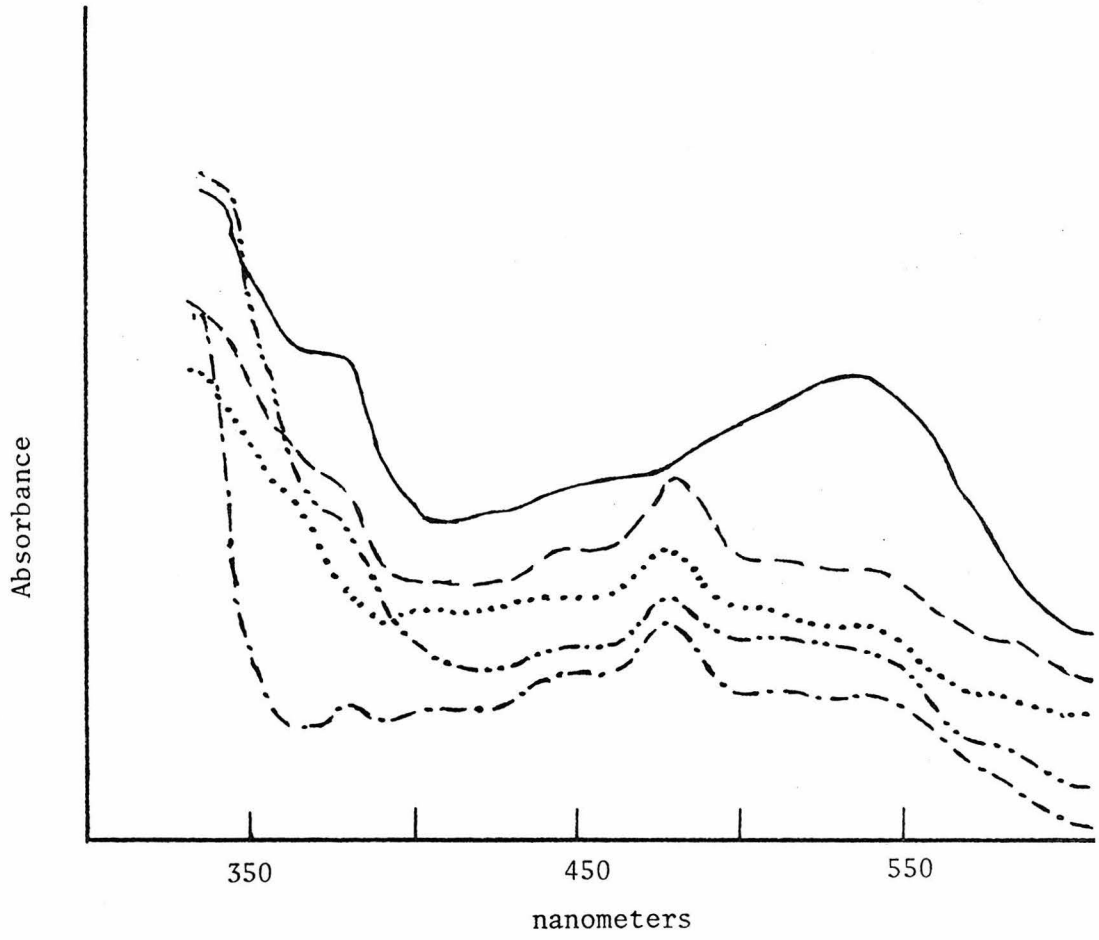



Figure 6: Effect of several substrate analogues on the absorption spectrum of enzyme-bound adenosylcobalamin. (—), enzyme, 30 units; $K_2HPO_4$, 3.5 μmoles; AdoCbl, $1.8 \times 10^{-3}$ μmoles; total volume 0.35 ml. Reference cell was identical minus AdoCbl. To the above sample and reference cuvettes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

of dioldehydrase) leaves apoenzyme which on addition of Ado-Cbl is catalytically active (up to 80% of the original activity can be recovered). Treatment with either reagent alone or both reagents sequentially does not remove enzyme-bound OH-Cbl. Table III gives details of this procedure, which is a modification of a procedure for exchanging OH-Cbl and Ado-Cbl with glycerol dehydrase (Schneider et al., 1970), and some representative results.

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

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

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

Table III. Reactivation of Several Forms of Inactive Dioldehydrase.

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

[a]Reaction mixture #3 contained 80 µmoles glycerol, otherwise the four reaction solutions were identical and contained: $K_2HPO_4$ buffer pH 8.0, 80 µmoles; Bovine serum albumin, 0.02 mg; and deoldehydrase, 17 units. Total volume 2.1 ml. Enzyme activity was determined by assaying a 10 µl aliquot for activity by a method described in the experimental section.

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

[c]Reactivation: Treatment A. Each of the four solutions, after inactivation, were subjected to dialysis for 12 hour periods against each of two changes of 2 liters of solution containing: $K_2SO_3$, 100 mmoles; $Mg(CH_3CO_2)_2$, 60 mmoles; 1,2-propanediol, 200 mmoles; and $K_2HPO_4$ buffer pH 8.0, 20 m moles. This was followed by dialysis for 3 hour periods against two changes of 2 liters of solution containing: 1,2-propanediol, 200 mmoles; and $K_2HPO_4$ buffer pH 8.0, 20 m moles.

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

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

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

Thus, although several molecules of glycerol appear to become very tightly associated with dioldehydrase apoenzyme the acquisition of more than one molecule of glycerol is strictly AdoCbl-dependent. Further investigation on this subject is under way.

# DISCUSSION

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

$$\text{SH} + \text{AdoCbl-E} \rightleftharpoons \underset{\text{(I)}}{\text{SH-AdoCbl-E}} \longrightarrow \underset{\text{(II)}}{\text{S}\bullet\ \begin{matrix}\text{HAdo}\\ \bullet\text{Cbl}\end{matrix}} \rightleftharpoons \underset{\text{(III)}}{?} \rightleftharpoons \underset{\text{(IV)}}{\text{PH}\ \begin{matrix}\cdot\text{Ado}\\ \cdot\text{Cbl}\end{matrix}} \rightleftharpoons \text{P} + \text{AdoCbl-E}. \qquad (9)$$

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

Four areas of experimental results of this work can be used to assess possible molecular mechanisms of inactivation and catalysis: (i) kinetics of catalysis and inactivation (II) kinetic isotope effects (iii) spectral observations of enzyme-bound cofactor before and after inactivation (iv)

stereospecificity of catalysis and inactivation. These four aspects were studied in considerably greater detail with glycerol as substrate-inactivator. However, results obtained with this substrate may also apply to the others, in view of their similar kinetic behavior.

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

Kinetic Isotope Effect. Results obtained with $D_5$-glycerol ($\frac{VmaxH}{VmaxD} \sim 8$) demonstrate that cleavage of a carbon-hydrogen bond is one of the important rate-contributing steps in the enzyme-catalyzed formation of product from glycerol. This suggests that the mechanism of catalysis is probably very similar to that of the normal substrate 1,2-propanediol. The isotope effect of 14 on inactivation suggests that the

mechanism of inactivation may in part be very similar to catalysis. However, we have verified that β-hydroxypropionaldehyde neither inhibits nor inactivates dioldehydrase holoenzyme. Inactivation, then, must occur somewhere between the initial binding of glycerol and the release of product.

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

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

The inactivation reaction may be the result of the formation of a highly reactive intermediate such as II or

III which, due to the modification of the substrate, lacks the proper steric or electronic configuration necessary for normal catalysis. Destruction of cofactor and/or enzyme by some noncatalytic reaction of the above intermediate may be responsible for inactivation.

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

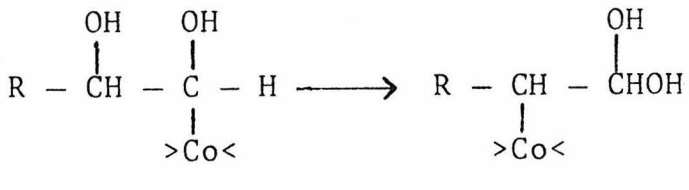
Of the inactivators listed in Table I only with 2,3-butanediol is inactivation accompanied by the transformation of enzyme-bound cofactor to enzyme-bound OH-Cbl at a rate similar to that of inactivation. With this inactivator, as in the case of oxygen inactivation, the formation of $Cbl^{II}$ cannot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

However, of the inactivators that do induce formation of $Cbl^{II}$, none exhibit a transformation of the $Cbl^{II}$ spectrum to that of OH-Cbl concomitantly with inactivation. The for-

mation of enzyme-bound OH-Cbl follows more rapidly after inactivation with ethylene glycol as substrate than with any of the other inactivators that induce $Cbl^{II}$ formation. This may be the result of a diminished ability of ethylene glycol, the smallest of these substrates, to protect the inactive enzyme·$Cbl^{II}$ complex from oxygen.

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

AdoCbl-dependent rearrangements have been postulated to take place via a transalkylation reaction (Intermediate




$III_a$ $III_b$

$III_a$ is presumably formed from intermediate II.), although no concrete evidence has yet been obtained for such a reaction occurring with any AdoCbl-dependent enzyme. The visible spectrum of glycerol-inactivated AdoCbl·enzyme may be due to a "trapped" intermediate such as $III_a$ or $III_b$. Intermediate $III_b$ is more likely in view of the observed lability of the intermediate, secondary alkylcobalamins being generally less stable than primary alkylcobalamins (Hogenkamp, 1975).

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

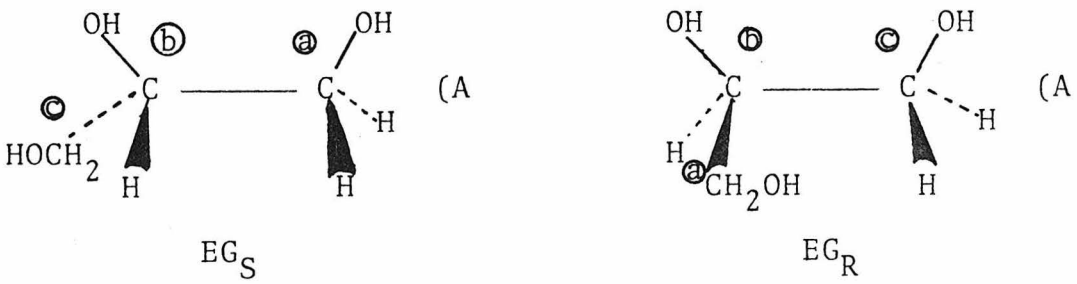
The presence of a reactive apoenzyme moiety at the active site is also indicated by the observation that removal of OH-Cbl from oxygen-inactivated holoenzyme does not re-

generate active apoenzyme. (This substrate-free inactivation has previously been attributed only to destruction of enzyme-bound cofactor which then remains irreversibly bound to the enzyme (Wagner et al., 1966).) In this respect the mechanisms of inactivation by oxygen and glycerol may be somewhat similar. A further similarity between glycerol and oxygen-inactivated holoenzyme is seen in Table III. Continued dialysis vs the $Mg^{2+}$ and $SO_3^{2-}$ solution past that required to remove enzyme-bound cobalamin restores some activity. Furthermore, the rate of the restoration appears to be about the same for both the glycerol- and oxygen-inactivated apoenzymes. This reactivation after prolonged dialysis is perhaps due to slow reduction of some oxidized species on the enzyme by $SO_3^{2-}$ and is the subject of continuing investigation.

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

cause, of inactivation. This indicates that the protein must play an active role in inactivation, and by implication, an active role in catalysis.

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

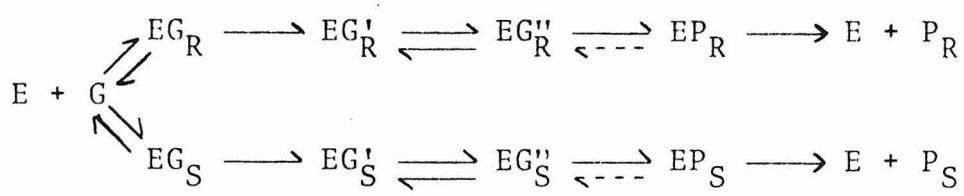



$EG_S$ represents glycerol bound to enzyme with the pro-S hydroxymethyl group at the site involved in hydrogen abstraction ("A"), while $EG_R$ represents the corresponding enzyme-glycerol complex with the pro-R hydroxymethyl group so oriented.

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

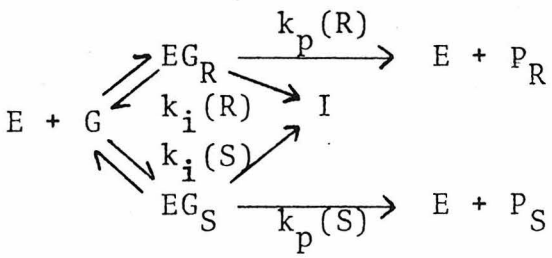
If one assumes the mechanism of the enzyme-catalyzed reaction with glycerol to be the same as that with 1,2-pro-

panediol, the minimum kinetic scheme that must be constructed is



(10)

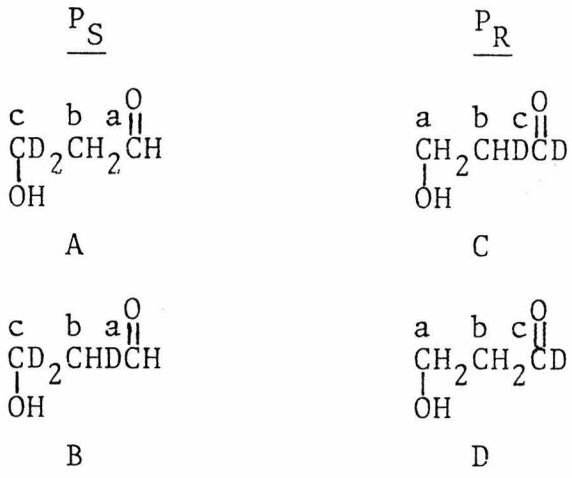
$EG_x$, $EG'_x$, $EG''_x$, and $EP_x$ are analogous to intermediates I-IV in eq 9. To present the following largely qualitative argument, we shall further abbreviate the kinetic scheme above to



(11)

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

Consider first the products formed from R-$D_2$-glycerol; four are possible:




Products A and B are derived from $EG_S$ while C and D are derived from $EG_R$. (Products B and D result from intermolecular hydrogen transfer. That this occurs has been demonstrated by Essenberg *et al.* (1971) and involves C-5' of cofactor as the intermediate hydrogen carrier.) $^1H$ nmr of the 2,4-dinitrophenylhydrazones of the above products indicates that better than 90% products C and D are formed. This result is particularly striking since products C and D should be disfavored relative to A and B due to kinetic isotope effects on product formation. This is strong evidence that product aldehyde originates almost entirely from $EG_R$.

