Kabasawa, I., and Hirs, C. H. W. (1972), *J. Biol. Chem. 247,* 1610.
Kimmel, M. T., and Plummer, T. H., Jr. (1972), *Anal. Biochem. 49,* 267.
Kornfeld, R., Keller, J., Baenziger, J., and Kornfeld, S. (1971), *J. Biol. Chem. 246,* 3259.
Kuhn, R., and Trischman, H. (1963), *Chem. Ber. 96,* 289.
Lee, Y. C., and Socca, J. R. (1972), *J. Biol. Chem. 247,* 5753.
Li, Y.-T., and Li, S.-C. (1968), *J. Biol. Chem. 243,* 3994.
Ng Ying Kin, N. M. K., and Wolfe, L. S. (1974), *Biochem. Biophys. Res. Commun. 59,* 837.
Nishigawa, M., Muramatsu, T., and Kobata, A. (1974), *Biochem. Biophys. Res. Commun. 59,* 638.
Nordén, N. E., Lundblad, A., Svensson, S., and Autio, S. (1974), *Biochemistry 13,* 871.
Phillips, N. C., Robinson, D., Winchester, B. G., and Jolly, R. D. (1974), *Biochem. J. 137,* 363.
Putnam, F. W., Florent, G., Paul, C., Shinoda, T., and Shimizu, A. (1973), *Science 182,* 287.
Reissig, J. L., Strominger, J. L., and Leloir, L. F. (1955), *J. Biol. Chem. 217,* 959.
Shimizu, A., Putnam, F. W., Paul, C., Clamp, J. R., and Johnson, I. (1971), *Nature (London), New Biol. 231,* 73.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Spik, G., Bayard, B., Fournet, B., Strecker, G., Bouquelet, S., and Montreuil, J. (1975), *FEBS Lett. 50,* 296.
Sugahara, K., Okumura, T., and Yamashina, I. (1972), *Biochim. Biophys. Acta 268,* 488.
Sukeno, T., Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1971), *Biochem. Biophys. Res. Commun. 45,* 219.
Sukeno, T., Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1972), *Biochemistry 11,* 1493.
Tarentino, A. L., and Maley, F. (1971), *Arch. Biochem. Biophys. 147,* 446.
Tarentino, A. L., and Maley, F. (1974), *J. Biol. Chem. 249,* 811.
Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1970), *J. Biol. Chem. 245,* 4150.
Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1972), *J. Biol. Chem. 247,* 2629.
Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1973), *J. Biol. Chem. 248,* 5547.
Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1974), *J. Biol. Chem. 249,* 818.
Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 591.
Toyoshima, S., Fukuda, M., and Osawa, T. (1972), *Biochemistry 11,* 4000.
Tsay, G. C., and Dawson, G. (1975), *Biochem. Biophys. Res. Commun. 63,* 807.
Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., and Weigele, M. (1972), *Science 178,* 871.
Walborg, E. F., Jr., Ray, D. B., and Öhrberg, L. E. (1969), *Anal. Biochem. 29,* 433.
Wolfe, L. S., Senior, R. G., and Ng Ying Kin, N. M. K. (1974), *J. Biol. Chem. 249,* 1828.

Mechanism of Action of Adenosylcobalamin: 3-Fluoro-1,2-propanediol as Substrate for Propanediol Dehydrase—Mechanistic Implications†

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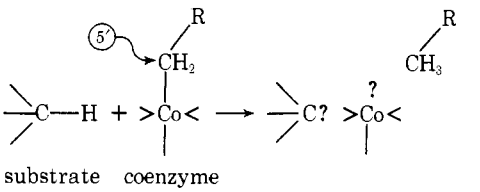
ABSTRACT: 3-Fluoro-1,2-propanediol has been found to be a substrate for propanediol dehydrase and has very similar binding and catalytic constants compared to the natural substrate. The only isolable products of the reaction are acrolein and inorganic fluoride; with 3-fluoro-3,3-dideuterio-1,2-propanediol 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.

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).



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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 rapdily 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 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 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 Determinations. 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 serum albumin, 0.02 mg. Total volume, 2 ml, 37°.

K_M Determinations. 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 had $[\alpha]^{25}D$ −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. 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]

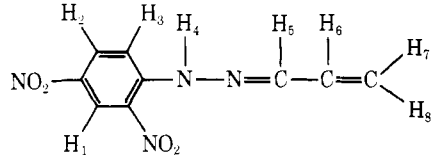
(RS)-3-Fluoro-1,2-propanediol. (*RS*)-3-Fluoro-1,2-propanediol was prepared by acid hydrolysis of epifluorohydrin (Aldrich Chemical Company) (Pattison and Norman, 1957).

(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 $[\alpha]D$ 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 potassium 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

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



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).

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% ^2H 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 ^1H 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 ^1H 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 ^1H NMR.

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

Table I

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).

