ANALOGUES OF 1,2-PROPANEDIOL, INACTIVATORS OF DIOLDEHYDRASE

I. STUDIES ON THE EFFECT OF 3-HALO-1,2-PROPANEDIOLS ON THE COENZYME B_{12} DEPENDENT DIOLDEHYDRASE REACTION.

II. STUDIES ON THE EFFECT OF AN EPOXIDE ON DIOLDEHYDRASE.

Thesis by
Mark Stuart Lamkin

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ABSTRACT

PART I

3-Chloro-1,2-propanediol, 3-bromo-1,2-propanediol, and 3-iodo-1,2-propanediol have been found to bind to dioldehydrase. Dissociation constants are approximately equal in each case. Binding occurs in a manner similar to that of the normal substrate, 1,2-propanediol. All three compounds behave as competitive inhibitors while 3-chloro-1,2-propanediol and 3-brom-1,2-propanediol inactivate the holoenzyme as well. This inactivation process is irreversible and may involve the alkylation of sulfhydryl residues of dioldehydrase. Of the above compounds, only 3-chloro-1,2-propanediol is converted to product; possibly β-chloropropionaldehyde is formed.

PART II

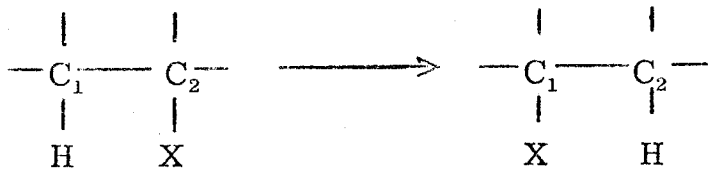
2,3-Epoxypropanol (glycidol) has been found to bind to dioldehydrase. Its calculated dissociation constant is 8.8×10^{-4} M. It binds to dioldehydrase even though it has but one hydroxyl group. 2,3-Epoxypropanol behaves as a competitive inhibitor. This compound also inactivates the holoenzyme. Unlike the compounds studied in Part I, it also inactivates the apoenzyme, although at a slower rate. Since epoxides are known to alkylate

sulfhydryl groups, the above result suggests the presence of exposed sulfhydryl residues.

INTRODUCTION

Dioldehydrase (R,S-1,2-propanediol hydrolyase, E.C. 4.2.1.28, from Klebsiella pneumoniae (ATCC 8724)) catalyzes the formation of propionaldehyde and acetaldehyde from 1,2-propanediol and ethylene glycol, respectively. One requirement for catalytic activity is the presence of a univalent cation. Numerous ions have been studied. The presence of potassium ions results in the greatest activity (Lee and Abeles, 1963). The enzyme has a broad pH profile with a maximum near pH 8.0. The enzyme is inactive outside the range of pH 6 to pH 10. The optimal temperature for the dioldehydrase reaction is 37°C.

Of greater importance is the absolute requirement of dioldehydrase for coenzyme B_{12}. Several other enzymes require coenzyme B_{12}, including glutamate mutase, methylmalonyl-CoA mutase, β-lysine mutase, ribonucleotide reductase, methionine synthetase, ethanolamine deaminase, and glycerol dehydrase. Except for the reductase and synthetase, the above enzymes catalyze reactions that involve 1,2 hydrogen shifts with simultaneous 2,1 shifts of unique functional groups. (Hogenkamp, 1968).



The formation of the aldehyde from the dioldehydrase reaction has been shown to involve the abstraction of a hydrogen

from the primary carbon of the vicinal diol (Browstein and Abeles, 1961). Hydrogen labelling experiments performed with dioldehydrase have demonstrated that the hydrogen transfer occurs intermolecularly. Subsequently, it has been postulated that the coenzyme B_{12} molecule participates as the hydrogen carrier (Abeles, 1965). The electronic nature of the coenzyme B_{12}-enzyme-substrate intermediates remain unresolved. Electron spin resonance experiments have been performed for numerous coenzyme B_{12} dependent enzymes. The results of these experiments support a radical mechanism (Babior et al., 1972, 1973; Hamilton et al., 1971, 1972; Orme-Johnson et al., 1973; Finlay et al., 1972; and Valinsky et al., 1973).

It has been previously reported that dioldehydrase reacts with only a very few diols (Lee and Abeles, 1963). In addition to 1,2-propanediol and ethylene glycol, 3-fluoro-1,2-propanediol and glycerol produce aldehydes when reacted with dioldehydrase and coenzyme B_{12} (Eagar et al., 1975; Toraya et al., 1976). In this study, 3-chloro-1,2-propanediol, 3-bromo-1,2-propanediol, 3-iodo-1,2-propanediol, and 2,3-epoxypropanol were examined to determine the effect on the reaction of various structural modifications of the normal substrate, 1,2-propanediol. Dissociation constants and inactivation rate constants were calculated and compared to determine possible reaction mechanisms.

PART I

Studies on the Effect of 3-Halo-1,2-propanediols on the Coenzyme B_{12} Dependent Dioldehydrase Reaction

INTRODUCTION

Current mechanisms for the hydrogen abstraction by coenzyme B_{12} have as an initial step the cleavage of the bond between the cobalt atom and the 5' methylene carbon atom of the deoxyadenosyl moeity of the coenzyme B_{12} molecule (Wagner et al., 1966). The electronic nature of the bond cleavage and hydrogen transfer still remain unresolved. At present, electron spin resonance evidence support a radical pathway for the dioldehydrase reaction (Finlay et al., 1972; Valinsky et al., 1973).

As previously stated, dioldehydrase has almost no activity for only slightly modified substrate analogues of 1,2-propanediol (Lee and Abeles, 1963; Toraya and Fukui, 1972). Studies using 3-fluoro-1,2-propanediol as a substrate have shown that this analogue behaves similarly to 1,2-propanediol. By changing the polar characteristics of possible substrates, one can get an idea of whether the mechanism has an ionic or a radical intermediate. The results of the 3-fluoro-1,2-propanediol experiments support a radical pathway (Eagar et al., 1975). In this study, 3-chloro-1,2-propanediol, 3-bromo-1,2-propanediol, and 3-iodo-1,2-propanediol have been used to examine the effect on the enzymatic reaction of substitutions of large electronegative atoms in the relatively uninvolved methyl group of the substrate.

EXPERIMENTAL SECTION

Enzyme Preparations. Dioldehydrase was obtained from Klebsiella pneumoniae (ATCC 8724) by a method similar to that reported by Lee and Abeles (1963). Dioldehydrase with a specific activity of at least 10 units/mg was used in all experiments. Substrate-free enzyme was prepared as previously reported (Frey et al., 1967).

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

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

3-Bromo-1,2-propanediol. 3-Bromo-1,2-propanediol was prepared by the acid hydrolysis of epibromohydrin which was purchased from Aldrich Chemical Company. The hydrolysis product was neutralized with potassium hydroxide to pH 8 and distilled under vacuum (Pattison and Norman, 1957).

3-Iodo-1,2-propanediol. 3-Iodo-1,2-propanediol was prepared by the addition of sodium iodide to 3-chloro-1,2-propanediol in nitromethane. The recrystallized product had a melting point between 49 and 50°C. (Rosenthal and Geyer, 1958).

1,1-Dideuterio-1,2-propanediol. 1,1-Dideuterio-1,2-propanediol was prepared by the reduction of ethyl lactate by lithium aluminum deuteride (Feiser and Feiser, 1967).

Yeast alcohol dehydrogenase. Yeast alcohol dehydrogenase

was purchased from Sigma Chemical Company.

Nicotine adenine dinucleotide. The disodium salt of the reduced form of nicotine adenine dinucleotide was purchased from Sigma Chemical Company. Solutions were made fresh due to the formation of inhibitory compounds.

Assays. Whenever possible, a continuous assay technique was utilized. The yeast alcohol dehydrogenase reaction was coupled to the dioldehydrase system under study (Sottocasa et al., 1971; Yakusheva et al., 1974). This assay was carried out in the sample compartment of a Beckman CIII spectrophotometer. The aldehyde product from the dioldehydrase reaction is reduced to the alcohol by reaction with yeast alcohol dehyrogenase and reduced nicotine adenine dinucleotide. Under the proper conditions the dioldehydrase reaction is rate limiting. The rate of the reaction can be followed by monitoring the concomitant oxidation of nicotine adenine dinucleotide. This oxidation can be observed by measuring the absorbance of nicotine adenine dinucleotide at 340 nm at various times.

RESULTS

Nature of the Reaction. The rate of conversion of 3-chloro-1,2-propanediol to product, as measured by the oxidation of nicotine adenine dinucleotide, decayed exponentially with time. The product was not acrolein as tested by an acrolein specific assay (Circle et al., 1945). 3-Bromo-1,2-propanediol and 3-iodo-1,2-propanediol did not react to form product (Fig. 1).

The three above compounds were found to act as competitive inhibitors of dioldehydrase. Dissociation constants were determined for each compound (Fig. 2-4). These values were very similar in magnitude to each other as well as to 3-fluoro-1,2-propanediol (Eagar et al., 1975) in spite of the large differences in the halogens' sizes (Van der Waals radii: hydrogen, 1.2Å; fluorine, 1.35Å; chlorine, 1.80Å; bromine, 1.95Å; and iodine, 2.15Å).

Two of the halogenated substrate analogues, namely 3-chloro-1,2-propanediol and 3-bromo-1,2-propanediol, were found to inactivate dioldehydrase. The inactivation could not be reversed by dialysis. The inactivation could be prevented by adding excess amounts of 1,2-propanediol. The inactivation rate constant of 3-bromo-1,2-propanediol was found to be approximately four times as large as that of 3-chloro-1,2-propanediol (Fig. 3, 5-6). Table I summarizes the calculated values of K_I, $k_{inactivation}$, and k_{cat} obtained for 3-chloro-1,2-propanediol, 3-bromo-1,2-pro-

panediol, and 3-iodo-1,2-propanediol.

The deuterium isotope effect on inactivation was measured for the above inactivators. The inactivator and either unlabelled 1,2-propanediol or 1,1-dideuterio-1,2-propanediol were allowed to react simultaneously with dioldehydrase and coenzyme B_{12}. The exposure of the inactivator to the resulting deuterium substituted coenzyme B_{12} produced only a slight change in the rate of inactivation as demonstrated by the ratios, $(k_H/k_D)_{inactivation}$, of 1.7 and 1.6 for 3-chloro-1,2-propanediol and 3-bromo-1,2-propanediol, respectively.

Spectral Observations of Intermediates. Visible spectra were taken of a reaction mixture of enzyme, coenzyme B_{12}, and inhibitor. Spectra were similar to those observed for glycerol inactivated dioldehydrase (Bachovchin et al., manuscript in preparation). Maxima were found near 540 nm and in the region between 350 and 383 nm (Fig. 7). These spectra are different from cyanocobalamin, aquocobalamin, hydroxycobalamin, and coenzyme B_{12}.