The observed kinetic isotope effects further support this conclusion. For example R-$D_2$-glycerol and RS-$D_2$-glycerol contain the same amount of deuterium. R-$D_2$-glycerol, however, exhibits a large isotope effect on product formation (6.8) while a much smaller effect (2.1) is observed with RS-$D_2$-glycerol. This clearly indicates that deuterium on carbon "c"

has a greater effect on product formation than deuterium on carbon "a".

The isotope effect of 8.0 observed with $D_5$-glycerol must represent the full extent to which an isotope effect can be expressed on catalysis, or more specifically, in light of the above arguments, on $k_p(R)$. The reduction of the isotope effect from 8.0 to 6.8 for R-$D_2$-glycerol, however, must be due to some contribution from hydrogen located on carbon "a". The contribution may be in the form of a small amount of product formation from the $EG_S$ form or possibly some very slow exchange between the deuterium of enzyme-bound coenzyme and hydrogen on the "a" carbon. Only a very small contribution (2%) from hydrogen on the "a" carbon is needed to account for the decrease of the isotope effect from 8.0 to 6.8.

Analogous arguments support the conclusion that inactivation occurs mainly via $EG_S$. For inactivation, $D_5$-glycerol shows an isotope effect of 14; this must also represent the full extent to which an isotope effect can be expressed. Were hydrogen at carbon "c" as important to inactivation as to catalysis, an isotope effect on inactivation much larger than the observed 1.8 would be exhibited by R-$D_2$-glycerol. Accordingly, hydrogen at carbon "a" must make a significant contribution to inactivation.

Does the isotope effect of 1.8 for R-$D_2$-glycerol reflect some inactivation via $EG_R$? There is an alternative explanation. When holoenzyme is inactivated by glycerol (no

deuterium) in the presence of 1,1-dideuterio-1,2-propanediol, an isotope effect is expressed on the inactivation rate (Bachovchin et al., manuscript in preparation). Thus, deuterium located on 1,2-propanediol can participate in the inactivation reaction and contribute to the expression of an isotope effect on $k_i$. The mechanism of this effect most likely involves the 5'-carbon of cofactor as the intermediate hydrogen carrier. The importance of this result to the present discussion is that a similar effect must occur with R-$D_2$-glycerol. The $EG_R$ binding conformation can behave as a deuterated substrate contributing deuterium to the coenzyme during catalysis. Moreover, that the coenzyme should be rich in deuterium follows from the much greater reactivity of the "c" carbon deuteriums in product formation and the much greater rate of catalysis compared to that of inactivation. Thus, it is very difficult to explain an isotope effect on inactivation as small as 1.8 with R-$D_2$-glycerol as inactivator unless inactivation occurs predominately or even exclusively via $EG_S$.

These conclusions are in stereochemical agreement with results obtained with 1,2-propanediol and 3-fluoro-1,2-propanediol (Eagar et al., 1975). With both of these substrates we have found that the "R" binding conformation is more favorable for catalysis than is the "S" binding conformation. Furthermore, substitution of fluorine at C-3 of (S)-1,2-propanediol reduces catalysis by a factor of two, while

the same modification of the (R)-isomer results in nearly no change in the catalytic rate. The present observation is that a hydroxyl group substituted at C-3 of (R)-1,2-propanediol has no detrimental effect on catalysis, while the same modification of the "S"-isomer is very deleterious to catalysis.

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

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

Replacement of the C-3 methyl group by hydrogen (ethylene glycol) results in a 20-fold increase in $K_M$ as well as in irreversible inactivation. Thus the methyl group plays an important role in both binding and in preventing

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

Substitution for hydrogen at C-3 of 1,2-propanediol by hydroxyl, thiol, fluorine, chlorine, methyl, or three fluorines substantially affects binding, catalysis, and inactivation. A quantitatively consistent picture relating these effects to the size or chemical nature of the substituent, however, does not emerge. A possible reason for this may be that each of the above analogues is in reality a mixture of two substrates, the (R)- and (S)-isomers.

A given substituent may have an entirely different effect on the binding, catalysis, and inactivation behavior of each isomer, as we have shown for 3-fluoro-1,2-propanediol (Eagar et al., 1975) and glycerol.

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

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

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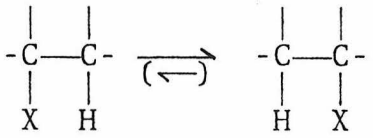
# PART III

## The Involvement of a 5'-Deoxyadenosine Intermediate Containing Three Equivalent Hydrogens in the Mechanism of Glycerol Inactivation of Propanediol Dehydrase Holoenzyme.

# INTRODUCTION

A number of enzymes requiring adenosylcobalamin (AdoCbl) catalyze 1,2-rearrangements of the type



where X can be alkyl, $NH_2$, or OH (Hogenkamp, 1968; Babior, 1975a, 1975b; Abeles and Dolphin, 1976). Propanediol dehydrase is an AdoCbl-dependent enzyme that catalyzes the rearrangement of both (R)- and (S)-1,2-propanediol to propionaldehyde and of ethylene glycol to acetaldehyde.

Until recently (Toraya et al., 1976; Bachovchin et al., 1977) it was thought that dioldehydrase did not act upon glycerol. This fact, until now, served to distinguish this enzyme from another very similar AdoCbl-dependent enzyme, glycerol dehydrase, which in addition to acting upon 1,2-propanediol and ethylene glycol also catalyzes the rearrangement of glycerol to β-hydroxypropionaldehyde.

We have recently demonstrated that glycerol is, in fact, very readily converted to the product aldehyde by dioldehydrase holoenzyme. However, glycerol also effects rapid and irreversible inactivation of dioldehydrase holoenzyme, thus explaining why catalytic activity was not detected previously (Lee and Abeles, 1963). Both the inactivation and catalysis

reactions were found to exhibit large primary deuterium isotope effects ($(\frac{k_H}{k_D})_p = 8$; $(\frac{k_H}{k_D})_i = 14$) indicating that hydrogen transfer is an important rate contributing step for inactivation as well as for catalysis. Experiments with various isotopically substituted glycerols have indicated that two distinct diastereomeric combinations of enzyme with glycerol are possible, "$EG_R$" and "$EG_S$". When glycerol is bound as "$EG_R$", a hydrogen is abstracted from the pro-R carbon and catalysis results at a rate actually greater than that observed with 1,2-propanediol. When bound "$EG_S$," inactivation follows hydrogen abstraction from the pro-S carbon (Bachovchin et al., 1977).

In addition, we have demonstrated that a number of slightly modified substrates are also irreversible inactivators of dioldehydrase and that these substrate-inactivators display a number of similarities to glycerol in their interaction with dioldehydrase holoenzyme. For example, an important feature common to all inactivators examined was the ability of 1,2-propanediol to serve as a pure competitive inhibitor of inactivation. This behavior was verified in particular with glycerol as inactivator; the relationship was found valid over a wide range of both glycerol and 1,2-propanediol concentrations (Bachovchin et al., 1977).

Interestingly, ethylene glycol and glycerol have been known to effect the irreversible inactivation of dioldehydrase and glycerol dehydrase holoenzymes, respectively,albeit at

slower rates than that of glycerol inactivation of dioldehydrase holoenzyme (Lee and Abeles, 1963; Poznanskaya et al., 1972). Thus, substrate-induced inactivation appears to be a rather common phenomenon associated with these enzymes, not at all limited to unnatural synthetic analogues of the normal substrates, and has as yet been very little studied. An understanding of the mechanisms of inactivation is, very probably, essential to the full understanding of the mechanism of catalysis with these AdoCbl-dependent enzymes.

Accordingly, we undertook to study this phenomenon, with particular emphasis on glycerol, as described above. This work further extends this investigation. Primarily, this paper presents and compares the kinetics of glycerol inactivation of dioldehydrase holoenzyme in the presence of 1,2-propanediol to that observed in the presence of 1,1-dideuterio-1,2-propanediol. The results obtained should contribute significantly to the understanding of the mechanism of glycerol inactivation of dioldehydrase in particular and to the mechanism of adenosylcobalamin dependent enzyme catalysis in general.

# EXPERIMENTAL

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

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

5'-Deoxyadenosine. 5'-Deoxyadenosine was prepared by the intermediate synthesis of 5'-indo-2',3'-isopropylidene-6-N-acetyladenosine by a previously reported method (Jahn, 1965; McCarthy et al., 1968). 5'-Deoxyadenosine was also prepared directly from 5'-iodo-5'-deoxyadenosine (Aldrich Chemical Company) by catalytic hydrogenation using previously described conditions (McCarthy et al., 1968). The materials obtained by both methods were identical.

Substrates. 1,2-Propanediol, 1,1-dideuterio-1,2-propanediol, glycerol, perdeuterioglycerol, (RS)-1,1-dideuterioglycerol, (R)-1,1-dideuterioglycerol, and 1-$^{14}$C-glycerol were obtained as previously described (Bachovchin et al., 1977).

1-$^{3}$H-glycerol was purchased from Amersham.

Determination of Inactivation Rates. Inactivation rates were determined using the coupled enzyme assay as described previously (Bachovchin et al., 1977).

Reaction mixture in general consisted of: yeast alcohol dehydrogenase, 15 units; NADH, 0.2 mM; dioldehydrase, from 0.05 units to 0.50 units; bovine serum albumin, 0.12 mg; potassium phosphate buffer, pH 8.0, 30 mM; adenosylcobalamin, 0.02 mM; and the desired amount of 1,2-propanediol and/or inactivator. Total volume 2.50 ml. The reaction mixture was incubated to 37°C with stirring. The inactivation reaction was started by the addition of AdoCbl and stopped by addition of 50 µl of a 6.0 M 1,2-propanediol solution. Enzyme activity (measured by the rate of production of propionaldehyde) was determined at a minimum of four different inactivation times, the slope of the corresponding semilog plot was taken as the observed inactivation rate constant ($k_{i,obs}$) for the given concentration of inactivator and 1,2-propanediol.

Inactivation by 1-$^3$H-glycerol. A typical solution was composed of the following: apoenzyme, 40 units; 1-$^3$H-glycerol 0.49 µCi/µmole, @.142 M); $K_2HPO_4$ buffer, 10 mM; AdoCbl, 0.025 mM; total volume 0.50 ml, 37°. In control experiments, the apoenzyme solution was either boiled for 2 min, or inactivated by addition of 0.01 ml saturated CN-Cbl solution prior to addition of AdoCbl. The holoenzyme-1-$^3$H-glycerol solution was allowed to react 1 hr at 37°C in the dark. 5'-Deoxyadenosine (0.15 ml of a 2 mg/ml solution) was added as carrier, and 0.05 ml of a saturated CN-Cbl solution was added to visualize subsequent chromatography. The solution was immersed in boiling $H_2O$ 2 min and then applied to a 2.6 x 26 cm column of

Bio-Gel P-2 (200-400 mesh) and eluted in 4 ml fractions with .015 N $NH_3$/.02% $NaN_3$ buffer in the dark. The nucleoside-containing fractions were pooled, acidified to pH 3.0 with 2N HCl, and applied to a 1 x 9 cm AG50W-X2 (200-400 mesh) column previously equilibrated with $10^{-3}$ M HCl. The column was washed with 100 ml $H_2O$ to remove radioactive glycerol. The nucleoside was eluted with 0.1 N $NH_3$, lyophilized, dissolved in a small amount of methanol, and applied to strips of Whatman 3MM paper or to silica gel plates. Paper chromatograms were developed by the descending method in the solvent systems given below; silica gel plates were developed by the ascending method.

| Method/Solvent | $R_f$ 5'-deoxyadenosine |
|---|---|
| Paper: | |
| n-BuOH:$H_2O$ (43:7) | 0.34 |
| n-BuOH:HAc:$H_2O$ (12:3:5) | 0.60 |
| n-BuOH:HAc:$H_2O$ (4:1:5) (organic phase) | 0.77 |
| 1M $NH_4Ac$:95%EtOH:$H_2O$ (9:20:1) | 0.75 |
| Silica Gel: | |
| Water-saturated nBuOH + 1% $NH_3$ | 0.35 |
| sec-BuOH:$H_2O$:$NH_4OH$ (50:18:7) | 0.27 |

The nucleoside was visualized by its ultraviolet extinction, and eluted from the chromatogram with methanol. The methanol was removed under vacuum, the residue taken up in 1 ml $H_2O$ and added to 10 ml scintisol complete or

PCS:Xylene (2:1), and assayed for radioactivity. Standardization was accomplished by using α-$^{3}$H- and α-$^{14}$C-toluene of known specific activities.

## RESULTS

Kinetics of Inactivation by Perdeuterioglycerol. The rate of inactivation of dioldehydrase holoenzyme by perdeuterioglycerol ($D_5$-Gly) was determined as a function of $D_5$-Gly concentration in the absence of 1,2-propanediol under anaerobic conditions at 37°C. As with glycerol, the rates of inactivation were at all times first-order in active enzyme. (The concentration of $D_5$-Gly could be assumed constant throughout the time course of the inactivation reaction; very little β-hydroxypropionaldehyde was formed compared to the initial concentration of $D_5$-Gly.) Accordingly, simple Michaelis-Menten equations suitably modified to account for decreasing active holoenzyme again apply (Bachovchin et al., 1977) and

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

where $[E_a]_o$ is the total amount of active enzyme present initially, $[E_a]$ is the total amount of enzyme, both free and bound to inactivator, remaining active at any time $\tau$. $k_{i,obs}$, The observed first-order rate constant for inactivation, is given by

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

where $k_i$ is the true first order rate constant for inactivation. $K_G$ is the dissociation constant of the enzyme-glycerol complex

$$K_G = \frac{[E_a][G]}{[E_aG]} .$$

A plot of $1/k_{i,obs}$ vs $1/[D_5\text{-Gly}]$ is linear (Fig. 1) and from the slope and intercept values of $k_i$ and $K_G$ for $D_5$-Gly were obtained. The resulting value of $K_G$ for $D_5$-Gly ($3.9 \times 10^{-3}$ M) is somewhat different from that of glycerol ($1.6 \times 10^{-3}$) obtained earlier by the same method (Bachovchin et al., 1977). The value of $k_i$ for $D_5$-Gly obtained from the y-intercept (0.085) gives an isotope effect of 15 on the inactivation rate constant. A value of 14 was reported earlier and was based on a comparison of the observed rate constants at 0.04 M glycerol and $D_5$-Gly in the absence of 1,2-propanediol. This is reasonable agreement, but, nevertheless the discrepancies can quantitatively be accounted for in terms of the differing contributions made by oxygen inactivation to $k_{i,obs}$ due to the different $K_G$ values.

Kinetics of Glycerol Inactivation. Competition with (RS)-1,1-Dideuterio-1,2-propanediol. The rate of inactivation in the presence of a fixed concentration of (RS)-1,1-dideuterio-1,2-propanediol ($D_2$-Diol) was determined as a

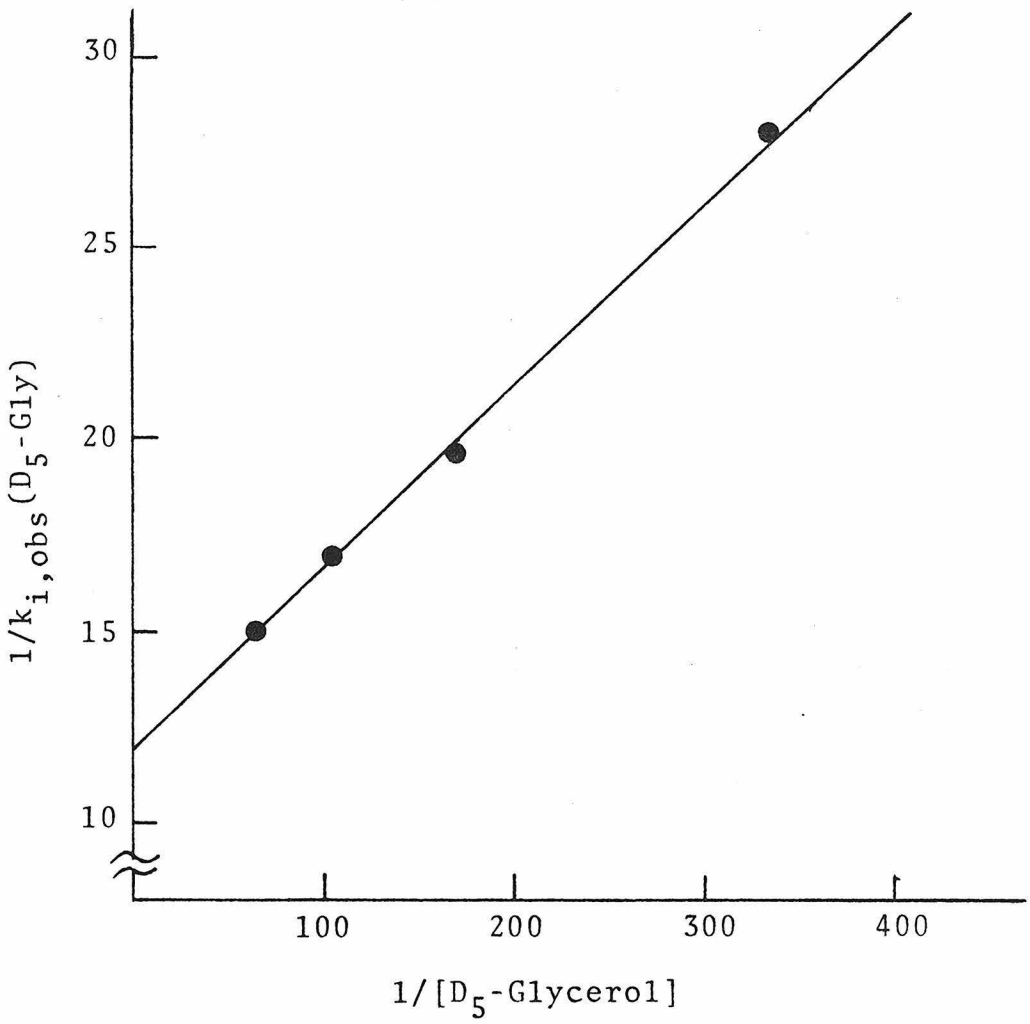



Figure 1. Plot of the reciprocal of the observed inactivation rate constant for substrate-free glycerol under anaerobic conditions versus the reciprocal of glycerol concentration. The slope and y intercept give $k_i = 0.085$ $K_G = 3.9 \times 10^{-3}$.

function of inactivator concentration for the following inactivators: glycerol ($H_2$-Gly), (RS)-1,1-dideuterioglycerol (RS-$D_2$-Gly); (R)-1,1-dideuterioglycerol (R-$D_2$-Gly); and perdeuterioglycerol ($D_5$-Gly). The fixed concentration of $D_2$-Diol chosen was $1.08 \times 10^{-4}$ M for two reasons. (1) This concentration is large enough for the assumption that $\Delta[S] = 0$ to be valid over the time interval during which inactivation rates were determined, (2) it is small enough to allow large rates of inactivation at reasonable concentrations of inactivator.

The results are illustrated in Figs. 2 and 3. Note that only with $D_5$-Gly (Fig. 2) as inactivator is the double reciprocal plot linear over the entire range of inactivator concentration. Moreover, in this case, the y-intercept is nearly identical to that obtained for the substrate-free case (Fig. 1). This indicates that equation 3 found to describe

$$k_{i,obs}(H_2\text{-Gly})_{H_2\text{-Diol}} = \frac{k_i(H_2\text{-Gly})}{1 + \frac{K_G(H_2\text{-Gly})}{[H_2\text{-Gly}]}\left(1 + \frac{[H_2\text{-Diol}]}{K_i(H_2\text{-Diol})}\right)} \quad (3)$$

the inactivation kinetics of $H_2$-Gly in the presence of $H_2$-Diol (Bachovchin et al., 1977) should again apply. Thus, the slope of the double reciprocal plot with $D_5$-Gly as inactivator should be given by

$$\text{slope} = \frac{K_G(D_5\text{-Gly})}{k_i(D_5\text{-Gly})}\left(1 + \frac{[D_2\text{-Diol}]}{K_i(D_2\text{-Diol})}\right)$$

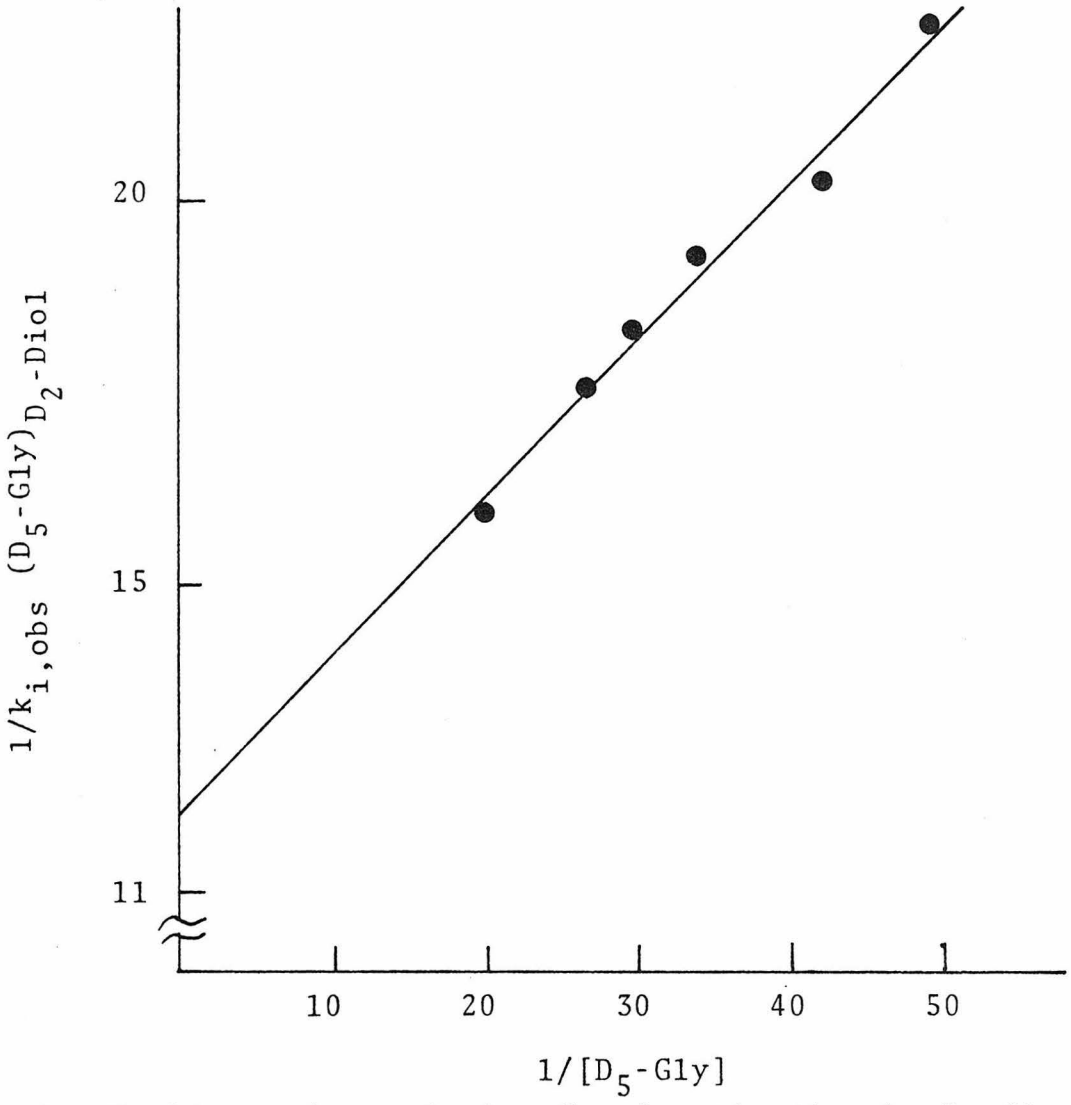



Figure 2. Double reciprocal plot for inactivation by $D_5$-Gly in the presence of $1.16 \times 10^{-4}$ $D_2$-Diol. Slope = 0.22. Intercept = 11.8.

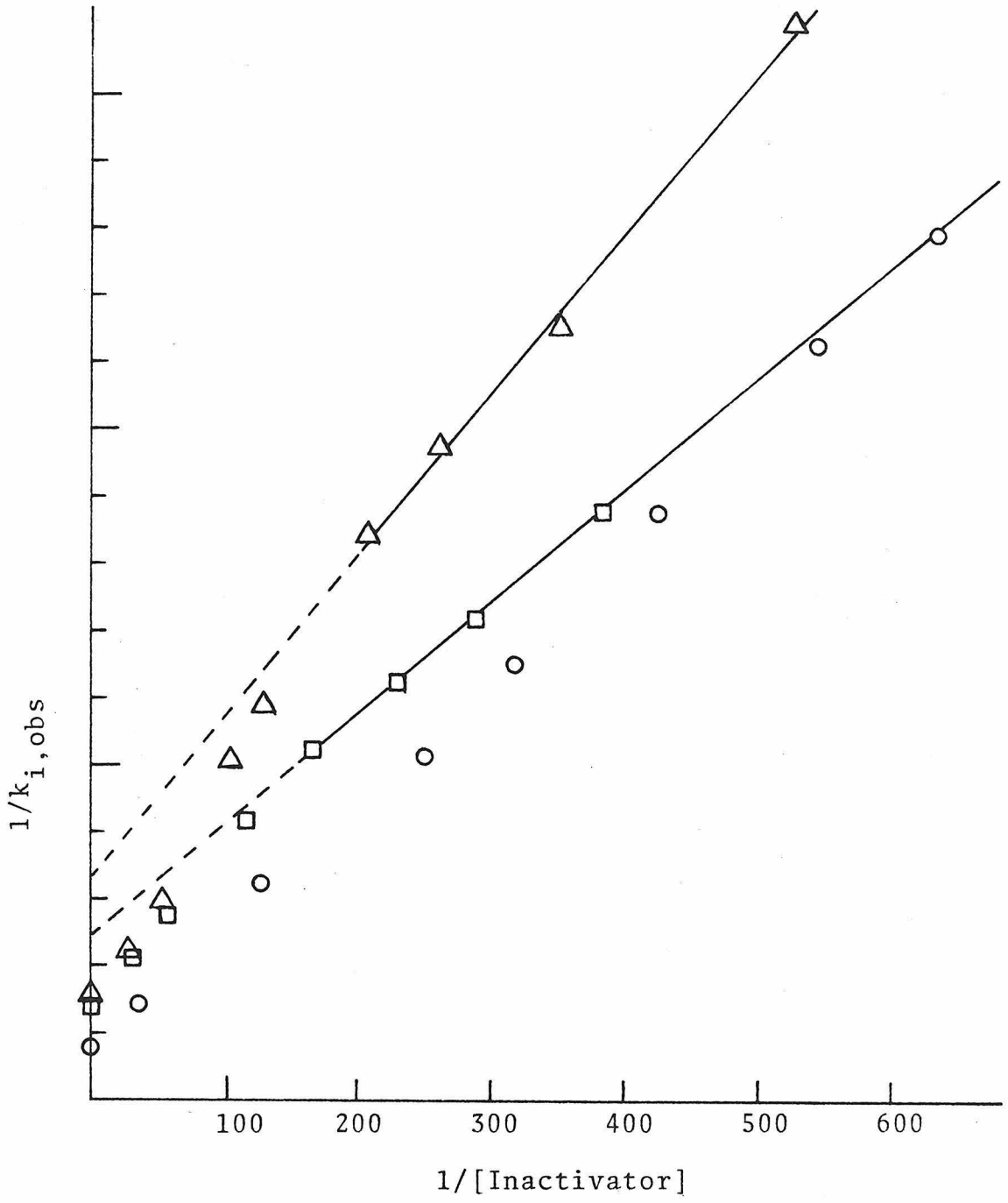



Figure 3. Plot of the reciprocal of the observed inactivation rate constant in the presence of $1.08 \times 10^{-4}$ M $D_2$-Diol versus the reciprocal of inactivator concentration. O, glycerol; □, R-$D_2$-glycerol; △, RS-$D_2$-glycerol. The values depicted at infinite inactivator concentration represent values obtained experimentally at 0.04 M inactivator in the absence of 1,2-propanediol.