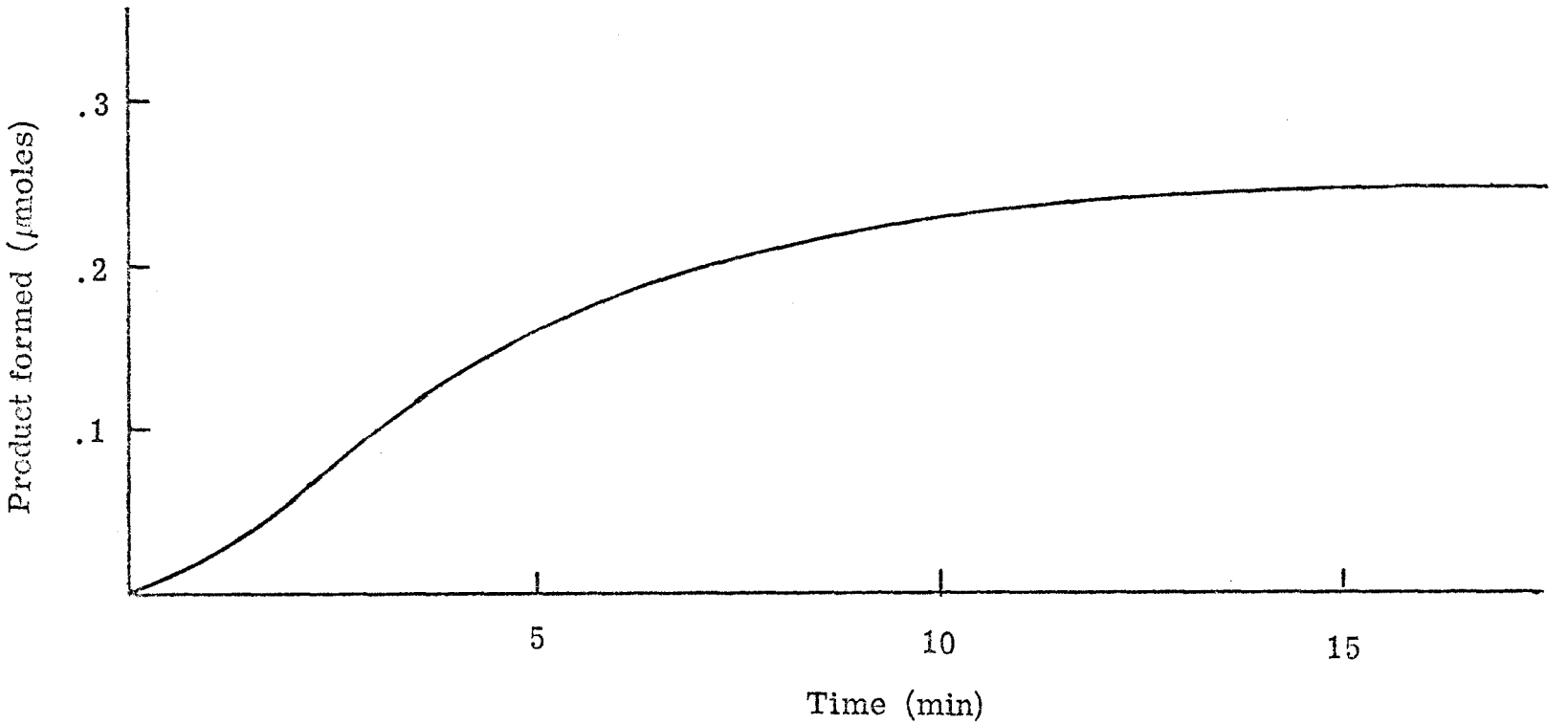


Fig. 1. Rate of product formation of 3-chloro-1,2-propanediol. Reaction mixture contains the following: apoenzyme, 2 units; potassium phosphate buffer, pH 8.0, 75 μmoles; coenzyme B_{12}, 40 γ; BSA, .02 mg; yeast alcohol dehydrogenase, 30 units; NADH, .5 mg; 3-chloro-1 2-propanediol, 1 mmoles.

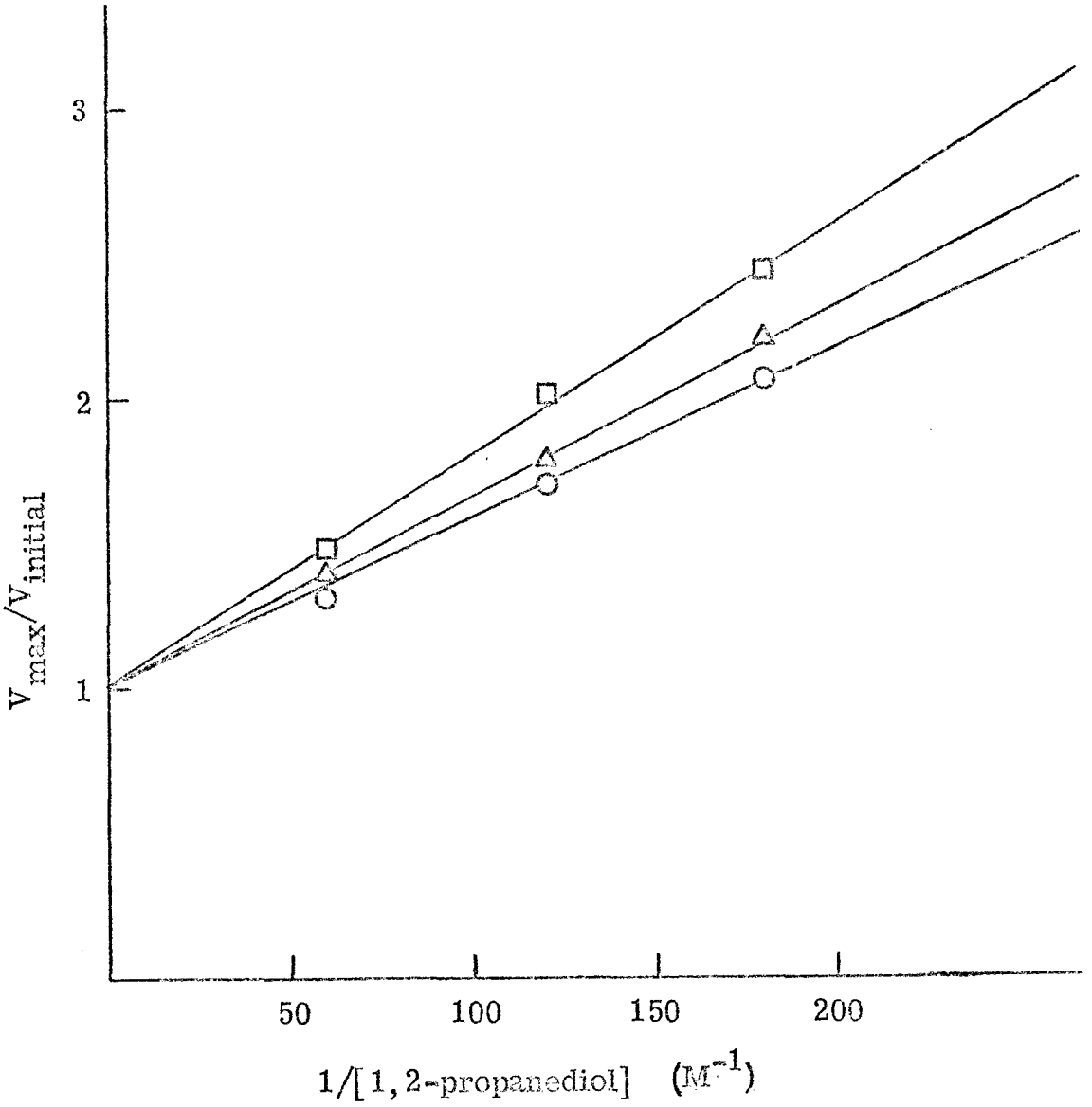


Fig. 2. Lineweaver-Burk plot for 3-chloro-1,2-propanediol in competiton with 1,2-propanediol. Reaction mixtures consist of the following: apoenzyme, .05 units; potassium phosphate, pH 8.0, 75 μmoles; coenzyme B_{12}, 40 γ; BSA, .02 mg; alcohol dehydrogenase, 30 units; NADH, .5 mg; varying amounts of 3-chloro-1,2-propanediol and 1,2-propanediol. ○ 8.33×10^{-2} M, △ 10^{-1} M, □ 1.33×10^{-1} M in 3-chloro-1,2-propanediol. The velocity is taken immediately after the start of the reaction.