Since the values of $K_G(D_5\text{-Gly})$ and $k_i(D_5\text{-Gly})$ are available from Figure 1, a value for $K_i(D_2\text{-Diol})$ can be calculated. The resultant calculated value is $3.0 \times 10^{-5}$ M. This is the same as the value of $K_i$ for $H_2$-Diol previously obtained in an analogous manner with glycerol as inactivator (Bachovchin et al., 1977). Thus there appears to be no deuterium isotope effect on the inhibition constant for propanediol.

Although the double reciprocal plot is linear over the entire concentration range only for inactivation by $D_5$-Gly, at low concentrations of inactivator the double reciprocal plots for $H_2$-Gly, R-$D_2$-Gly, and RS-$D_2$-Gly all appear to become linear (Fig. 3). Note that $H_2$-Gly and R-$D_2$-Gly share the same line at low concentrations while RS-$D_2$-Gly defines a line with a different slope and intercept. Applying equation 3 to these linear regions the slope should be given by

$$\text{slope} = \frac{K_G(G)}{k_i(G)} \left(1 + \frac{[D_2\text{-Diol}]}{k_i(D_2\text{-Diol})}\right) ,$$

where G can be either $H_2$-Gly, R-$D_2$-Gly, or RS-$D_2$-Gly. Taking the y intercept (obtained by extrapolation of the linear region to infinite inactivator concentration) to represent the reciprocal of the respective $k_i$, and using the value of $K_i$ for $D_2$-Diol determined above a value of $K_G$ for each inactivator can be calculated. The resultant calculated value in each case is $1.6 \times 10^{-3}$ M. This is the same value previously determined for $H_2$-Gly independent of the presence of

1,2-propanediol (Bachovchin et al., in press). Thus, although $D_5$-Gly exhibits a value for $K_G$ somewhat different than that for $H_2$-Gly, both R-$D_2$-Gly and RS-$D_2$-Gly appear to exhibit no deuterium isotope effect on $K_G$.

The variation of the inactivation rate constant with concentration of inactivator can more informatively be illustrated as a plot of the expressed isotope effect vs 1/[Inactivator]. The isotope effect was calculated by comparing the experimentally determined observed inactivation rate constant at each concentration of the above inactivators in the presence of $D_2$-Diol to the observed rate constant that would obtain for the corresponding concentrations of $H_2$-Gly and $H_2$-Diol. For example, the isotope effect on inactivation at a given concentration of R-$D_2$-Gly would thus be defined as

$$\left(\frac{k_H}{k_D}\right)_i = \frac{k_{i,obs(H_2\text{-Gly})_{H_2\text{-Diol}}}}{k_{i,obs(R\text{-}D_2\text{-Gly})_{D_2\text{-Diol}}}} \quad , \qquad (4)$$

where [Gly] = [R-$D_2$-Gly] and [$H_2$-Diol] = [$D_2$-Diol], $k_{i,obs}$-$(R\text{-}D_2\text{-Gly})_{D_2\text{-Diol}}$ is the experimentally determined observed inactivation rate constant at the particular concentration of R-$D_2$-Gly in the presence of $D_2$-Diol, and $k_{i,obs}(H_2\text{-Gly})_{H_2\text{-Diol}}$ is the corresponding rate constant for $H_2$-Gly in the presence of $H_2$-Diol and is given by equation 3. The values of $k_i$ and $K_D$ for $H_2$-Gly were previously determined and are listed in Table I for comparison.

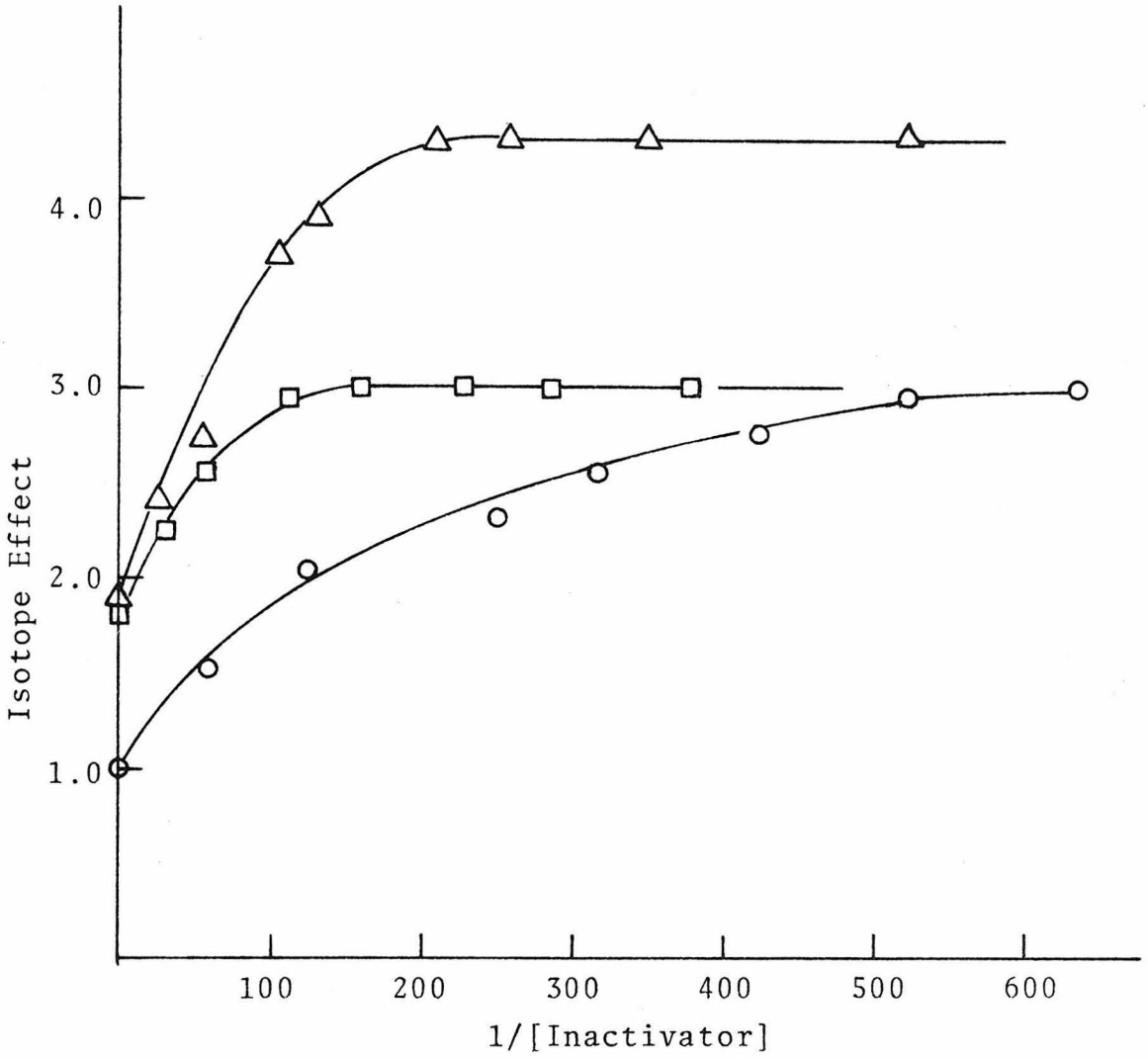



Figure 4. Plot of the isotope effect on the observed inactivation rate constant versus the reciprocal of inactivator concentration in the presence of $1.08 \times 10^{-4}$ M $D_2$-Diol. ○, $H_2$-Gly; □, R-$D_2$-Gly; △, RS-$D_2$-Gly. The isotope effects were calculated from the corresponding experimentally determined values of $k_{i,obs}$ as described in the results section.

Kinetics of Glycerol Inactivation. Competition with (RS)-1,2-propanediol. The rate of inactivation in the presence of a fixed concentration of $H_2$-Diol was determined as a function of inactivator concentration for the following inactivators: RS-$D_2$-Gly; R-$D_2$-Gly; and $D_5$-Gly. (The corresponding experiment with $H_2$-Gly as the inactivator has already been presented (Bachovchin et al., 1977) and is given by equation 3 together with the values of $k_i(H_2\text{-Gly})$ and $K_G(H_2\text{-Gly})$ listed in Table I.) The results are illustrated in Fig. 5 as a plot of the expressed isotope effect on inactivation vs the reciprocal of the inactivator concentration. The isotope effect was calculated as described in the previous section.

Isolation and Identification of 5'-deoxyadenosine. When dioldehydrase holoenzyme is inactivated by 1-$^3$H-glycerol, tritium is transferred to the nucleoside moiety of AdoCbl. The nucleoside cochromatographs with authentic 5'-deoxyadenosine on silica gel and Whatman 3MM paper in a number of solvent systems. The amount of tritium associated with 5'-deoxyadenosine is directly proportional to the amount of holoenzyme used; control experiments using 1-$^{14}$C-glycerol or heat-inactivated apoenzyme resulted in no radioactivity associated with 5'-deoxyadenosine. A least-squares fit of $^3$H dpm in 5'-deoxyadenosine as a function of the amount of enzyme indicates that 5'-deoxyadenosine is enriched in tritium by a factor of approximately 2.1 over substrate, on

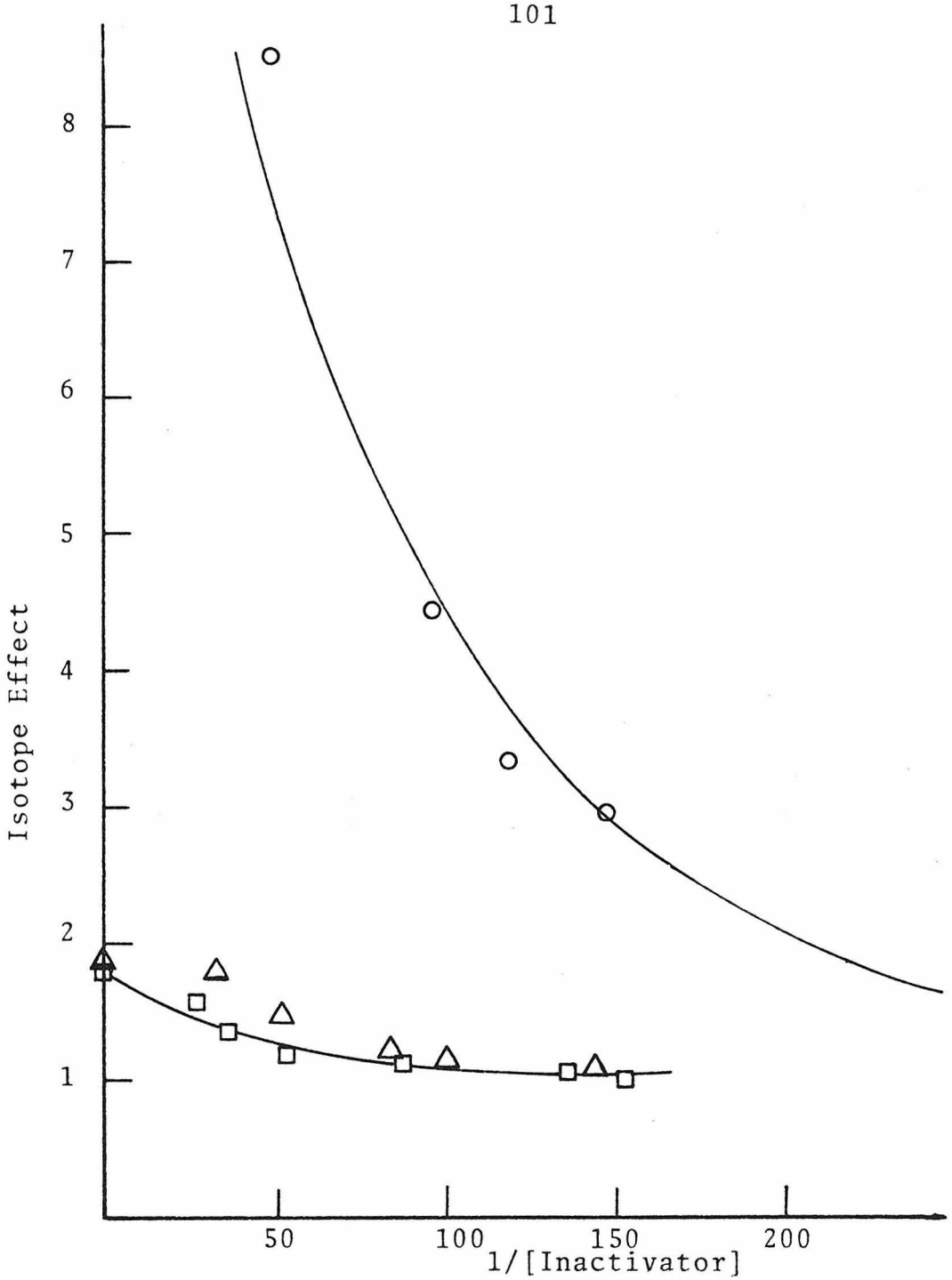



Figure 5. Plot of the isotope effect on the observed rate of inactivation versus the reciprocal of inactivator concentration in the presence of $1.16 \times 10^{-4}$ M $H_2$-Diol. ○, $D_5$-Gly; △, RS-$D_2$-Gly; □, R-$D_2$-Gly. The isotope effects were calculated from the corresponding experimentally determined values of $k_{i,obs}$ as described in the results section.

Table I. Comparison of the limiting values of the inactivation rate constants and resulting isotope effects.

| | Substrate Free | | $D_2$-Propanediol | | 1,2-Propanediol | |
|---|---|---|---|---|---|---|
| | $k_i$, $min^{-1}$ | $(k_H/k_D)_i$ | $k_i$, $min^{-1}$[a] $1.08 \times 10^{-4}$ $D_2$-Diol | $\frac{k_H}{k_D}$ | $k_i$[a] | $(k_H/k_D)_i$ |
| Glycerol | 1.3 | 1.0 | 0.43 | 3.0 | 1.30 | 1.0 |
| R-$D_2$-Glycerol | 0.72 | 1.8 | 0.43 | 3.0 | 1.30 | 1.0 |
| RS-$D_2$-Glycerol | 0.68 | 1.9 | 0.30 | 4.3 | <1.30 | >1.0 |
| $D_5$-Glycerol | 0.093 | 15.0 | 0.087 | 15.0 | ≈0.90 | ≈1.4 |

[a]The $k_i$ values listed in the competition with substrate experiments are the constant $k_{i,s}$ fitting equation 3 at low inactivator concentrations.

a per-mole basis. This, of course, assumes quantative recovery of enzyme generated 5'-deoxyadenosine.

## DISCUSSION

Equation 3 accurately describes the kinetics of inactivation when both inactivator and inhibitor are isotopically "homogeneous" with respect to transferrable hydrogens (i.e., $H_2$-Gly with $H_2$-Diol, and $D_5$-Gly with $D_2$-Diol). However, such is not the case for the isotopically "mixed" cases (i.e., $H_2$-Gly, RS-$D_2$-Gly, R-$D_2$-Gly with $D_2$-Diol, and $D_5$-Gly, RS-$D_2$-Gly, R-$D_5$-Gly, with $H_2$-Diol). An explanation for this is immediately suggested by an inspection of the results, i.e., that the "inhibitor" can contribute a hydrogen (or deuterium) to the inactivation pathway. With this hypothesis no effect on the rate of inactivation ($k_i$) would be expected for the "homogeneous" cases. With the "mixed" cases, however, this process might be expected to cause a variation in $k_i$ with the concentration of inactivator and thus could explain the non-linearity of the double reciprocal plots for these "mixed" cases.

A mechanism whereby this may occur is readily constructed drawing upon the current postulated mechanism of catalysis and known experimental facts. Catalysis by dioldehydrase holoenzyme, similar to that of other AdoCbl-dependent enzymes, involves two hydrogen transfer steps, (1) from C-1 of substrate to enzyme-bound cofactor (2) from cofactor to C-2 of

product (Abeles, 1971). This net 1,2-hydrogen migration occurs intermolecularly as well as intramolecularly and involves C-5' of the cofactor as the intermediate hydrogen carrier (Essenberg et al., 1971). Evidence for the formation of 5'-deoxyadenosine as an intermediate and evidence for at least partial equivalency of the C-5' hydrogens, although not conclusive, is now quite extensive (Babior, 1975 b). Moreover, the isotope effect on the second hydrogen transfer step (cofactor-product) is much larger than that for the first hydrogen transfer step and thus accumulation of isotopic hydrogen at the 5'-carbon of the cofactor occurs during catalysis. Enrichment in tritium in the enzyme bound AdoCbl by as much as 20-fold over substrate has been reported to occur during catalysis (Essenberg et al., 1971). Thus a mechanism exists for the "inhibitor" of inactivation to contribute a hydrogen (or deuterium) to the inactivation pathway and requires only that inactivation occur at some point on the catalytic pathway after the formation of 5'-deoxyadenosine.

Changes in $K_G$ or $K_i$ (equation 3) with concentration of inactivator should also be considered as an explanation for the nonlinearity of the double reciprocal plots in the mixed cases. However, this explanation appears to be unlikely. The inhibition constant, $K_i$, for 1,2-propanediol is totally unaffected by substitution of deuterium. While $D_5$-Gly does exhibit an isotope effect on $K_G$, RS-$D_2$-Gly and

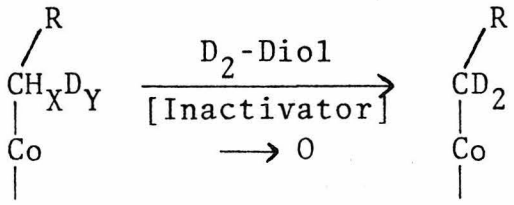
R-$D_2$-Gly do not. (The reason for this is not clear but may be due to the fifth deuterium located at C-2, or alternatively, it may be due to the combined effect of five deuterium substituents whereas RS-$D_2$-Gly, and R-$D_2$-Gly each possess only two.) Moreover, the effects observed when $H_2$-Diol protects against inactivation by $D_5$-Gly are too large to explain in terms of effects on $K_G$ and $K_i$. Thus, an intermolecular effect on $k_i$ remains as the best explanation.

In the following discussion we shall argue that not only does transfer of hydrogen from cofactor contribute to the observed inactivation rate constant, thereby implicating the presence of an intermediate such as 5'-deoxyadenosine in the inactivation pathway, but that transfer of hydrogen from such a cofactor intermediate containing three equivalent hydrogens is the rate-determining step in the inactivation process. This hypothesis is supported by and can account for all of the inactivation kinetic data presented here. In particular the limiting value of the isotope effects observed at low inactivator concentrations (Figs. 4 and 5) can be quantitatively accounted for by this hypothesis.

Consider first, the kinetics of the "mixed" cases with $D_2$-Diol as the inhibitor of inactivation. At infinite concentrations of inactivator, $D_2$-Diol should be effectively excluded from the holoenzyme and the rate of inactivation should and does extrapolate to that observed for the free inactivator (Figs. 3 and 4). (The rates of inactivation and

corresponding isotope effects observed for each inactivator in the absence of propanediol are listed in Table I.) However, as the concentration of inactivator is decreased, $D_2$-Diol becomes increasingly subject to catalysis and, consequently, the enzyme-bound AdoCbl becomes richer in deuterium. Thus, the increasing isotope effect with decreasing inactivator concentration can be explained (Fig. 4).

However, Fig. 4 illustrates that the isotope effect on inactivation does not continually increase with decreasing inactivator concentration, but reaches a limiting maximum value for each inactivator. This experimental finding can also be accounted for in terms of the above hypothesis. Considering the much larger effect on the second hydrogen transfer step (cofactor — product) one might reasonably expect that as the concentration of inactivator is decreased a point should be reached where the transferable hydrogens located on the enzyme-bound AdoCbl become effectively replaced with deuterium.



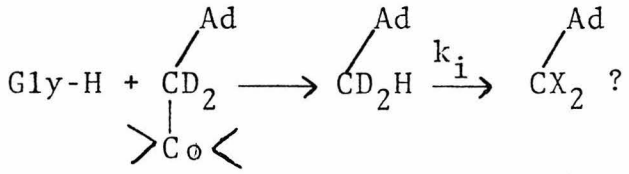

(X and Y represent the isotopic composition of the cofactor that obtains at infinite inactivator concentration.) Thus at this point no further increase in the isotope effect with

decreasing inactivator concentration would be possible and a maximum value for the isotope effect for each inactivator would obtain, thus accounting for the experimental observation illustrated in Fig. 4.

The inactivator concentration at which this maximum limiting value is expressed differs somewhat for each inactivator. With R-$D_2$-Gly, this "saturation" effect is seen to occur at relatively large concentrations of R-$D_2$-Gly. With RS-$D_2$-Gly a somewhat lower concentration is required while with $H_2$-Gly this saturation effect occurs only at very low concentrations of this inactivator. That this order should obtain is also reasonable in terms of the proposed hypothesis. We have previously shown that glycerol is also a substrate for dioldehydrase holoenzyme and that catalysis occurs predominately from the pro-R-end of the glycerol molecule (Bachovchin et al., 1977). R-$D_2$-Gly contains only deuterium on the pro-R-carbon and thus catalysis with this glycerol as substrate should further aid in enrichment of the enzyme-bound AdoCbl with deuterium. RS-$D_2$-Gly, on the other hand, contains both deuterium and hydrogen in equal amounts on the pro-R-carbon and should be somewhat less effective in this regard, while $H_2$-Gly, containing only hydrogen, should retard the saturation of the enzyme-bound AdoCbl by deuterium. Thus the above observation is qualitatively predicted by the proposed hypothesis.

The maximum limiting values of the isotope effects are

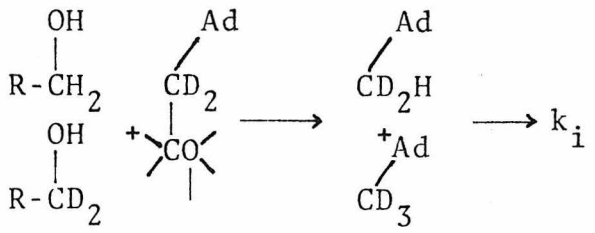
seen from Fig. 4 to be 3.0 for both $H_2$-Gly and R-$D_2$-Gly while for RS-$D_5$-Gly the experimental value is 4.3. These values are nearly quantitatively accounted for by the proposed hypothesis. Consider glycerol. At very low concentrations of glycerol the inactivator "finds" the enzyme-bound AdoCbl saturated with deuterium



Since glycerol contains only hydrogen for abstraction the resulting 5'-deoxyadenosine moiety must be '$CD_2H$. Using 15 as the value for the primary isotope effect and assuming the three hydrogens have an otherwise equal chance to be removed in this rate determining step one calculates that the isotope effect should be

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

This agrees reasonably well with the experimental value. With R-$D_2$-Gly, again only hydrogen can be contributed since inactivation occurs predominately from the "pro-S end" (Bachovchin et al., 1977) and the calculated value is the same as that for glycerol, as is the experimental value. However, with RS-$D_2$-Gly as the inactivator both deuterium and hydrogen can be contributed and thus two 5'-deoxyadenosine moieties are possible



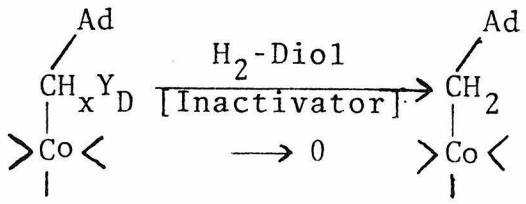
Assuming an equal contribution to the isotope effect from both of the above species one calculates that the isotope effect in this case should be

$$\left(\frac{k_H}{k_D}\right)_i = \left[\left(\frac{.5}{\left[\left(\frac{2}{3}\right)\left(\frac{1}{15}\right)+\left(\frac{1}{3}\right)(1)\right]^{-1}}\right)+\frac{.5}{15}\right]^{-1} = 4.5 \ ,$$

also in good agreement with the experimental value.

A number of experimental points were obtained for each inactivator in the "saturated" region. With $k_i$ constant eq 3 should again apply and, in fact, the double reciprocal plots of these points are linear with slopes and intercepts indicating the appropriate new values of $k_i$ while reassuringly indicating no effect on either $K_G$ or $K_i$ for each inactivator (Fig. 3). Thus the proposed hypothesis qualitatively or quantitatively accounts for all of the features of the experimental results presented in Figs. 3 and 4.

Consider now, the corresponding "mixed" cases with $H_2$-Diol serving as the inhibitor of inactivation. In these cases, the deuterium content of enzyme-bound AdoCbl should decrease with decreasing concentration of inactivator and the isotope effect should accordingly also decrease.




The experimental results are in agreement with this contention (Fig. 5). Note in particular the effect that occurs with $D_5$-glycerol as inactivator. At large concentrations of $D_5$-glycerol the isotope effect approaches 15, but with decreasing $D_5$-glycerol concentration the isotope effect decreases dramatically.

Again, one might expect that at some point, as the concentration of inactivator is lowered, the enzyme-bound AdoCbl will, as before, become saturated. However, this time the saturation is with hydrogen, rather than deuterium and a limiting <u>minimum</u> value for the isotope effect would obtain. The results in Fig. 5 do suggest that such a limit is approached for each inactivator. However, in practice, meaningful experimental data in the "constant $k_i$" region cannot be obtained for a number of reasons (such as changing inhibitor concentration due to more rapid catalysis among others).