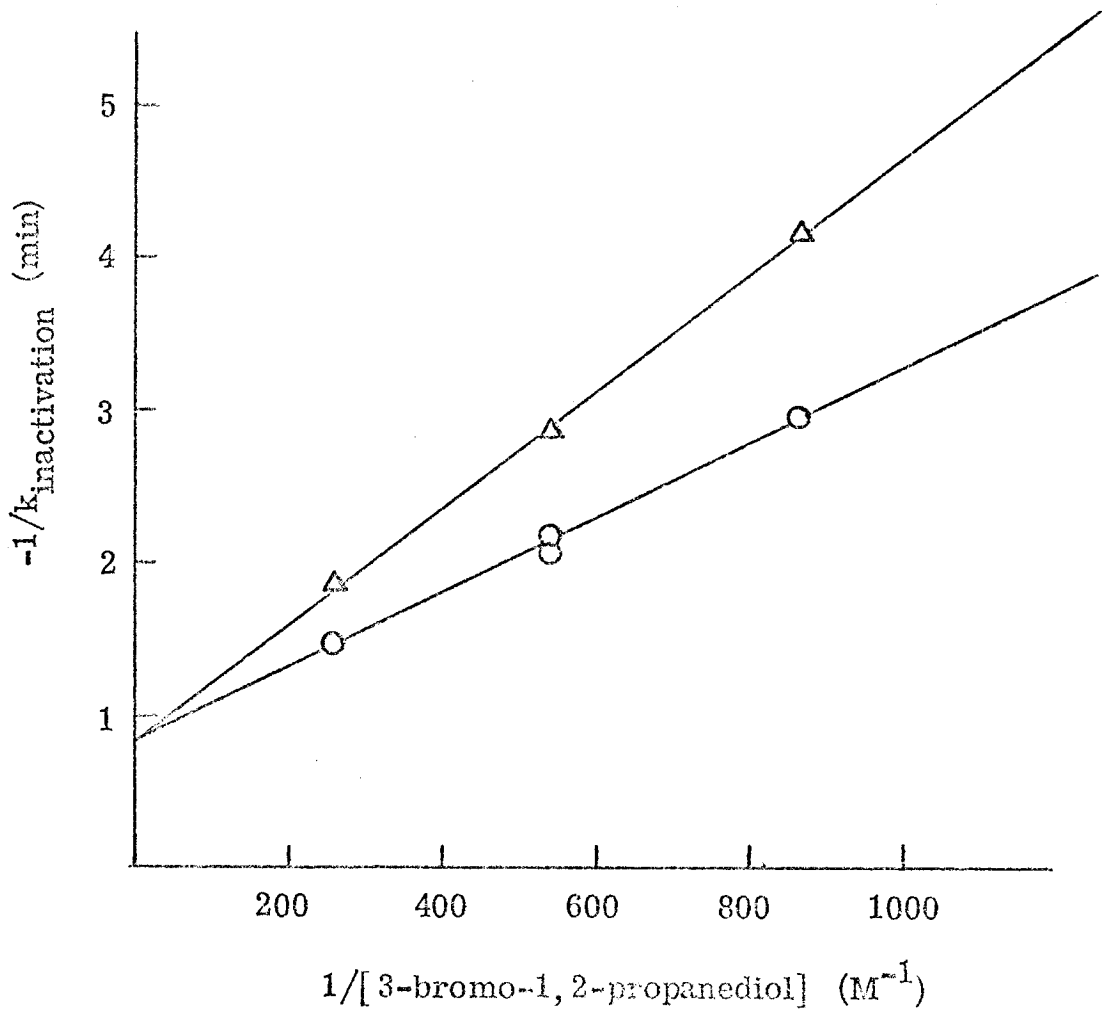


Fig. 3. Lineweaver-Burk plot for 3-bromo-1,2-propanediol in competition with 1,2-propanediol. Reaction mixtures consist of the following: apoenzyme, .05 units; potassium phosphate, pH 8.0, 75 μmoles; coenzyme B_{12}, 40 γ; BSA, .02 mg; alcohol dehydrogenase, 30 units; NADH, .5 mg; varying amounts of 2-bromo-1,2-propanediol and 1,2-propanediol. For each point on the graph a plot of ln % activity versus time of inactivation was determined. O 4.22×10^{-3} M and Δ 8.18×10^{-3} M in 1,2-propanediol. Y-intercept is the reciprocal of the substrate-free inactivation rate constant.

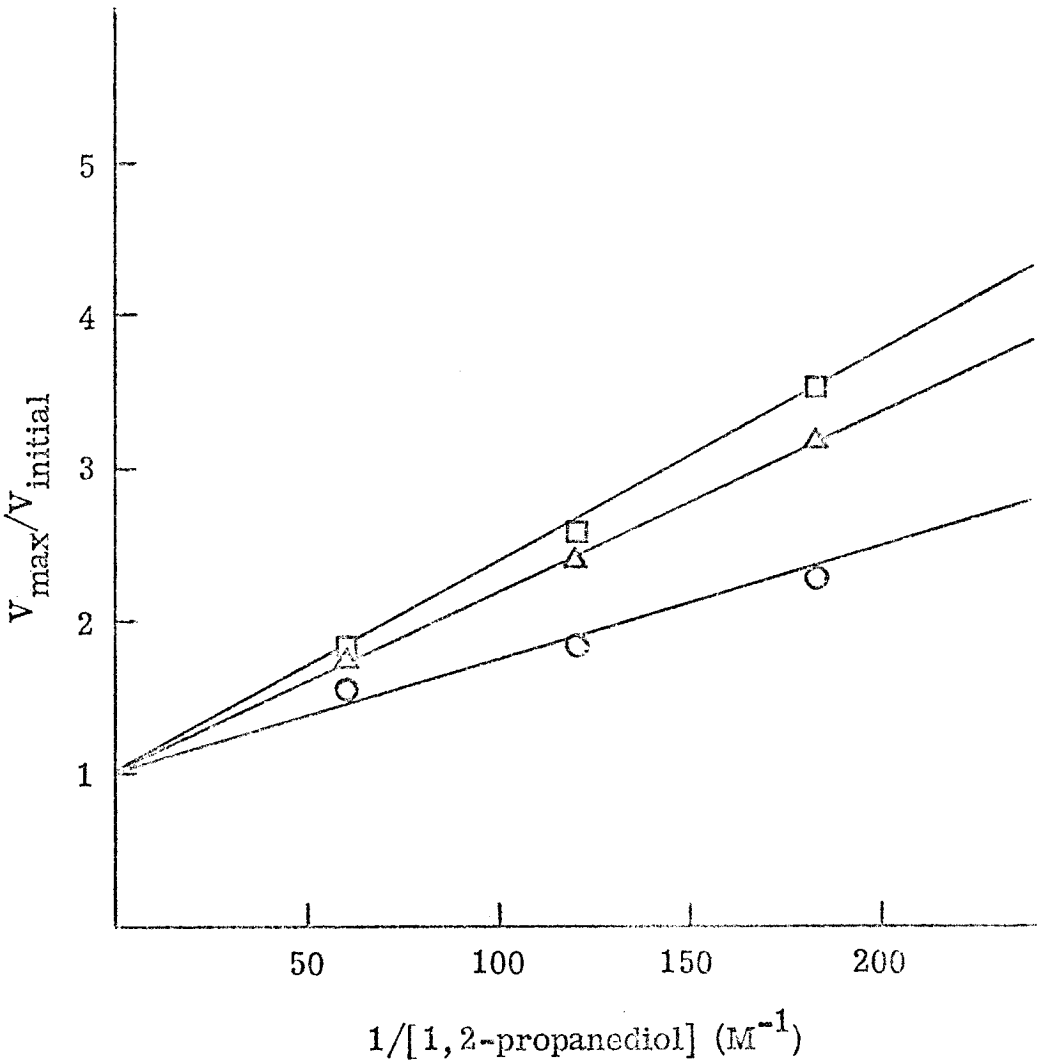


Fig. 4. Lineweaver-Burk plot for 3-iodo-1,2-propanediol in competition with 1,2-propanediol. Reaction mixtures consist of the following: apoenzyme, .05 units, potassium phosphate, pH 8.0, 75 μmoles; coenzyme B_{12}, 40 γ; BSA, .02 mg; alcohol dehydrogenase, 30 units; NADH, .5 mg; varying amounts of 3-iodo-1,2-propanediol and 1,2-propanediol. ○ 9.28×10^{-2} M, △ 1.32×10^{-1} M, and □ 1.65×10^{-1} M in 3-iodo-1,2-propanediol. Velocities were measured immediately after the start of the reaction.

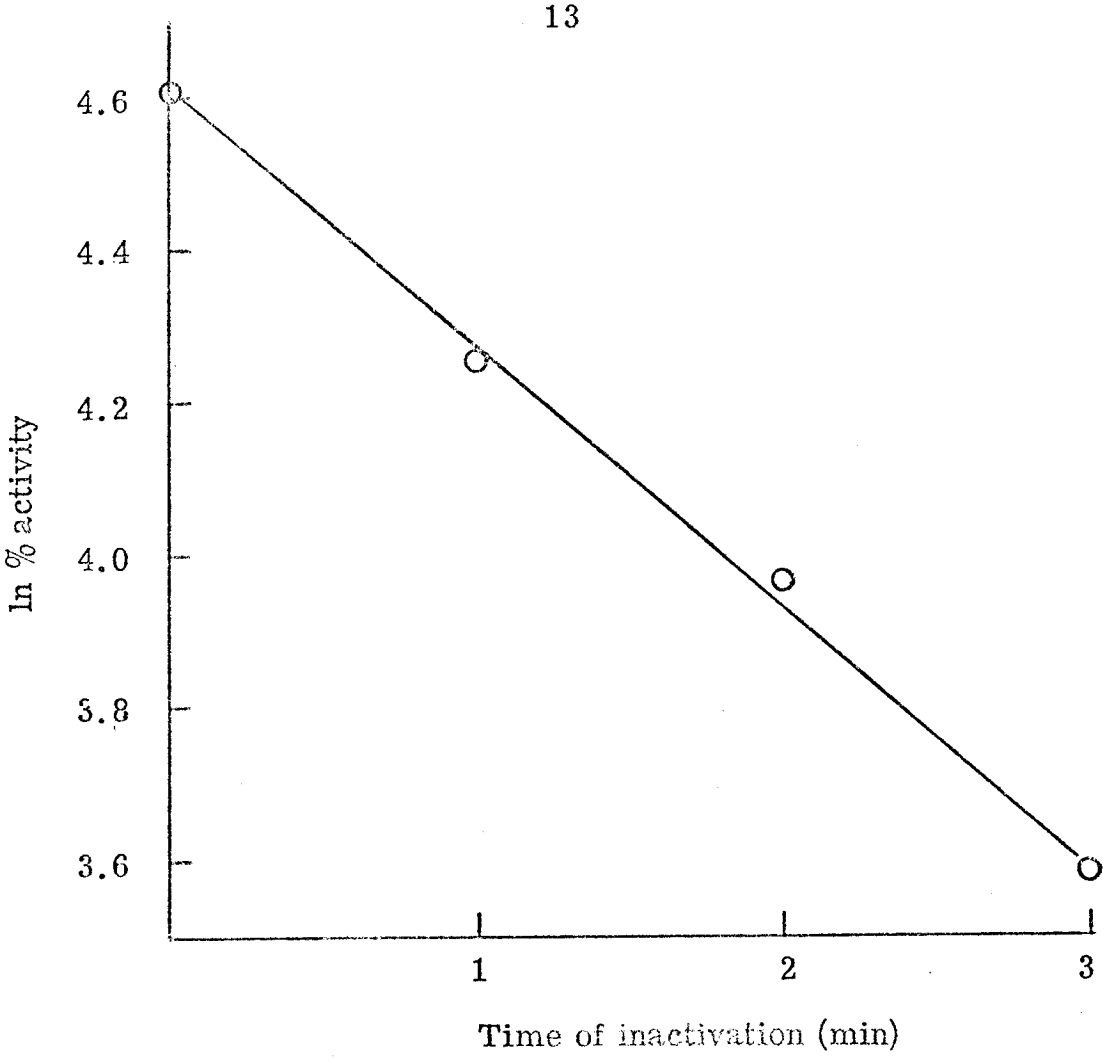


Fig. 5. Rate of inactivation for 3-chloro-1,2-propanediol. Reaction mixtures consist of the following: apoenzyme, .05 units; potassium phosphate, pH 8.0, 75 μmoles; coenzyme B_{12}, 40 γ; BSA, .02 mg; 3-chloro-1,2-propanediol, .5 mmoles; alcohol dehydrogenase, 30 units; NADH, .5 mg. Inactivation was stopped by the addition of saturating amounts of 1,2-propanediol.