Nevertheless, limiting minimum values for the isotope effects expected for each of these inactivators can be calculated based on the above described hypothesis, and these values are found to agree rather well with the values approached experimentally (Fig. 5). For example, R-$D_2$-glycerol possesses only hydrogen at the pro-S carbon and can thus

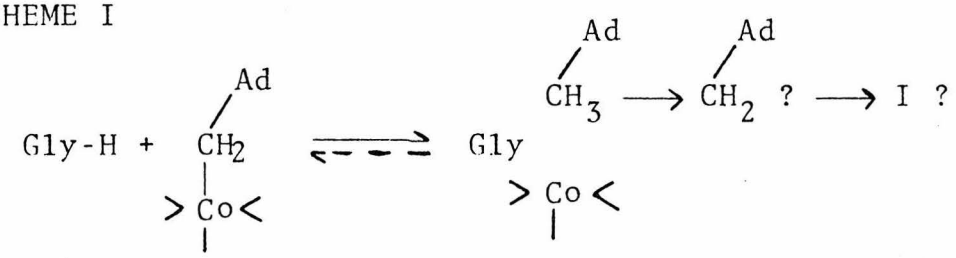
generate only a $'CH_3$ intermediate. The calculated value is thus 1.0 for the isotope effect. The calculated value for RS-$D_2$-glycerol is only slightly greater (1.1). With both of these inactivators the experimental value appears to approach 1.0 as the limiting minimum value. With $D_5$-glycerol, on the other hand, only deuterium can be contributed and the composition of the intermediate in the limiting case must be $'CH_2D$. The calculated value of the isotope effect for this intermediate is 1.4. This represents a very large change from the value of 15 observed at infinite $D_5$-glycerol concentration and presents the theory with a challenging test. Significantly, the experimental data indicates that such a large change does in fact occur. Moreover, a value of somewhat greater than 1.0 appears to be the experimental limit giving very good agreement with the calculated value (Fig. 5).

## SUMMARY AND CONCLUSIONS

The results presented here strongly support the occurrence of an intermediate such as 5'-deoxyadenosine on the inactivation pathway, and furthermore strongly indicates that transfer of hydrogen from a 5'-deoxyadenosine intermediate containing those equivalent hydrogens is the rate determining step in the inactivation pathway. This hypothesis is supported by its ability to quantitatively account for the magnitude of the limiting value of the isotope effects observed at low inactivator concentrations with both $H_2$-Diol

and $D_2$-Diol serving as "inhibitors" of inactivation. Moreover, this hypothesis accounts qualitatively for the manner in which $k_i$ varies with inactivator concentration for the isotopically "mixed" case and for the non-variance of $k_i$ for the isotopically "homogeneous" cases. Accordingly, we conclude that the mechanism of inactivation, like catalysis, involves as a first step abstraction of hydrogen from C-1 of glycerol and transfer to the 5'-carbon of the cofactor. At some point following this transfer the three hydrogens on the 5'-carbon become "equivalent" and inactivation occurs either concomitantly with or subsequently to the rate determining step of hydrogen transfer from the 5'-carbon.

SCHEME I




Whether this hydrogen transfer step involves transfer back to C-2 of glycerol (as catalysis) or whether it involves transfer to some other group or species associated with the enzyme or coenzyme remains as yet undetermined, as does the exact nature of the inactivated holoenzyme complex. The identification of the nucleoside in the inactivated complex as 5'-deoxyadenosine suggests that the 5'-deoxyadenosine moiety which obtains after the second hydrogen transfer step (Scheme I) is capable of acquiring a third hydrogen, possibly either from substrate

or a nearby amino acid residue since intact AdoCbl cannot be recovered following glycerol inactivation (Bachovchin et al., 1977).

The significance of these results for the mechanism of catalysis is that a 5'-deoxyadenosine moiety containing three "equivalent" hydrogens has long been speculated upon as an intermediate in catalysis. The results here clearly establish the kinetic feasibility of such an intermediate in the mechanism of glycerol inactivation; a mechanism which is very similar to and may in fact be identical to the mechanism of catalysis; with the difference being that enzyme-bound "product" from "$EG_S$" effects the irreversible inactivation of the holoenzyme.

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# PART IV

## Hydrogen Transfer From Enzyme-Bound Adenosylcobalamin as a Partial Rate Limiting Step in Catalysis.

# INTRODUCTION

Adenosylcobalamin-dependent propanediol dehydrase catalyzes the rearrangement of both (R)- and (S)-1,2-propanediol to propionaldehyde and ethylene glycol to acetaldehyde. The mechanism of this rearrangement involves two hydrogen transfer steps, substrate to cofactor, and cofactor to substrate; the overall reaction exhibits a large primary deuterium isotope effect (10-12)(Abeles, 1972; Eagar et al., 1975). Based on results with isotopically labeled substrates Essenberg et al. (1971) have concluded that the first hydrogen transfer step is the rate-limiting step in catalysis.

Very rarely, however, in enzymatic catalysis can a single step be considered completely rate determining (Northrop, 1975). More often, the velocity of the reaction is dependent upon several "rate-contributing" or partially rate-limiting steps. Results are presented here which support the supposition that the second hydrogen transfer step in the dioldehydrase catalyzed rearrangement of 1,2-propanediol is at least "partially rate determining."

## EXPERIMENTAL

Enzyme Preparations. Propanediol dehydrase ((R,S)-1,2-propanediol hydro-lyase; EC 4.2.1.28) was obtained from Klebsiella pneumoniae (ATCC 8724) by a procedure similar to that of Lee and Abeles (1963). Enzyme with a specific activity of between 25 and 50 unit/mg was used for all determinations.

Adenosylcobalamin was purchased from Sigma Chemical Company.

(RS)-1,1-Dideuterio-1,2-propanediol was prepared by lithium aluminum deuteride reduction of dl-ethyl lactate (Fieser and Fieser, 1967). Deuterium content was at least 98% as determined by proton NMR. The isotope effect on catalysis was 13, somewhat greater than the value of 10-12 reported by Abeles (1972).

Rate Determinations. The rate of propionaldehyde production was determined using the coupled-enzyme assay by monitoring the decrease in absorbance at 340 nm. Reaction mixtures consisted of: dioldehydrase, ~0.1 unit, $NADH_2$, 0.5 mg; yeast alcohol dehydrogenase, ~5 units; bovine serum albumin 0.4 mg; 0.01 M $K_2HPO_4$ buffer, pH 8.0; 80 μg AdoCbl; and the desired amounts of substrate. Total volume 2.5 ml. The reaction mixtures were incubated at 37°C with stirring and the reaction was started by the addition of AdoCbl.

## RESULTS

The rate of catalysis was determined as a function of the mole fraction of deuterated and non-deuterated 1,2-propanediol (1,1-dideuterio-1,2-propanediol and 1,2-propanediol). The rates of catalysis observed at any given composition of substrates were linear for at least the initial ten minutes except for a brief lag following addition of the AdoCbl. The results are illustrated in Fig. 1.

## DISCUSSION

Consider the following scheme:

$$E + S_H \rightleftharpoons ES_H \xrightarrow{k_H} E + P$$

$$E + S_D \rightleftharpoons ES_D \xrightarrow{k_D} E + P \quad ,$$

where $S_H$ and $S_D$ represent 1,2-propanediol and 1,1-dideuterio-1,2-propanediol, respectively, $ES_H$ and $ES_D$ are the enzyme substrate complexes and $k_H$ and $k_D$ are the corresponding rate constants for product formation. The resultant rate equation for the overall observed rate of catalysis is

$$\frac{dp}{dt} = k_P H + k_P D$$

$$= \frac{k_H E_o}{1 + \frac{K_M(S_H)}{[S_H]}\left(1 + \frac{[S_D]}{K_i(S_D)}\right)} + \frac{k_D E_o}{1 + \frac{K_M(S_D)}{[S_D]}\left(1 + \frac{[S_H]}{K_i(S_H)}\right)} \qquad (1)$$

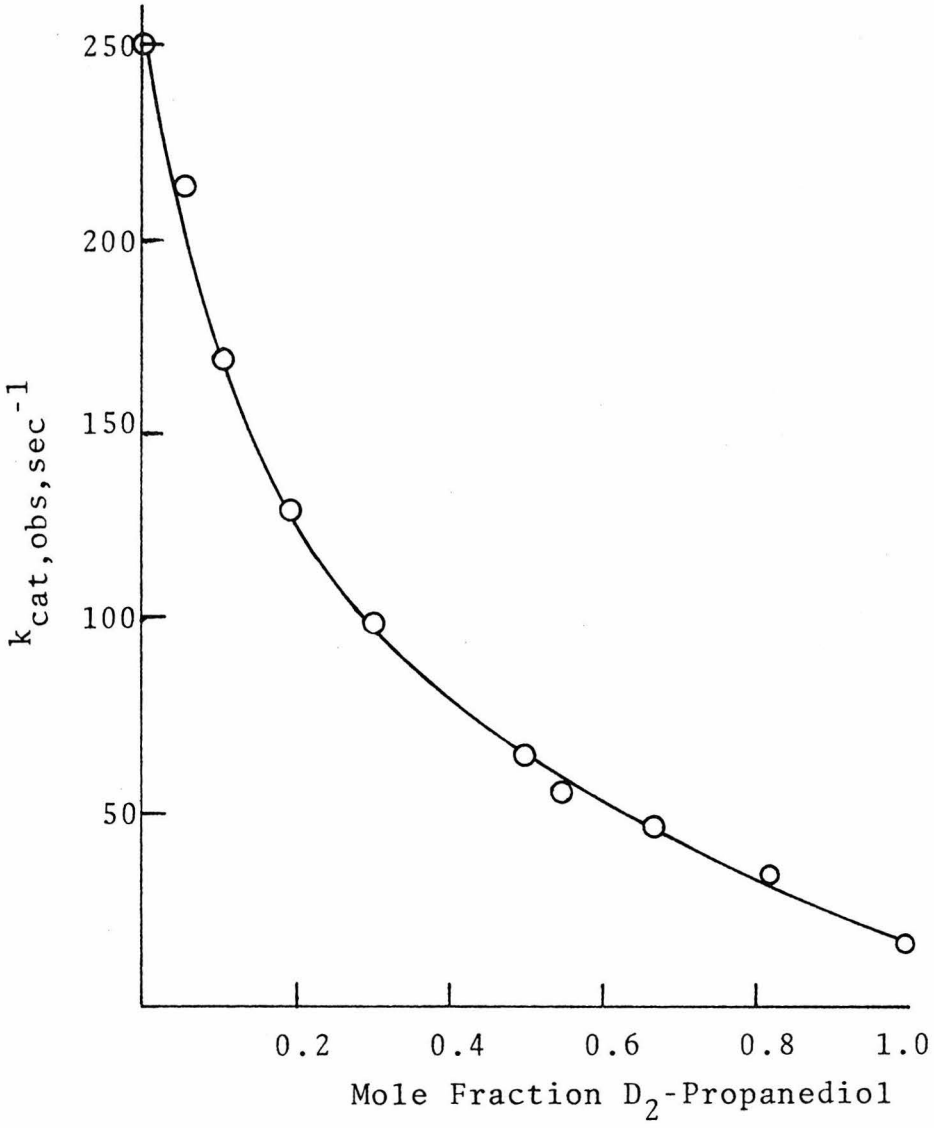


Figure 1. Variation of the observed catalytic constant with deuterium content of the substrate. Total substrate concentration = $5 \times 10^{-3}$ M.

$k_i(S_H)$ and $k_i(S_D)$ are the inhibitor constants for 1,2-propanediol and 1,1-dideuterio-1,2-propanediol and are a measure of the total amounts of enzyme "bound" as $ES_H$ and $ES_D$, respectively. $K_M(S_H)$ and $K_M(S_D)$ are the usual, Michaelis-Menten or Briggs Haldane constants for each of these substrates.

There are several possible explanations for the behavior of the observed rate of catalysis illustrated in Fig. 1. First, if $K_i(S_H) > K_i(S_D)$ an initial rapid decrease in the rate with increasing mole fraction deuterated substrate, followed by a more gradual decrease as 1.0 mole fraction deuterated substrate is approached would be expected. However, we have previously shown that $K_i(S_H) = K_i(S_D)$ (Bachovchin et al., manuscript in preparation). In addition, it seems probable that for at least the undeuterated case $K_i = K_M$ (Bachovchin et al., 1977). Thus it is difficult to account for the observed behavior in these terms.

An alternate explanation, which we favor, is that a decrease in the catalytic constant ($k_H$) governing the reaction with the undeuterated substrate occurs with increasing mole fraction 1,1-dideuterio-1,2-propanediol. We have previously reported evidence for such an intermolecular isotope effect in the mechanism of glycerol inactivation (Bachovchin et al., manuscript in preparation). The possibility that such a phenomenon accounts for the data in Fig. 1 is supported by the fact that the second hydrogen transfer step has been shown to exhibit a much greater isotope effect

than the first (Essenberg et al., 1971). Thus, deuterium would be expected to accumulate at the 5'-carbon of enzyme-bound cofactor. Moreover, the deuterium content of the enzyme-bound cofactor would be expected to increase very rapidly initially with increasing 1,1-dideuterio-1,2-propanediol followed by a much lesser increase as the cofactor becomes "saturated" with deuterium. This, then, provides an explanation for the data in Fig. 1 and requires only that the second hydrogen transfer step (cofactor → product) be at least partially rate determining. If this should prove to be the case, a kinetic "handle" on the nature of the intermediate hydrogen carrier that obtains in the mechanism of catalysis (presumably 5'-deoxyadenosine) would be provided.

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# PROPOSITION 1

There are nine adenosylcobalamin dependent hydrogen transfer enzymes that have to date been identified. However, investigation of these enzymes has been seriously hampered by their generally low availability. All of these enzymes are at present obtained through classical purification procedures that require many days of laborious attention and result in a few mg of semipure apoenzyme representing only a fraction of the amount of apoenzyme present in the initial crude preparations. A specific adsorbent utilizing adenosylcobalamin covalently attached to agarose has been reported and has proven valuable in the purification of adenosylcobalamin dependent ribonucleotide reductase (Yamada and Hogenkamp, 1972). Gram quantities of this enzyme are now available.

This specific adsorbent, however, has not proven applicable to the purification of the hydrogen transfer enzymes. There are several reasons for this: (1) in general the hydrogen transfer enzymes undergo irreversible inactivation when bound to AdoCbl unless substrate is present, and it is very difficult to maintain saturating substrate concentrations during the purification procedure when active holoenzyme is continually converting the substrate into product. (2) AdoCbl is more difficult to remove from the hydrogen transfer

enzymes. In addition, the procedure provides an added inconvenience; AdoCbl is light sensitive and the agarose-AdoCbl absorbent must be protected from light.

There are several derivatives of the active cofactor (e.g. OH-Cbl and CN-Cbl) that have a very strong and specific affinity for these enzymes (Babior, 1975); with dioldehydrase, for instance, the binding of both OH-Cbl and CN-Cbl is irreversible (Lee and Abeles, 1963). These derivatives offer the advantages of not being light sensitive or catalytically active. Until recently, however, these derivatives were considered irreversible inactivators since there existed no known method for restoring catalytic activity to the apoenzyme after binding OH-Cbl or CN-Cbl. This thesis reports a technique for removing OH-Cbl from dioldehydrase that results in better than 80% recovery of active apoenzyme. It is proposed that an OH-Cbl affinity absorbent be developed exploiting this technique for removing and recovering active apoenzyme from bound OH-Cbl for the purification of adenosylcobalamin dependent dioldehydrase. The successful development of such an affinity absorbent for dioldehydrase will very likely prove applicable to the purification of the other adenosylcobalamin dependent hydrogen transfer enzymes, as well as aiding in the discovery and isolation of other as

yet unknown adenosylcobalamin dependent enzymes.

CN-Cbl attached to insoluble supports has been successfully applied to the purification of Vitamin $B_{12}$ binding proteins such as transcobalamin II, human granulocyte vitamin $B_{12}$-binding protein and intrinsic factor (Allen and Magerus, 1972a; 1972b; 1972c; Allen and Mehlman, 1973). Purification 2 million-fold was achieved in the case of transcobalamin II demonstrating the potency of this technique. However, the removal of these vitamin $B_{12}$ binding proteins from this affinity adsorbent requires extensive dialysis against 7.5 M guanidine-HCl, a method that would clearly irreversibly alter the structure of any of the hydrogen transfer enzymes. This procedure also results in the destruction of more than 50% of the vitamin $B_{12}$ binding ability of these proteins; thus the method described above may also prove applicable to this field.

The successful development of an affinity absorbent utilizing OH-Cbl for the purification of dioldehydrase depends on linking the OH-Cbl to an insoluble support in such a way that the OH-Cbl will be free to interact with dioldehydrase in a manner similar to its interaction free in solution.

In view of the relatively large size of dioldehydrase (m.w. 250,000; Essenberg et al., 1971) steric constraints should be expected to play an important role. Thus the OH-Cbl will probably need to be a sufficient distance from the solid matrix to minimize steric-interference from the solid matrix in the binding process. This can be accomplished

through the use of a spacer group or "arm" connecting the insoluble support to the OH-Cbl. Also, the spacing of the OH-Cbl itself on the solid matrix will probably need to be some distance to minimize steric interference to the binding of dioldehydrase by OH-Cbl from adjacent OH-Cbl. This can be accomplished by linking the OH-Cbl to the solid matrix under conditions that should assure a relatively low density of bound OH-Cbl.

There are a number of functional groups on the OH-Cbl molecule that have been used or can be used as points of attachment. Functional groups that have been used include the amide side chains (Allen et al., 1974) and the phosphate group (Olesen et al., 1971). Other possibilities include direct attachment to the cobalt through the formation of a cobalt-carbon or cobalt-sulfur bond (Pratt, 1972). Although this last possibility has the disadvantage of resulting in a light sensitive linkage it has two potential advantages. The interaction with dioldehydrase permitted by this linkage may be very good and the light sensitivity may be turned into an advantage by providing a way of removing apoenzyme-OH-Cbl from the insoluble support. The recovery of active apoenzyme by the procedure reported in this thesis should at this point present no problems.

A number of solid supports successfully applied to the building of affinity absorbents and the isolation of enzymes

include cellulose, Sephadex, polyacrylamide, agarose and Sepharose (Jakoby, 1971). Finding the combination of solid support, spacer groups, and point of attachment to OH-Cbl that will give the desired results may require a trial and error process.

Once the right combination of the above factors is found dioldehydrase will readily be separable from the crude sonicate by column or batch procedures. The problem then becomes one of recovering active holoenzyme. One possible solution is to recover the apoenzyme bound to OH-Cbl free in solution. This could be accomplished by adding OH-Cbl to the buffer that induces the dissociation of OH-Cbl from dioldehydrase and eluding dioldehydrase bound to free OH-Cbl. The OH-Cbl could then be removed by dialysis as described in this thesis. Alternatively the OH-Cbl could be attached to the solid matrix by a spacer group containing a labile linkage (Nexo, 1974). After the protein has been adsorbed and other undesired proteins washed free the linkage can be cleaved by application of mild heat or pH change and the dioldehydrase could then be obtained bound to freed OH-Cbl. This technique would have the advantage that the enzyme could be maintained at relatively high concentrations. This is desirable since dioldehydrase undergoes irreversible inactivation at low protein concentration.

The development of an affinity absorbent for dioldehydrase as described above is clearly feasible and would if

successful, prove to be not only invaluable to the further study of this adenosylcobalamin dependent enzyme, but to the study of other known, and possibly as yet unknown adenosylcobalamin dependent hydrogen transfer enzymes, and thus of the mechanism of action of adenosylcobalamin itself.

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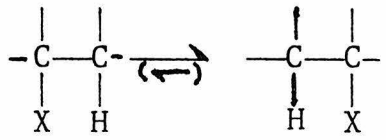
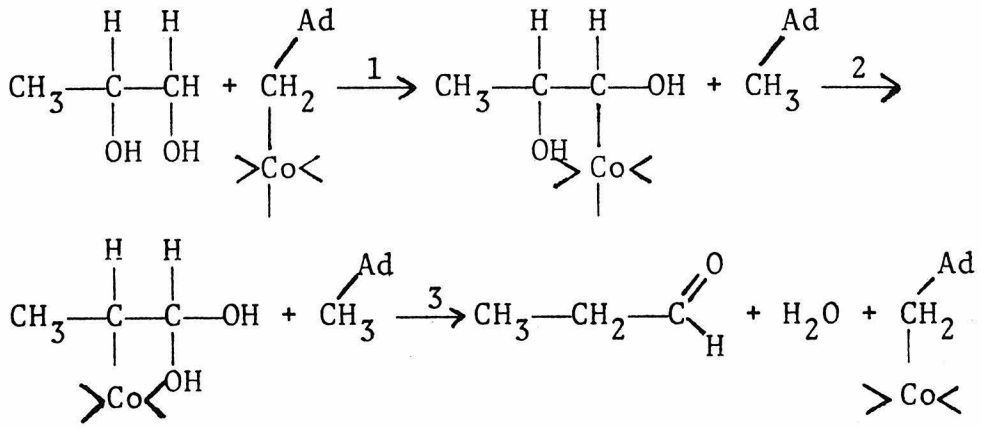
PROPOSITION II

There are nine known adenosylcobalamin dependent hydrogen transfer enzymes which catalyze rearrangements of the type



where X can be alkyl, $-NH_2$, or OH (Hogenkamp, 1968; Babior, 1975a, 1975b; Abeles and Dolphin, 1976). The currently favored minimum mechanistic scheme for these rearrangements is illustrated below using the rearrangment of 1,2-propanediol to propionaldehyde catalyzed by dioldehydrase (one of the most extensively studied of these enzymes) as a model (Essenberg et al., 1971)

SCHEME I



The first part of the reaction sequence involves the abstraction of a hydrogen from C-1 and transfer to the 5'-carbon of the cofactor which presumably, becomes a methyl group, and the formation of a cobalt carbon bond to C-1 of the substrate. It is not intended to indicate in Scheme I that step 1 occurs in a concerted process. Step 2 involves the migration of the cobalt from C-1 to C-2 of the substrate in what has been called a transalkylation reaction, while the OH group migrates from C-2 to C-1. The third and final part involves the return of hydrogen from the 5'-carbon of the cofactor to C-2 of substrate, the reformation of active coenzyme, and the enzymatic dehydration at C-1.

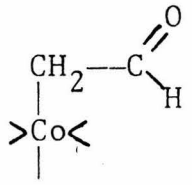
While biochemists have concerned themselves with the mechanisms of hydrogen transfer and the role of 5'-deoxyadenosine as an intermediate (Babior, 1975a), model systems chemists have concentrated on aspects of the interaction of the substrate with the cobalt. As a result, the mechanism of hydrogen transfer and the role of 5'-deoxyadenosine in the enzymatic reaction appears to be fairly well understood, and, the literature has become virtually inundated with model systems and studies of the mechanisms of model systems that undergo reactions relevant to the proposed transalkylation reaction in the enzyme catalyzed reaction (see for example: Schrauzer et al., 1970; Brown, 1973; Silverman and Dolphin, 1975; Dowd et al., 1975). However, although there is ample precedent for it from model systems,

there is as yet not a single thread of evidence for the formation of a substrate alkylcobalamin as an intermediate in any adenosylcobalamin dependent enzyme catalyzed reaction.

This thesis reports results demonstrating the presence of an unknown alkyl or thiocobalamin bound to glycerol inactivated holoenzyme. TCA denaturation and precipitation of the protein results in the recovery of OH-Cbl only. Thus, it is very unlikely that this alkylcobalamin is adenosylcobalamin and must instead be a species with the 5'-deoxyadenosyl ligand replaced by substrate or by an amino acid side chain. It is proposed that this question be answered and the nature of the ligand identified i.e. if an amino acid side chain, what is the amino acid, if substrate, what is the nature of the substrate cobalamin and how does it relate to the mechanisms of catalysis and inactivation.

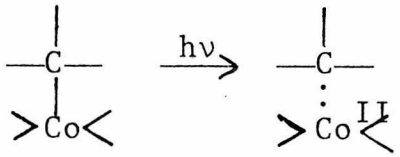
As an initial approach it is proposed that enzymatic hydrolysis of the glycerol inactivated holoenzyme would be most productive. This could be carried out under very mild conditions and is therefore not likely to disrupt possibly labile carbon-cobalt or carbon-sulfer bonds (Hirs, 1967). If an amino acid side chain is liganded to the cobalt, the cobalamin species should be relatively stable and readily isolable by conventional techniques. The cobalamin isolated will most likely not be liganded to a single amino acid but to a peptide fragment. The amino acid actually involved in

ligation may be identified by a number of methods, since sensitive methods exist for the isolation and identification of amino acids and peptides (Hirs, 1967). However, if some form of glycerol is liganded to the cobalt the cobalamin species may or may not be isolable. If the substrate cobalamin is isolable it can of course be identified by more or less conventional techniques (Pratt, 1972). However, there is a strong possibility that a substrate cobalamin will be exceedingly labile once free in solution. Secondary alkylcobalamins, for example, are very unstable and difficult to synthesize and isolate (Hogenkamp, 1975; Pratt, 1972) as is formylmethylcobalamin (Silverman et al., 1974),



a proposed intermediate in the reaction of ethylene glycol with dioldehydrase holoenzyme. An approach which may prove productive in this case would be to inactivate dioldehydrase holoenzyme with $^{14}$C-glycerol, remove the labeled product and substrate glycerol by gel filtration and recover the intact but inactivated holoenzyme -$^{14}$C-glycerol complex. Experiments can now be designed to ascertain the nature of the substrate cobalamin. Photolysis, for example, should result in the release of $^{14}$C-label if glycerol is the ligand. The conditions under which photolysis is carried out could

be varied to gain further information. Photolysis of an alkylcobalamin results in homolytic cleavage of the cobalt-carbon bond. Anaerobic and aerobic conditions may yield




different photolysis products which could then be characterized to yield information about the original alkylcobalamin. Free radical trapping agents such as thiols could be employed and may also yield information about the original alkylcobalamin.

Whether the 5'-deoxyadenosyl ligand proves to be replaced by an amino acid side chain or by substrate the result will be interesting and informative. Very little is known about the structure of the active sites of these enzymes and the nature of the amino acids located there. Thus the isolation and identification of a cobalamin species liganded to an amino acid would represent an advance in this respect. If on the other hand the cobalamin is found to be liganded with glycerol, it would represent the first evidence for the occurrence of a substrate-cobalt bond in an enzymatic system.

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# PROPOSITION III

One of the most interesting and intriguing questions in molecular biology today is how external stimuli such as light, heat, chemicals, etc. get translated into a biological response. It is generally agreed that receptor molecules, which are usually proteins, serve as the initiating point for the response; the interaction of a receptor with its effector causing a change that can then be transmitted. The nature of this change and the mechanism for its transmission are, at present, topics for investigation.

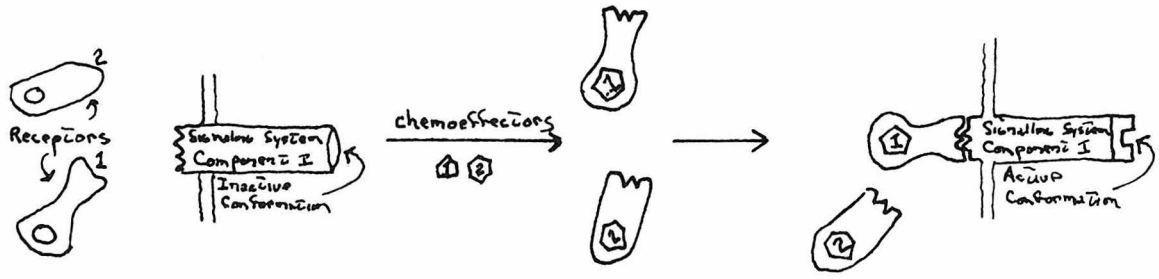
It is now well established that bacteria exhibiting chemotactic behavior possess specific chemoreceptors (Adler, 1969). Moreover, a number of chemotactic binding proteins have been isolated, identified, and shown to be connected with the chemotactic response (Hazelbauer and Adler, 1971; Anraku, 1968; Kalchar, 1971; Boos, 1972; Aksamet and Koshland, 1972; Adler et al., 1973).