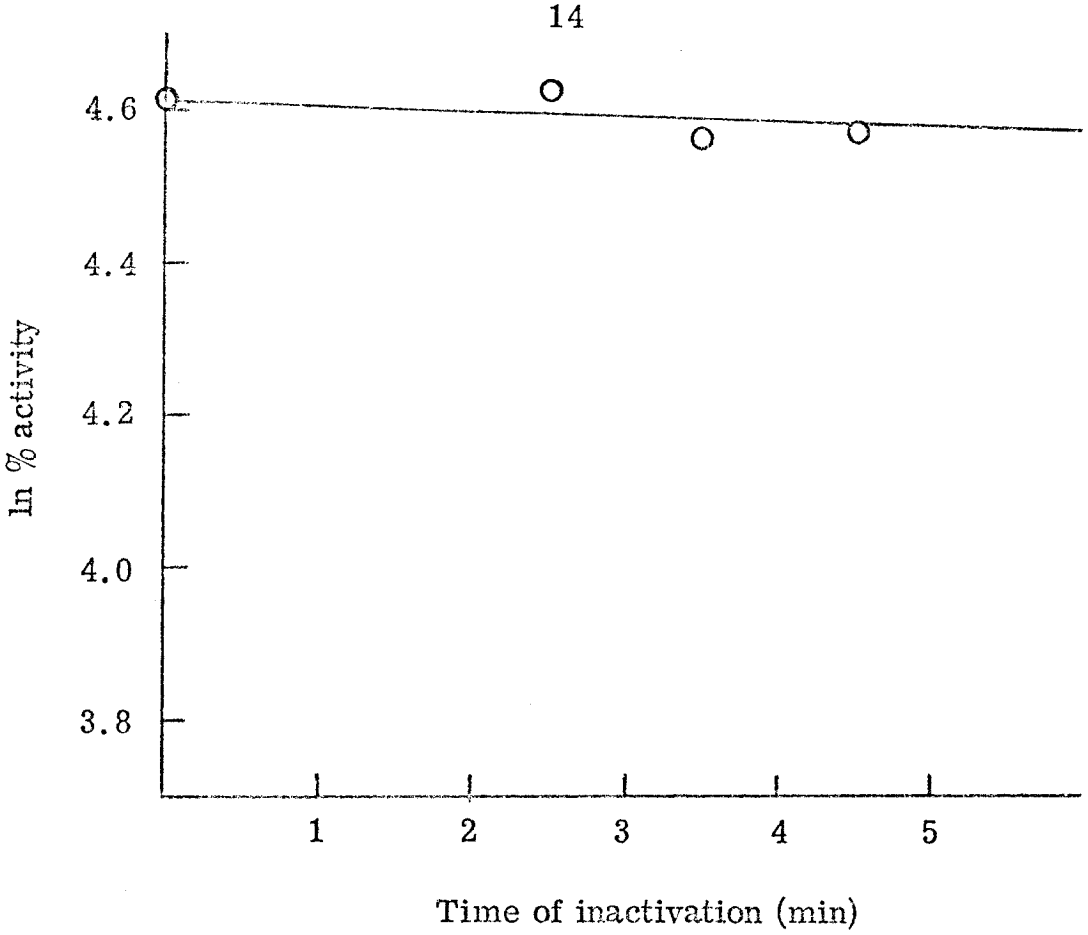


Fig. 6. Rate of inactivation for 3-iodo-1,2-propanediol. Reaction mixtures consist of the following: apoenzyme, .05 units; potassium phosphate, pH 8.0, 75 μmoles; coenzyme B_{12}, 40 γ; BSA, .02 mg; 3-iodo-1,2-propanediol, .5 mmoles; alcohol dehydrogenase, 30 units; NADH, .5 mg. Inactivation was stopped by the addition of saturating amounts of 1,2-propanediol.

TABLE I

Substrate analogue	$K_I \times 10^4$ (M)†	k_{inact} (min^{-1})	k_{cat} (sec^{-1})‡
3-chloro-1,2-propanediol	4.39	.34	10.5
3-bromo-1,2-propanediol	5.35	1.21	0
3-iodo-1,2-propanediol	4.06	0	0

† K_I was determined using 3.0×10^{-5} M as the K_M for 1,2-propanediol.

‡ k_{cat} was determined from the total product formed before complete inactivation of the enzyme, in the absence of substrate, based on a molecular weight of 250,000 daltons and a specific activity of 60 units/mg. (One unit of enzyme is defined as the amount required to produce one micromole of propionaldehyde in one minute at 37°C.)

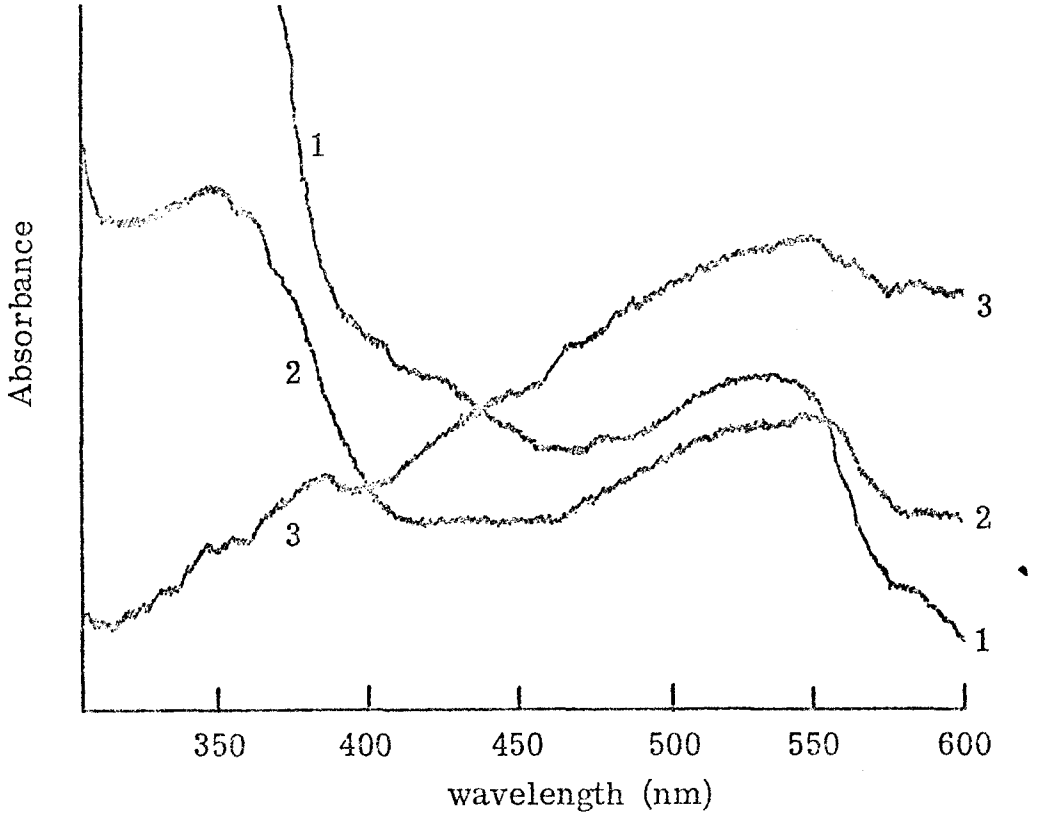


Fig. 7. Difference spectra for enzyme-coenzyme B_{12}-substrate analogue complexes. Reference and sample optical cells consist of the following: apoenzyme, 25 units; potassium phosphate, pH 8.0, 100 μmoles; sample cell also contained coenzyme, 2.8 γ and substrate analogue. 1 3-chloro-1,2-propanediol, 2 3-bromo-1,2-propanediol, and 3 3-iodo-1,2-propanediol.

DISCUSSION

Competition for One Site Followed by Inactivation. Results indicated that the substrate analogues behaved as complete exclusive inhibitors acting on the free enzyme; that is to say, they are competitive inhibitors. A complication developed when it was noticed that the inhibitors also inactivated dioldehydrase. Below are the reaction that take place.

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

$$E + I \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EI \xrightarrow{k_7} E_i \qquad \text{where}$$

E = enzyme, S = substrate, ES = enzyme-substrate complex, P = product, I = inhibitor, EI = enzyme-inhibitor complex, and E_i = inactive form of enzyme.

The rate of the reaction is the formation of product, so

$$v = \frac{d[P]}{dt} = k_3[ES]$$

Applying the usual steady state approximation to [ES] one gets:

$$\frac{d[ES]}{dt} = k_1[E][S] - k_2[ES] - k_3[ES] = 0$$

and where

$$[ES] = \frac{k_1[E][S]}{k_2 + k_3} = \frac{[E][S]}{K_M} \qquad K_M = \frac{k_2 + k_3}{k_1}$$

Whenever possible, the initial rates were measured so that the inactivation rate, k_7, did not enter into the reaction. Then one may apply the equilibrium approximation to [EI]:

$$\frac{d[EI]}{dt} = k_5[E][I] - k_6[EI] = 0$$

and where

$$[EI] = \frac{k_5}{k_6}[E][I] = \frac{[E][I]}{K_I} \qquad K_I = \frac{k_6}{k_5}$$

Since $[E]_t = [E] + [ES] + [EI] = [E]_o$, where $[E]_t$ = concentration of active enzyme,

$$[E] = [E]_t \left\{1 + \frac{[S]}{K_M} + \frac{[I]}{K_I}\right\}^{-1}$$

Substituting into the rate equation, one gets

$$v_{initial} = \frac{k_3}{K_M}[E][S] = \frac{k_3[E]_t}{\frac{K_M}{[S]}\left\{1 + \frac{[S]}{K_M} + \frac{[I]}{K_I}\right\}}$$

$$v_{initial} = \frac{k_3[E]_t}{\frac{K_M}{[S]}\left\{1 + \frac{[I]}{K_I}\right\} + 1} = \frac{v_{max}}{\frac{K_M}{[S]}\left\{1 + \frac{[I]}{K_I}\right\} + 1}$$

Rearranging terms one gets the Lineweaver-Burk form:

$$\frac{1}{v_{initial}} = \frac{K_M}{v_{max}}\left\{1 + \frac{[I]}{K_I}\right\}\frac{1}{[S]} + \frac{1}{v_{max}}$$

Thus the binding constant, K_I, can be determined from the slope of a linear plot of $1/v_{initial}$ as a function of $1/[S]$ for a given

inhibitor concentration.

$$K_I = \frac{[I]}{\frac{\text{slope}}{V_{max} K_M} - 1}$$

In the case of 3-bromo-1,2-propanediol, initial rates could not be measured accurately, so another slightly varied method was used. In this case, the inactivation rate was formally considered as the catalytic rate of the above derivation. Thus 3-bromo-1,2-propanediol is the substrate and 1,2-propanediol is the inhibitor since it inhibits the inactivation reaction. The inactivation of the enzyme is a first order process. This has been confirmed by plotting the logarithm of the percent of residual activity as a function of the period of inactivation. The plot is linear. So $\ln (v/v_{max}) = -kt$;

$$\frac{d \ln(v/v_{max})}{dt} = -k$$

By determining the inactivation constant as a function of inactivator concentrations at constant 1,2-propanediol concentrations, a Lineweaver-Burk plot analogous to the catalysis reaction may be made. The inactivator's binding constant may be determined from the slope of a line.

$$K_I = \frac{\text{slope } K_M}{k_{max}([S] + K_M)}$$

The inactivation rate constant, k_p, for both inactivators reported

are calculated as the y-intercept of a double reciprocal plot of 1/k versus 1/[inactivator].

Coupled Enzyme Systems. It is known that yeast alcohol dehydrogenase converts many aldehydes to alcohols at pH 8 at 37°. Conditions may be adjusted so that there are two sequential pseudo-first order reactions when alcohol dehydrogenase and nicotine adenine dinucleotide (NADH) are added to the dioldehydrase assay mixture. The necessary conditions are when the reduced form of nicotine adenine dinucleotide and coenzyme B_{12} are in excess. Also, by using an excess of alcohol dehydrogenase, the catalytic rate of the dioldehydrase reaction can be made rate limiting. All assays were carried out with at least 100 times excess of alcohol dehydrogenase as well as with saturating amounts of 1,2-propanediol or one of its analogues. The reactions are as follows:

$$S + E_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_1S \xrightarrow{k_3} E_1 + P_1 \qquad \text{(Reaction A)}$$

$$P_1 + E_2 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_2P_1 \xrightarrow{k_7} E_2 + P_2 \qquad \text{(Reaction B)}$$

where S = 1,2-propanediol, E_1 = dioldehydrase, P_1 = propionaldehyde, E_2 = yeast alcohol dehydrogenase, and P_2 = n-propanol.

There is a considerable lag before the steady state has been reached. During this pre-steady state period, the rate of Reaction A is greater than the rate of reaction B. The observed rate, the rate of Reaction B, increases exponentially until both reaction

rates are equal. The time lag is a function of the Michaelis parameters of yeast alcohol dehydrogenase (Gutfreund, 1965).

$$\tau_{\frac{1}{2}} = \frac{K_M}{v_{max}} \ln 2$$

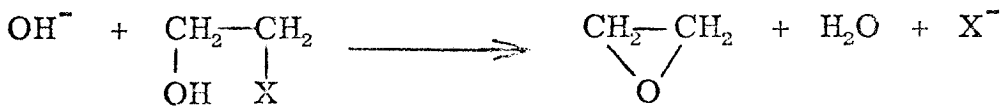
K_M for propionaldehyde is 2.5×10^{-3} M (Bruemmer and Roe, 1971). Typically .05 units of dioldehydrase was used. Therefore, v_{max} corresponds to .05 μM/min. These values give a $\tau_{\frac{1}{2}}$ of about two seconds. Therefore, all measurements were taken after at least thirty seconds (fourteen half lifes) to ensure that the rate observed is equal to the rate of the dioldehydrase reaction.

The possibility that yeast alcohol dehydrogenase may be inactivated was checked. Alcohol dehydrogenase and the inactivator were in sufficient excess so that their concentrations did not change significantly. Therefore, inactivation of alcohol dehydrogenase would not interfer with the measurements of the inactivation of dioldehydrase.

Reaction Mechanism. Unfortunately, no simple trend could be found relating either the dissociation constant or the inactivation constant to the nature of the halogen atom. Inactivation rate constants increase from the fluoro-substituted to the chloro-substituted to the bromo-substituted 1,2-propanediol, but decreases to approximately zero for the iodo-substituted compound. There is essentially no difference in dissociation constants for these substrate analogues.

The fact that the choice of halogen has no effect on binding, whereas it does have an effect on inactivation, may suggest that binding occurs in a normal manner; then a conformational change occurs within the bound substrate analogue. Thus, although 3-iodo-1,2-propanediol binds to dioldehydrase, the iodine atom may sterically prevent the conformational change required for inactivation.

It is known that β-halogenated ethanols form epoxides under basic conditions to produce ethylene oxide.



The possibility that γ-halogenated diols may undergo similar epoxidations to form 2,3-epoxypropanol may be of value in explaining the inactivation of dioldehydrase. Epoxides may react with alcohols, thiols, and amines in the active site of the enzyme. Barron et al. (1952) have observed the alkylation of sulfhydryl groups of yeast alcohol dehydrogenase.

One possibility is that the pH 8 buffer in which reactions are done is sufficiently basic for the formation of 2,3-epoxypropanol in solution. This epoxide may add to a reactive group in the binding site of dioldehydrase (Reaction A, Fig. 8). Rate constants for the formation of ethylene oxide from β-halogenated ethanols have been determined. At 0°C, the rates of epoxidation are rather fast for 2-bromoethanol (.987 min^{-1}) and 2-iodoethanol (.902 min^{-1}) compared to 2-chloroethanol (.0168 min^{-1}) (McCabe

and Warner, 1948). This ordering of the rates of epoxidation is in disagreement with the ordering of the rates of inactivation observed for dioldehydrase. In addition, if epoxide formation occurs as a result of the basic conditions, one would expect some reaction to occur between dioldehydrase and the resulting 2,3-epoxypropanol in the absence of coenzyme B_{12}. Results indicate that inactivation does not occur in the absence of coenzyme B_{12}. Thus, it appears clear that Reaction A is not a plausible mechanism for inactivation. Hydrolysis of 2,3-epoxypropanol produces glycerol, an inactivator of dioldehydrase (Toraya et al., 1976). However, since the rate of hydrolysis of ethylene oxide to ethylene glycol is extremely slow (McCabe and Warner, 1948), glycerol is not the inactivating species in this case.

Since 2,3-epoxypropanol is not the inactivator and glycerol is not formed significantly in the reaction, it appears that the halogenated propanediols, themselves, are responsible for inactivation. Haloacetates react with amino acid side chains including sulfhydryl groups. Similarly, the haloglycols may react to form alkylated enzyme (Reaction B, Fig. 8). If the 3-halo-1,2-propanediols bind in two orientations, pro-R and pro-S, in one orientation, the halogen atom may be near a sulfhydryl group in the binding site so that an S_N2 reaction may take place. The resulting alkylated enzyme would be inactive. Since bromine is a better leaving group than chlorine, the relative rates of inactivation by 3-bromo-1,2-propanediol and 3-chloro-1,2-propanediol agree

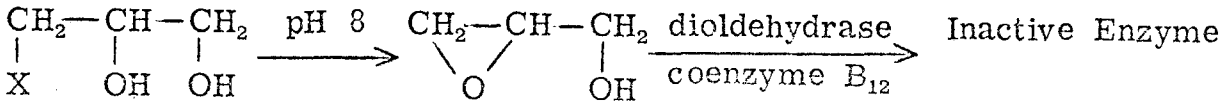
with this mechanism. Although iodine is a better leaving group than bromine, perhaps iodine's size prevents it from binding in the inactivation orientation. Since the inactivation is coenzyme B_{12} dependent, perhaps coenzyme B_{12} induces a conformational change in the enzyme that allows proper alignment of the sulfhydryl group and the halogen atom of the inactivator. Since haloacetates are somewhat specific for sulfhydryl groups at pH 8, perhaps there are no exposed sulfhydryl groups before coenzyme B_{12} binds to dioldehydrase.

In Reaction B, binding takes place followed by nucleophilic substitution. Coenzyme B_{12} is required to induce a conformational change in the enzyme thus exposing essential sulfhydryl groups. On the other hand, hydrogen abstraction may occur as fast as the substitution. This raises the possibility of a third mechanism of inactivation. Depending upon the orientation of the bound substrate analogue, hydrogen abstraction may occur at different positions. For the substrate bound in the product forming orientation, abstraction occurs at the C_1 position. For substrate analogues bound in the other orientation, abstraction may occur at the C_2 position (Fig. 9).

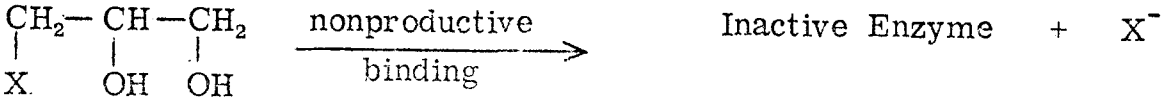
Depending upon the choice of halogen atoms, different reactions may occur following hydrogen abstraction. These reactions are shown in Figure 10. Chlorine will rearrange as well as eliminate from alkyl radicals. If the rate of the dehydration process is comparable to the rates of chlorine migration and chlorine elimination, then product is sometimes formed from one of the

binding orientations. Otherwise, inactivation occurs by the reaction of the resulting alkene with the sulfhydryl group. On the other hand, bromine and iodine are eliminated from alkyl radicals faster than they rearrange. Therefore, no product is observed for these two substrate analogues. One would expect both bromine and iodine to be potent inactivators of dioldehydrase. However, the non-inactivating character of 3-iodo-1,2-propanediol remains unexplained.

The next step in studying the inactivation process is to determine if the inactivating agent is specific for one amino acid. Using labelled substrate analogues, the enzyme is inactivated. After hydrolysis, chromatography and labelling analysis should help in determining which amino acid residues may be near the active site.

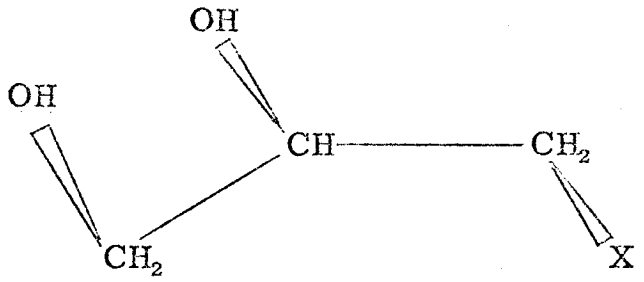


Reaction A (Epoxidation)

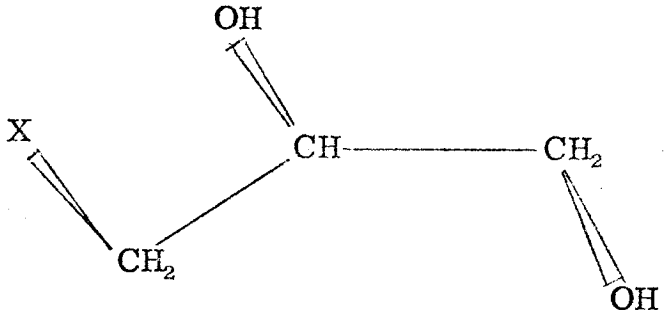


Reaction B (S_N2)

Fig. 8. Mechanisms of inactivation by 3-halo-1,2-propanediols.



Product forming orientation



Inactivating orientation

Fig. 9. Possible binding orientations of substrate analogues.