Specifically, the ribose and galactose chemotactic receptors have been isolated and identified from Escherichia coli (Anraku, 1968) and from Salmonella typhimurium (Aksamet and Koshland, 1974). The S. typhimurium galactose binding protein binds one molecule of galactose and evidence that this protein is the true receptor for galactose chemotaxis

has recently been obtained by Strange and Koshland (1976). The ribose binding protein was similarly characterized earlier (Aksamet and Koshland, 1974; Aksamet and Koshland, 1972).

Although both the galactose and ribose receptors bind other sugars, the purified galactose binding protein shows no affinity for ribose and the purified ribose binding protein exhibits no affinity for galactose (Strange and Koshland, 1976). Somewhat peculiarly, in view of the above, ribose was found to inhibit the in vivo galactose chemotactic response in Salmonella typhimurium. The same result was found in E. Coli. Moreover, galactose was likewise found to inhibit the in vivo ribose chemotactic response (Strange and Koshland, 1976).

Based on this result the author proposes the following model




The chemoeffectors, galactose and ribose when bound to their corresponding receptors induce a conformational change in the receptor molecules. The receptors then compete for the component I molecule in the signaling system. The inhibition

observed then, arises not because the sugars compete for the same receptor molecules, but because the receptor molecules, when bound to their chemoeffectors, compete for the same component I molecule. In support of this model mutants lacking the ribose binding protein were found to exhibit no ribose inhibition, and most recently a mutant was found possessing both the ribose and galactose binding proteins but exhibited neither ribose nor galactose chemotaxis. Furthermore, fluorescence data has been obtained which appears to support the occurrence of a conformational change in both the galactose and ribose binding proteins on binding to galactose and ribose respectively (Strange and Koshland, 1976).

It is proposed that experiments can be conducted to determine if such a conformational change does indeed occur. The purified ribose and galactose receptors both free and bound to chemoeffector can be used to induce an immune response. Antisera specific for the pure galactose and ribose receptors as well as antisera specific for the corresponding receptors bound to their respective chemoeffector can be prepared by conventional techniques (Williams and Chase, 1967). The degree of a cross reactivity exhibited by each preparation of antisera should yield significant information. For example, the antisera prepared against the ribose binding protein bound to ribose could be tested for cross reactivity against the galactose binding protein, both in the presence and absence of galactose. If the antisera exhibits a greater

degree of interaction with the galactose binding protein-galactose complex than with the pure galactose binding protein it can be taken as evidence for not only a conformational change but a conformational change that gives rise to antigenic determinants the same as or very similar to those on the ribose binding protein-ribose complex and thus provide support for the proposed model. The reverse experiment could, of course, also be done to yield complimentary information. Evidence for the occurrence of a conformational change only on binding of the chemoeffector can be obtained by comparing the cross reactivities of the antisera raised against each receptor with that raised against the receptor bound to the corresponding chemoeffector and vice versa.

The experiments proposed here should be able to detect a conformational change in the chemotactic receptors on binding their respective chemoeffectors and furthermore, determine if the galactose and ribose receptors bound to their respective chemoeffectors exhibit antigenic determinants in common that they do not possess in the absence of the chemoeffector. Positive results would lend great support to the model proposed by Strange and Koshland (1976).

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# PROPOSITION IV

Every immunologically competent vertebrate organism is capable of producing a seemingly infinite assortment of antibody molecules with specificities that appear to be virtually limitless. The question of the origin of this diversity remains unresolved and there is at present a great deal of controversy generated by two extreme points of view, represented by the germ line theory and the somatic mutation theory.

Antibody molecules are composed of constant and variable regions. There appears to be a limited number of different constant segments a particular organism can generate, i.e. there are four or five different constant segments associated with the light chains and around ten different constant regions associated with the heavy chains (Eisen, 1974). Each constant segment is transmitted from generation to generation in a Mendelian fashion and thus appears to be coded for by structural genes in the germ line. The number of different variable segments that a particular individual can generate on the other hand, is enormously greater (Eisen, 1974; Nisonoff et al., 1975). Moreover, it now seems well established that the variable regions are associated with the antibody combining sites and are thus the basis for antibody specificity and diversity (Capra et al., 1971; Titani, 1965; Wu and Kabat,

1970).

Proponents of the germ line theory maintain that structural genes exist in the germ line for all the variable sequences an individual can synthesize while advocates of the somatic mutation theory argue that only a small number of variable germ line genes actually exist and diversity arises through random somatic mutations and/or recombinations.

There are many pros and cons to both theories. Briefly, the principal advantages of the germ line theory are that the diversity of antibody molecules can be explained in terms of conventional mechanisms such as gene duplication, mutation, and selection, and that the total number of genes required could probably be accomodated in the vertebrate genone. The chief disadvantage of this theory is that it has difficulty accounting for the existence of antibodies specific for antigens that probably never existed in the past evolutionary history of the species. The chief advantage of the somatic mutation theory, on the other hand, is that it provides economy yet the potential for tremendous diversity. Moreover, a mechanism exists for selecting and expanding a particularly favorable mutation (clonal selection theory). The chief criticism of this theory is that it cannot account for the vast number of variable sequences that exist at the onset of immunological competence (Eisen, 1974; Nisonoff et al., 1975).

Everyone agrees that mutations and recombinations,

although infrequent, do occur. The real question is then, can an organism utilize these random mutations to generate new or more effective antibody molecules. In the following, proposals for experiments are made that may answer this question.

The existence of idiotypes and antiidiotypic antisera is a relatively recent discovery (Oudin and Michel, 1969). Antiidiotypic antisera can be generated in a number of ways. For instance, antisera can be raised to an antigen in a particular individual and then injected into another individual of the same species and allotype. The anti-antibodies generated will often be specific for the antibody combining site of the injected antiserum. It is generally believed that idiotypes are associated with the variable amino acid sequences that are responsible for the uniqueness of each antibody molecule (Eisen, 1975; Nisonoff et al., 1975).

Idiotypic cross-reactions among antibodies of a given specificity raised from different species are exceedingly rare, i.e. antiidiotypic antibodies to a particular antibody will very rarely react with another antibody of the same specificity from a different species. Antibodes from different individuals of an inbred strain often do exhibit extensive idiotypic cross reactions. However, there are instances where idiotypic cross reactions are very weak or non-existant. For example, antiidiotypic antibodies prepared against anti-p-azophenyl-

arsonate antibodies from a number of inbred strains of mice (C57BL, DBA/2J, SWR/J, RF/J, ΔKR, and C57BR/cdj) do not exhibit appreciable cross reactivity (Nisonoff et al., 1975). This absence of idiotypic cross reactivity in inbred mice has been very little studied.

Thus, it is proposed that a number of antigens be used to raise antibodies in these strains of mice and that the resulting antibodies screened for idiotypic cross reactivity. The results of the cross reactivity tests should then be quantitated and correlated with the nature of the antigen. If for example, antigens that probably could not have existed in the past evolutionary history of the species such as newly synthesized organic molecules should prove to generate antibodies with very low idiotypic cross reactivity compared to that observed with other antigen the operation of a somatic mutation mechanism would be indicated.

If such experiments should reveal evidence for the operation of somatic mutation in these particular situations they could be followed up by a number of other kinds of experiments. For example, the cells producing antibody molecules exhibiting the above low idiotypic cross reactivity in a particular individual could be destroyed. (A possible method to accomplish this would be to inject relatively large quantities of highly radioactive antiidiotypic antibodies.) The amount of time required for the individual to mount a new response to the given antigen should be determined along

with the idiotypic cross reactivity properties of the antibodies. A lag time in the return of an immune response and low idiotypic cross reactivity with the antibodies from the first response would further support the operation of a somatic mutation mechanism.

Once a set of antigens are defined that generate antibodies in an inbred strain with very low idiotypic cross reactivity, information from various other kinds of experiments could be obtained and could prove useful. The response to these antigens as a function of the age of the individual, for example, should be determined. If very young, barely immunologically competent individuals should exhibit a restricted response, or little or no response to these antigens, it would indicate the absence of the appropriate specificities in the germ line and a requirement for a certain amount of time to generate the proper specificities. A second type of experiment would be to determine if the progeny of a given pair of these individuals should generate antibodies that will exhibit idiotypic cross-reactivity with either or both of his parents.

Although apparently difficult to accomplish, antiidiotypic antibodies have been successfully produced from mice of the same inbred strain (Sirisinha and Eisen, 1972). These antiidiotypic antibodies were produced against myeloma proteins with a specificity for DNP. DNP does not generate

antibodies with low idiotypic cross reactivity in inbred strains of mice. An interesting experiment would be to determine if antiidiotypic antibodies could be generated from mice of the same strain with greater ease if antibodies exhibiting low cross reactivity (as above) were used as the immunogen. A positive result would be a strong indication of the occurrence of different determinants on the antibodies of "genetically identical" individuals and thus lend support to the utilization of random somatic mutations as a mechanisms for the production of improved or new specificities.

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# PROPOSITION V

Nuclear magnetic resonance has already proven to be a very powerful tool in many areas of chemistry and biology. Applications of $^{1}H$, $^{13}C$, $^{19}F$, and $^{31}P$ magnetic resonance to the study of biochemical processes are numerous. Most recently $^{15}N$ magnetic resonance has come under scrutiny in regard to potential applications to biochemical problems (Gust et al., 1975). Nitrogen is ubiquitous in biological systems and is often closely associated with or directly involved in biochemical processes of interest; thus the feasibility of $^{15}N$ magnetic resonance holds great promise.

Of particular interest in this regard is the feasibility of applying $^{15}N$ magnetic resonance to the study of the ring nitrogens in porphyrins and corrins. The porphyrin ring is implicated in many important biological processes such as light absorption (Chlorophyll), oxygen transport (Hemoglobin and myoglobin), electron transfer (cytochromes), and enzyme catalysis (peroxidase and catalase)(Marks, 1969; Smith, 1975). The corrin ring system is similar to that of porphyrins in several respects and is involved in a number of important enzyme catalyzed reactions (Babior, 1975).

The $^{15}N$ spectrum of 95% $^{15}N$ enriched pheophytin a has been obtained on a Bruker HFX-90 E spectrometer operating at 9.12 MHz and using a 60 sec pulse delay. The chemical shifts of

the ring nitrogens and proton coupling constants were determined. The $^{15}N$ spectrum of chlorophyll a however, could not be obtained. The reason was attributed to the elimination of the only efficient relaxation mechanism for the nitrogen nuclei when Mg replaces the two central protons. However, the chemical shifts of the ring nitrogens in chlorophyll a were obtained indirectly by double resonance experiments (Boxer et al., 1974). The $^{15}N$ spectrum of cyanocobalamin was recently examined at the natural abundance level at 18.25 MHz using the Bruker WH-180 (Gust et al., 1975). Although it was possible to obtain seven well resolved resonances for the seven amide nitrogens, the ring nitrogen were not observed. Again the lack of signal was attributed to long relaxation times of the ring nitrogen in addition to small NOE values and coupling to cobalt.

It is proposed that the potential application of $^{15}N$ magnetic resonance to the study of these ring nitrogens be explored further with particular attention given to the use of paramagnetic species for improving the sensitivity of this method. Two general areas should be explored: (1) the use of paramagnetic species to reduce the relaxation times of the ring nitrogens making more rapid signal accumulation possible (Farnell et al., 1972), and to eliminate possibly unfavorable NOE's arising from proton decoupling (LeMar, 1974), (2) the potential for dynamic nuclear polarization as a means of enhancing the signal (Hausser and Stehlik, 1968). Since

porphyrins and corrins a normally found coordinated to metals that readily undergo reversible oxidation reductions reactions resulting in net exchange between paramagnetic and diamagnetic states e.g., $Co^{II} \rightleftharpoons Co^{III}$; $Fe^{III} \rightleftharpoons Fe^{II}$ (Pratt, 1972; Smith, 1975) it may be possible to utilize these metals as the paramagnetic species. However, other more conventional techniques may also give the desired results.

Farnell et al. (1972), have examined the effect of tris (acetylacetonato)chrominum III on the signal intensities of the $^{15}N$ signal from benzonitrile, 4-bromobenzonitrile, and 4-toulunitrile. Concetrations of the paramagnetic species as high as 0.1 M were found to result in favorable enhancements of the signal to noise (approximately 5-fold) due to the reduction in relaxation times. Thus, this paramagnetic species may be one to examine initially.

Particularly intriguing, however, is the possibility for large enhancements of the $^{15}N$ signals from a dynamic nuclear polarization experiment. Electron overhauser effects of 400 have been observed for the proton resonance in several systems (Carrington and McLachlan, 1967). Large effects have also been observed on $^{13}C$, $^{31}P$, and $^{19}F$ signals in liquids (Hausser and Stehlik, 1968). For example a net signal enhancement of -1200 was observed in the $^{13}C$ NMR spectrum of 1,2-bis-diphenylene-phenylalkyl radical in benzene. No dynamic nuclear polarization work has as yet been reported on the $^{15}N$ nucleus. If effects as large as 1000 are possible $^{15}N$ becomes approximately

equal to proton in sensitivity for equal number of nuclei, and with $^{15}N$ enrichment it should be possible to observe the $^{15}N$ signal in biochemical systems.

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