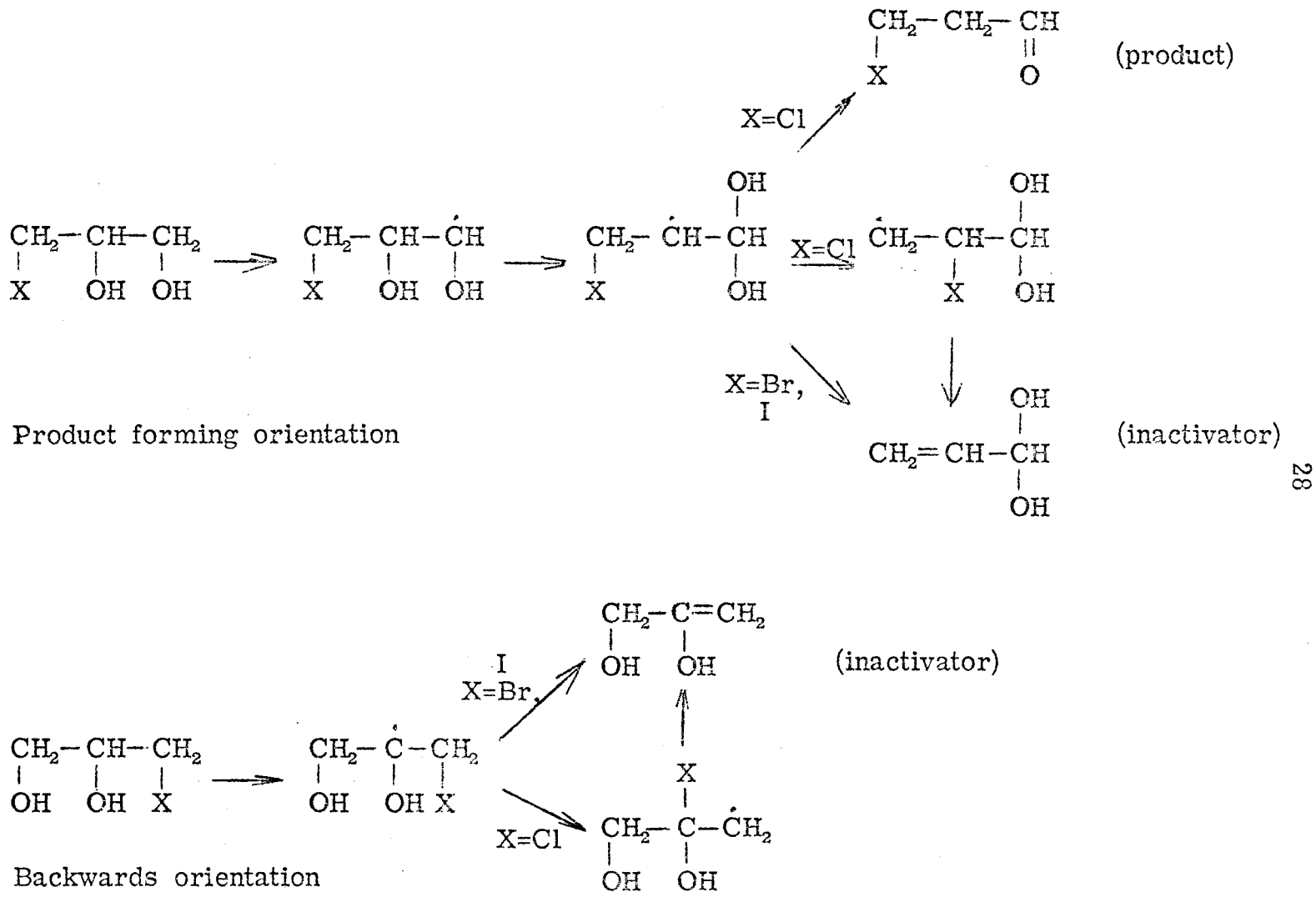


Fig. 10. Mechanisms for inactivation by 3-halo-1,2-propanediols.

PART II

Studies on the Effect of an Epoxide on Dioldehydrase

INTRODUCTION

A number of similar compounds have been found to bind to dioldehydrase. Among these compounds are 1,2-propanediol and ethylene glycol (Lee and Abeles, 1963), 3-fluoro-1,2-propanediol (Eagar et al., 1975), glycerol, 1,2-butanediol, and 2,3-butanediol (Toraya et al., 1976), and 3-chloro-1,2-propanediol, 3-bromo-1,2-propanediol, and 3-iodo-1,2-propanediol. All of these compounds have at least two adjacent hydroxyl groups; that is to say, they are glycols. The effect of the substitution of electron withdrawing groups on the methyl group of 1,2-propanediol was to increase their dissociation constants. However, some of these substrate analogues also inactivate dioldehydrase.

Two different mechanisms for inactivation have been presented in Part I. Alkylation by the solvent dependent epoxide has been ruled out. In this study, 2,3-epoxypropanol, an epoxide similar to 1,2-propanediol, is examined, first, to test its binding capacity for dioldehydrase, and second, to examine its inactivating properties.

EXPERIMENTAL SECTION

Dioldehydrase was obtained as previously mentioned in Part I. Coenzyme B_{12}, nicotine adenine dinucleotide, and yeast alcohol dehydrogenase were purchased from Sigma Chemical Company.

2,3-Epoxypropanol. 2,3-Epoxypropanol (glycidol) was prepared by the treatment of 3-chloro-1,2-propanediol with concentrated sodium hydroxide at 15°C. The product was distilled at 60°C under vacuum (Jones, 1973).

Assays. The coupled assay method described in Part I was used to determine the binding constant of 2,3-epoxypropanol. This assay had to be abandonned during inactivation studies since the compound reacts to inactivate alcohol dehydrogenase. A modification of the 2,4-dinitrophenylhydrazone assay of Lee and Abeles (1963) was utilized. In general, the reaction was allowed to proceed for a desired time and stopped by the addition of .1 ml of 2N hydrochloric acid. Thirty minutes after the addition of .1 ml of DNP solution (100 mg 2,4-dinitrophenylhydrazine, .4 ml concentrated hydrochloric acid diluted to 25 ml in carbonyl-free methanol), .5 ml pyridine and .1 ml potassium hydroxide solution (5 g potassium hydroxide in 5 ml water diluted to 25 ml in carbonyl-free methanol) were added. The tubes were centrifuged. Twelve minutes after the last addition, the absorbances were read at 475 nm. Assay solutions were made similar to those for the

coupled enzyme assay except alcohol dehydrogenase and nicotine adenine dinucleotide were not added.

RESULTS

Nature of the Reaction. 2,3-Epoxypropanol reacts with dioldehydrase in the presence of coenzyme B_{12} to form product. This rate of catalysis decays exponentially with time. However, the rate constant for product formation could not be measured accurately by our present technique. The product has tentatively been identified as β-hydroxypropionaldehyde by thin layer chromatography of its 2,4-dinitrophenylhydrazone derivative on silica gel with chloroform. (Fig. 1)

2,3-Epoxypropanol exhibits inhibitory properties. Its calculated dissociation constant is 8.8×10^{-4} M. (Fig. 2). Thus, 2,3-epoxypropanol is one compound that binds to dioldehydrase, yet it is not a glycol.

In addition to being a competitve inhibitor, 2,3-epoxypropanol inactivates dioldehydrase. Inactivation occurs in the presence of coenzme B_{12} as well as in the absence of coenzyme B_{12}. The rate of inactivation of the holoenzyme is ten times faster than the rate of inactivation of the apoenzyme (Fig. 3,4). Table II summarizes the values determined for the inactivation rate constants for several inactivators of the holoenzyme and apoenzyme.

Spectral Observation of the Intermediate. The visible spectum was observed for a mixture of dioldehydrase,

coenzyme B_{12}, and 2,3-epoxypropanol as in Part I. The resulting spectrum is very similar to those reported in Part I. (Fig. 5).

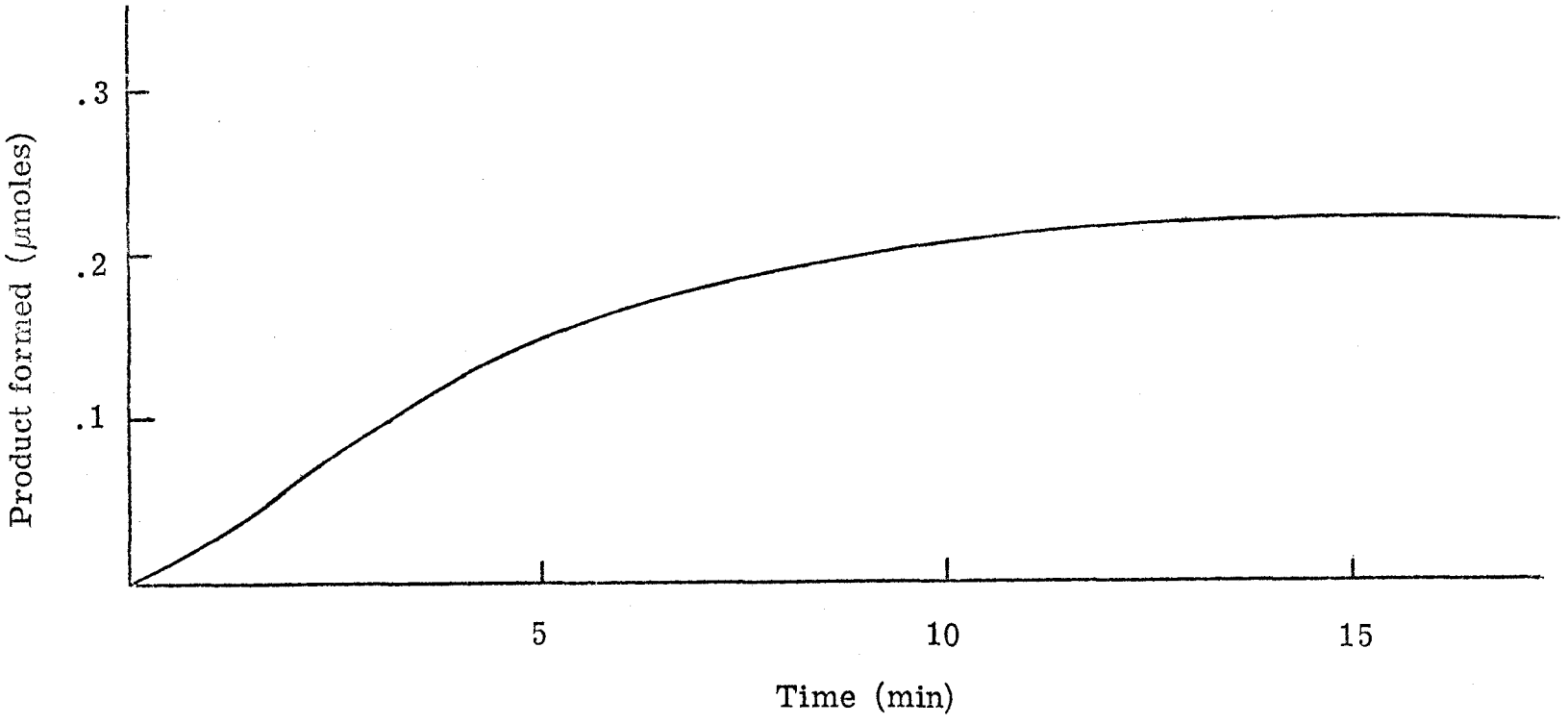


Fig. 1. Rate of product formation of 2,3-epoxypropanol. Reaction mixture contains the following: apoenzyme, 3 units; potassium phosphate, pH 8.0, 75 μmoles; coenzyme B_{12}, 40 γ; BSA, .02 mg; yeast alcohol dehydrogenase, 30 units; NADH, .5 mg; 2,3-epoxypropanol, 1 mmoles.

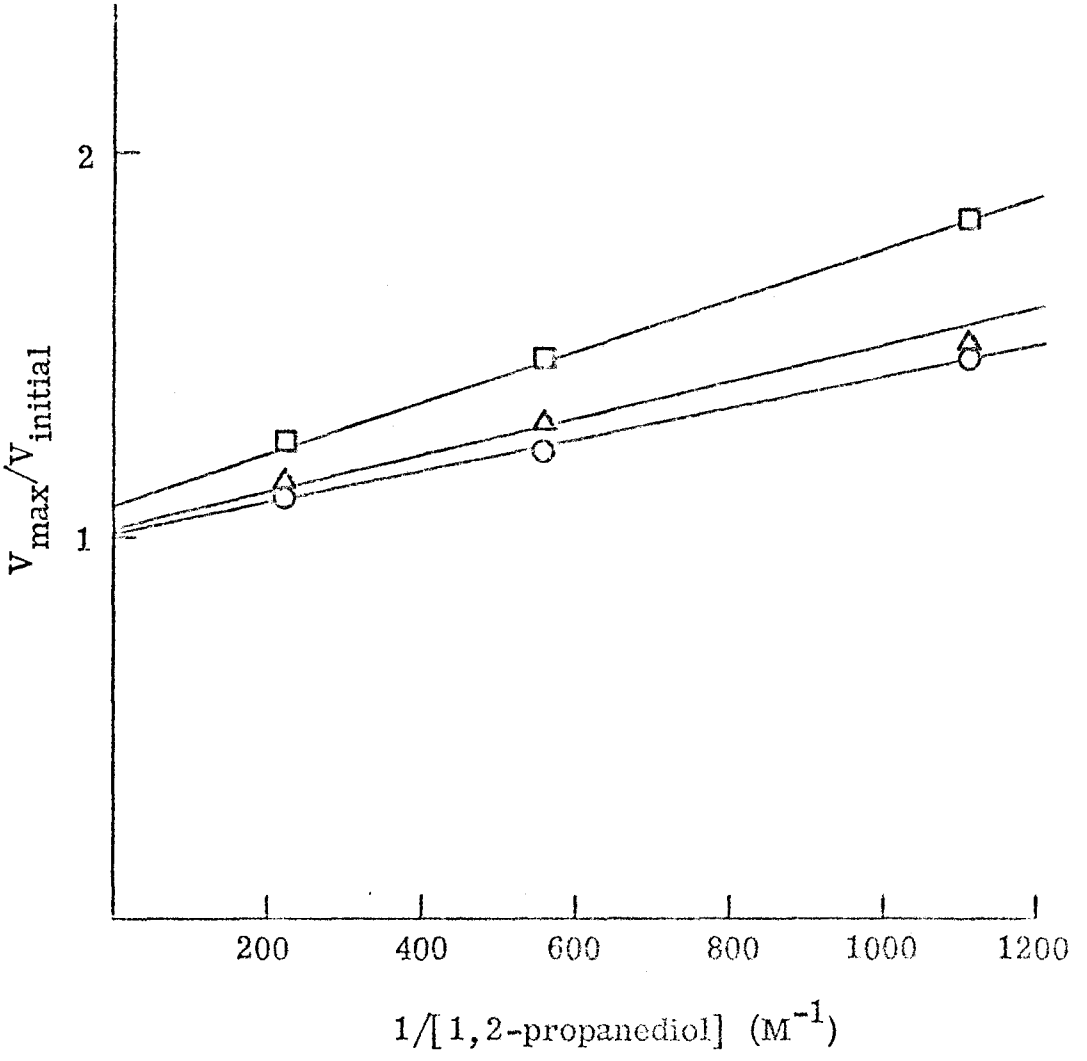


Fig. 2. Lineweaver-Burk plot for 2,3-epoxypropanol in competion with 1,2-propanediol. Reaction mixtures consist of the following: apoenzyme, .05 units; potassium phosphate, pH 8.0, 75 μmoles; coenzyme B_{12}, 40 γ; BSA, .02 mg; alcohol dehydrogenase, 30 units; NADH, .05 mg; varying amounts of 2,3-epoxypropanol and 1,2-propanediol. ○ 1.07×10^{-2} M, △ 1.43×10^{-2} M, and □ 2.14×10^{-2} M in 2,3-epoxypropanol. The velocity is measured immediately after the start of the reaction.

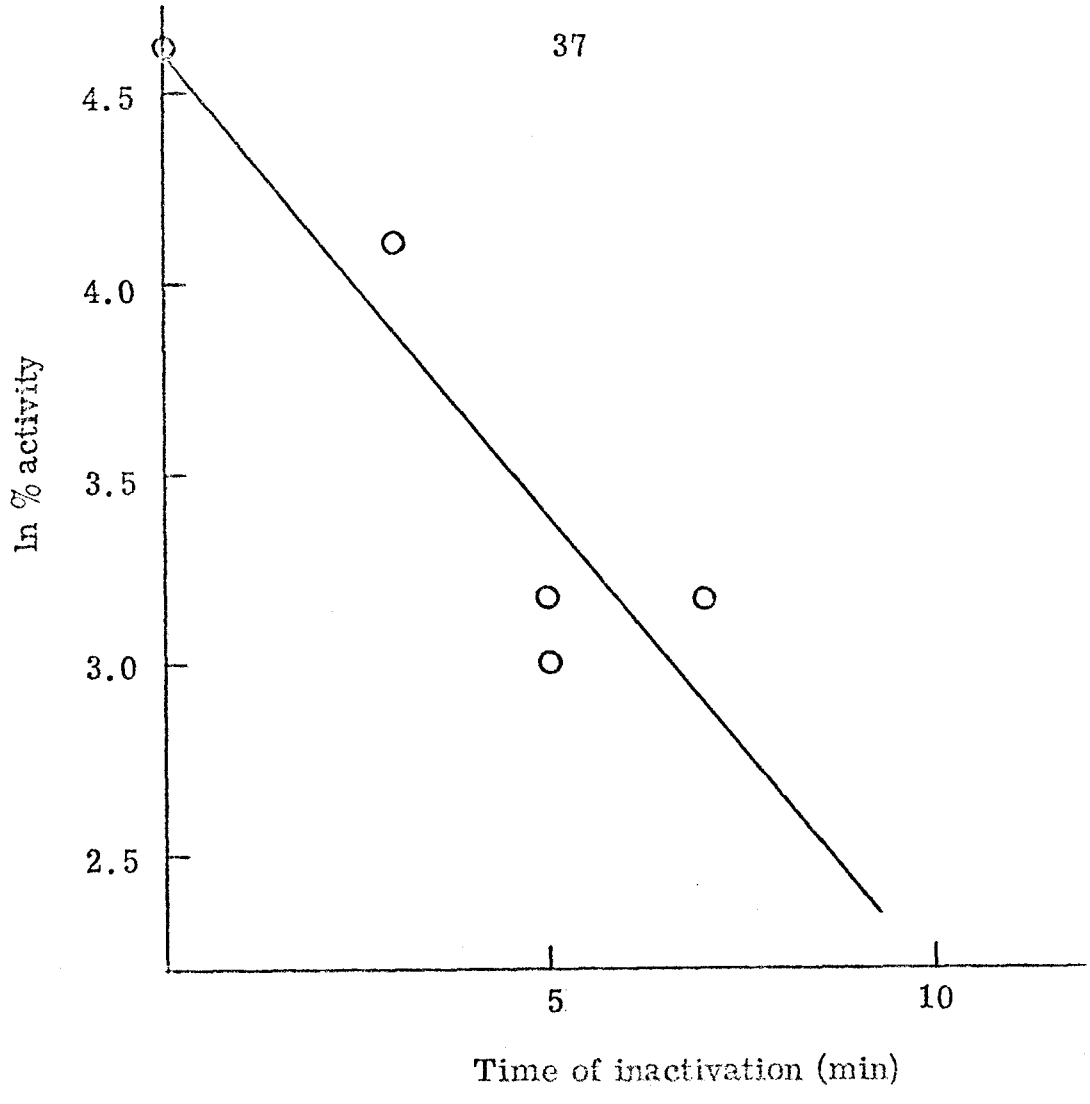


Fig. 3. Rate of inactivation for 2,3-epoxypropanol in the presence of coenzyme B_{12}. Reaction mixtures consist of the following: apoenzyme, .05 units; potassium phosphate, pH 8.0, 60 μmoles; coenzyme B_{12}, 40 γ; 2,3-epoxypropanol, .5 mmoles; BSA, .02 mg. Inactivation was stopped by the addition of saturating amounts of 1,2-propanediol. Rates were determined by measuring the amount of propionaldehyde formed at various times following various periods of inactivation using the DNP assay.

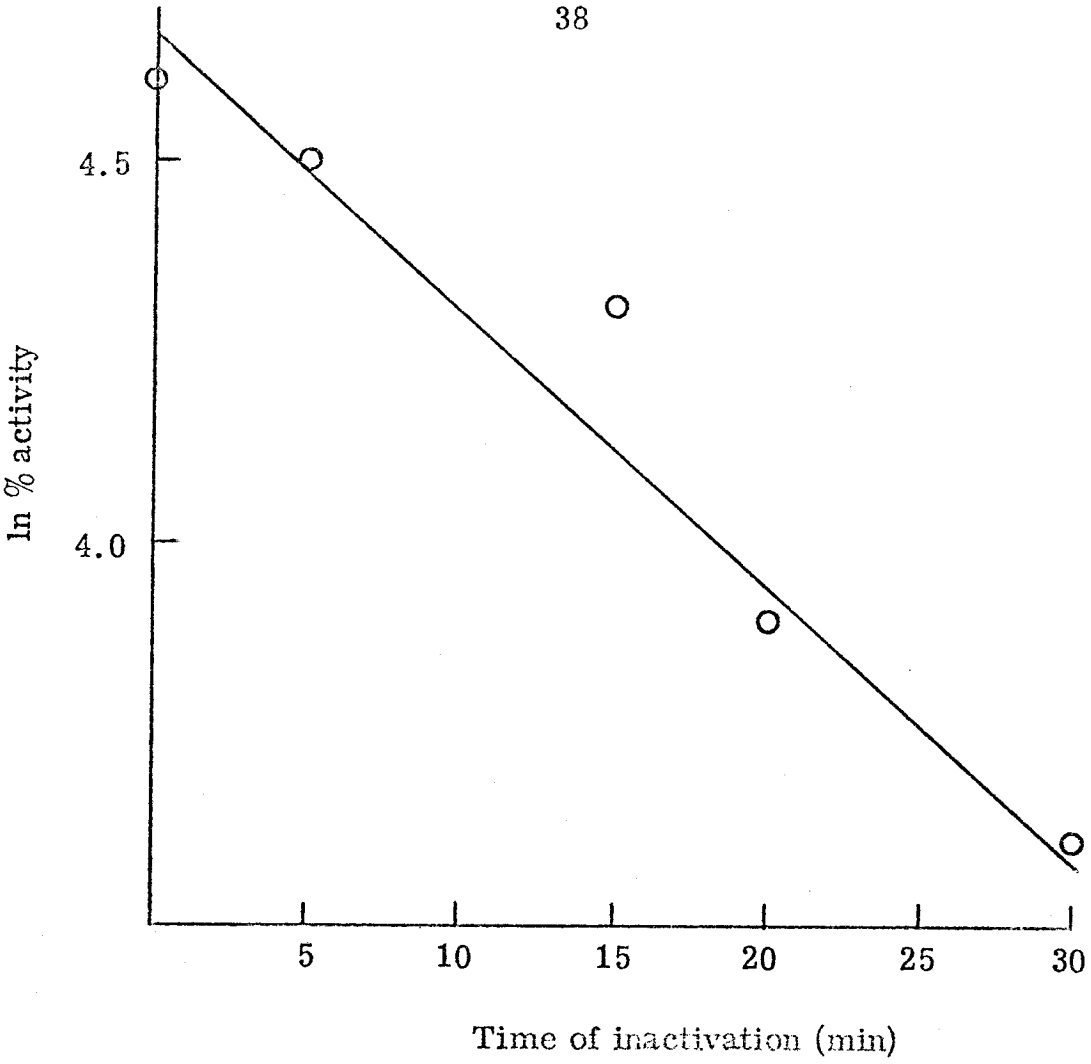


Fig. 4. Rate of inactivation for 2,3-epoxypropanol in the absence of coenzyme B_{12}. Reaction mixtures consist of the following: apoenzyme, .05 units; potassium phosphate, pH 8.0, 60 μmoles; 2,3-epoxypropanol, .5 mmoles; BSA, .02 mg. Inactivation was stopped by the addition of saturating amounts of 1,2-propanediol and 40 γ of coenzyme B_{12}. Rates were measured by measuring the amount of propionaldehyde formed at various times after various periods of inactivation using the DNP assay.

TABLE II

Substrate analogue	k_{inact} (holoenzyme)	k_{inact} (apoenzyme)
3-chloro-1,2-propanediol	0.34	0
3-bromo-1,2-propanediol	1.21	0
3-iodo-1,2-propanediol	0	-
2,3-epoxypropanol	0.24	0.034

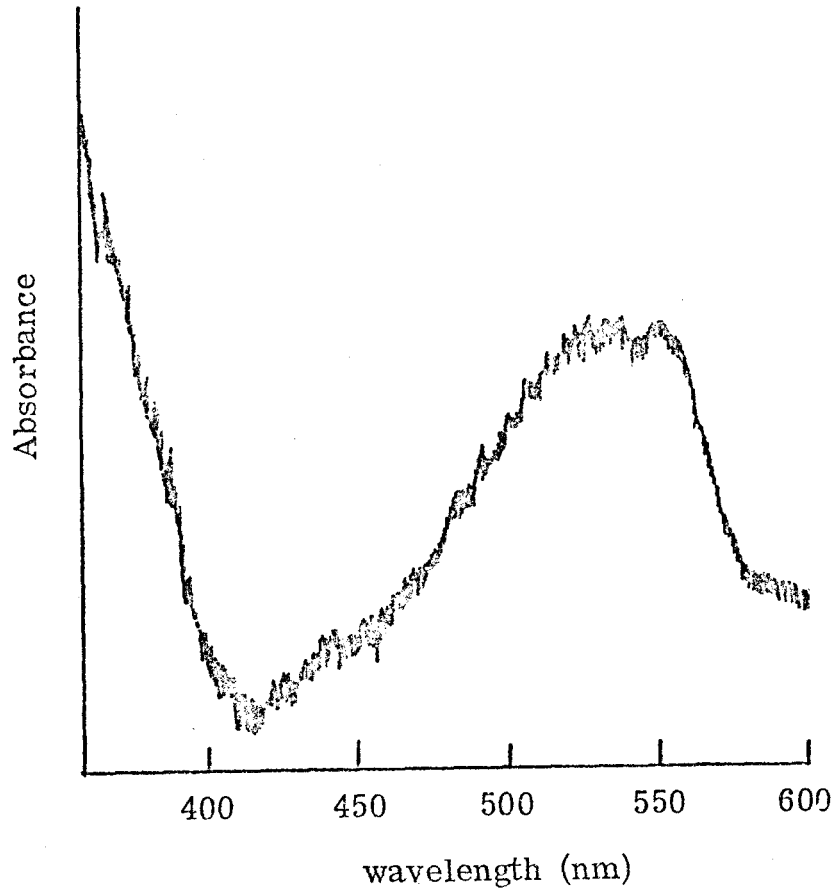


Fig. 5. Difference spectrum for enzyme-coenzyme B_{12}-2,3-epoxypropanol complex. Reference and sample optical cells consist of the following: apoenzyme, 30 units; potassium phosphate, pH 8.0, 100 μmoles; sample cell also contained coenzyme B_{12}, 2.8 γ and 2,3-epoxypropanol, .5 mmoles.

DISCUSSION

Binding. The fact that 2,3-epoxypropanol binds to dioldehydrase may not be too surprising. Many glycols bind to the enzyme. This might suggest the importance of the hydroxyl group for binding. Although the hydroxyl groups may be important for binding, the fact that 2,3-epoxypropanol binds may suggest that hydrogen bonding involves the hydroxyl oxygen atoms. Close examination of 1,2-propanediol and 2,3-epoxypropanol results in the following observation. When the C_1 and C_2 atoms and the two oxygen atoms are coplanar, the various interatomic distances and bond angles are approximately equal for both compounds. Therefore, the enzyme sees very little difference between the two molecules and binds both compounds.

Even though both compounds ressemble each other, the dissociation constant for the epoxide is approximately twenty times greater than the glycol's. The fact that the oxide ring may be mistaken as a hydroxyl group suggests the possibility of binding in two different orientations. In one orientation, catalysis may occur. In the other orientation, inactivation may occur.

Inactivation. As stated in Part I, ethylene oxide is a sulfhydryl reagent. The fact that 2,3-epoxypropanol inactivates dioldehydrase would lead one to suspect that it also alkylates sulfhydryl groups. Since it inactivates the apoenzyme as well as the holoenzyme, unlike the inactivating compounds studied in Part

I, it appears that 2,3-epoxypropanol inactivates the enzyme via a different mechanism from that of 3-bromo-1,2-propanediol and 3-chloro-1,2-propanediol.

The fact that inactivation occurs much more slowly in the absence of coenzyme B_{12} than in the presence of coenzyme B_{12} suggests that 2,3-epoxypropanol may alkylate exposed reactive residues as well as those residues in the binding site. To test this possibility one might use two inactivators, one in the absence of the other, to check the specificity of the above compounds. In one set of experiments, the enzyme is completely inactivated by unlabelled 3-bromo-1,2-propanediol. After dialysis, the enzyme is allowed to react with labelled 2,3-epoxypropanol. In an opposing set of experiments, the enzyme is allowed to react with unlabelled 2,3-epoxypropanol followed by labelled 3-bromo-1,2-propanediol. The relative amount of labelling retained on the enzyme would give some indication as to the degree of specificity of the epoxide versus the brominated 1,2-propanediol.

The possibility that the epoxide ring may react with coenzyme B_{12} has been ruled out. Inactivated enzyme was treated with magnesium acetate and potassium sulfite. This treatment enables the coenzyme moeity to be dialyzable (Schneider et al., 1970). Subsequent addition of coenzyme B_{12} yielded no reactivation. It seems unlikely that coenzyme B_{12} would be transformed to the dihydroxypropylcobalamin. This transformation would occur if coenzyme B_{12_S} was present, yet no evidence exists for the

presence of coenzyme B_{12_S} in the enzymatic reaction. Therefore, either coenzyme B_{12} is covalently bound to the enzyme or the enzyme, itself, is modified by 2,3-epoxypropanol.

CONCLUSIONS

A number of competitive inhibitors of dioldehydrase have now been found to inactivate the enzyme as well. One class of inactivators includes 3-chloro-1,2-propanediol and 3-bromo-1,2-propanediol. These compounds inactivate the holoenzyme. Coenzyme B_{12} is required for inactivation. Another class of inactivators includes 2,3-epoxypropanol. The epoxide inactivates the apoenzyme as well as the holoenzyme.

It has been postulated that the first class of inactivators, the halogenated diols, react with sulfhydryl groups in the active site either by an S_N2 reaction or a radical mechanism. Both mechanisms assume that the substrate analogue may bind in an orientation different from the orientation required for catalysis.

Recent studies with glycerol suggest that the orientation of the molecule in the binding site determines whether catalysis occurs or inactivation occurs. Kinetic studies using various deuterium labelled glycerols suggest that catalysis takes place when glycerol binds in the pro-R configuration while inactivation takes place when it binds in the pro-S configuration (Bachovchin et al., manuscript in preparation).

Other substrates for dioldehydrase inactivate the enzyme. These substrates include ethylene glycol (Lee and Abeles, 1963) and 2,3-butanediol (Toraya et al., 1976). These compounds inactivate dioldehydrase at much slower rates than the other

inactivators. Perhaps these compounds bind in nonproductive orientations. Perhaps their slow inactivation rates may be explained by their lack of good leaving groups required for the alkylation process.

It has been shown that binding constants and rate constants depend upon the stereoisomer used (Eagar et al., 1975). In the studies of the 3-halo-1,2-propanediols, racemic mixtures were used. In these cases, each stereoisomer is in competition with the other. Kinetic studies of the optically active substrate analogues may be helpful in determining true inactivation rate constants. These values may help in confirming the backwards binding required for inactivation.

2,3-Epoxypropanol reacts with both the apoenzyme and the holoenzyme. However, their inactivation rates are different. Therefore, some reactive groups are more accessible after co coenzyme B_{12} binds to dioldehydrase. It appears clear that at least two different areas of the holoenzyme are inactivated- the binding site and the exposed groups. It would be interesting to examine the rates of inactivation of ethylene oxide on the apoenzyme and the holoenzyme and compare these rates to those obtained for 2,3-epoxypropanol.

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