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 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 an 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 I 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 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 acrole-

Table II

	ΔG (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.

in 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 5×10^{-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 I.

1,2-Propanediol. The modified Dnp assay for propionaldehyde alone allowed meaningful initial velocities to be obtained at initial concentration only as low as 1×10^{-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 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

$$V_{(RS)} = k_{cat}[[E_0]/(1 + \bar{K}_S[S] + \bar{K}_R[R])][\bar{K}_S[S] + \bar{K}_R[R]] = [[E_0]/(1 + \bar{K}_S[S] + \bar{K}_R[R][k_3\bar{K}_S[S] + k_6\bar{K}_R[R]]$$

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

$$V_{(RS)} = k_{cat}[[E_0][RS]/(2 + \bar{K}_S[RS] + \bar{K}_R[RS]][\bar{K}_S + \bar{K}_R] = [[E_0][RS]/(2 + \bar{K}_S[RS] + \bar{K}_R[RS])[k_3\bar{K}_S + k_6\bar{K}_R]$$

whence

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

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

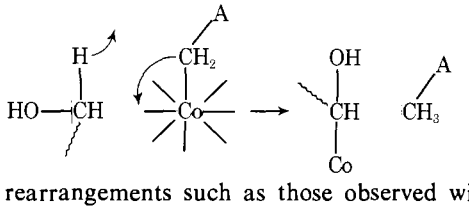
Reaction Mechanism. The observation of a kinetic isotope effect of about 13 observed when deuterium is substituted for hydrogen at C-1 of 3-fluoro-1,2-propanediol indicates that hydrogen removal from C-1 is one of the major rate-determining steps in the enzymatic reaction of the fluorodiol, which is 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 (Steitwieser 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 $\frac{1}{3}$ 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 adenosylcobalamine–enzyme complexes. (The observed rearrangement was an analogue of that of β-methylitaconate $\rightleftharpoons$ α-methylglutarate: Dowd et al., 1975.) The detailed mechanism of such rearrangements remains unresolved though cobalt–π 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).

References

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

Babior, B. M., Moss, T. H., and Gould, D. C. (1972), *J. Biol. Chem. 247,* 4389.

Babior, B. M., Moss, T. H., Orme-Johnson, W. H., and Beinert, H. (1973), *J. Biol. Chem. 249* 4537.

Baker, C. G., and Meister, A. (1951), *J. Am. Chem. Soc. 73,* 1336.

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

Clark, D. T., and Lilley, D. M. J. (1970), *Chem. Commun.,* 603.

Curtin, D. Y., Gourse, J. A., Richardson, W. H., and Rinehart, K. L. (1959), *J. Org. Chem. 24,* 93.

Dowd, P., Shapiro, M., and Kang, K. (1975), *J. Am. Chem. Soc. 97,* 4755.

Fieser, L. E., and Fieser, M. (1967), *Reagents for Organic Synthesis,* New York, N.Y., Wiley, p 584.

Finlay, T. H., Valinsky, J., Mildvan, A. S., and Abeles, R. H. (1972), *J. Biol. Chem. 248,* 1285.

Fredericks, P. S., and Tedder, J. M. (1960), *J. Chem. Soc.,* 144.

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

Frey, P. A., Karabatsos, G. L., and Abeles, R. H. (1965), *Biochem. Biophys. Res. Commun. 18,* 551.

Galiba, I., Tedder, J. M., and Watson, R. A. (1966), *J. Chem. Soc.,* 1321.

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

Gottwald, L. K., and Kun, E. (1965), *J. Org. Chem. 30,* 877.

Hamilton, J. A., Tamao, Y., Blakley, R. L., and Coffman, R. E. (1972), *Biochemistry 11,* 4696.

Hamilton, J. A., Yamada, R., Blakley, R. L., Hogenkamp, H. P. C., Looney, F. D., and Winfield, M. E. (1971), *Biochemistry 10,* 347.

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

Hutchins, R. O., Maryanoff, B. E., and Milewski, C. (1971), *Chem. Commun.,* 1097.

Huyser, E. S. (1965), *Adv. Free-Radical Chem. 1,* 77.

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

Karrer, P., Portman, P., and Suter, M. (1948), *Helv. Chim. Acta 31,* 1617.

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

Lee, H. A., Jr., and Abeles, R. H. (1962), *Symp. Bid. 15,* 310.

Lloyd, W. J., and Harrison, R. (1971), *Carbohydr. Res. 20,* 133.

Martin, R. H., Lampe, F. W., and Taft, R. W. (1966), *J.*

Am. Chem. Soc. 88, 1353.
Orme-Johnson, W. H., Beinert, H., and Blakley, R. L. (1973), *J. Biol. Chem. 249,* 2338.
Pattison, F. L. M., and Norman, J. J. (1957), *J. Am. Chem. Soc. 79,* 2315.
Reichstein, T., Peddin, A., and Grussner, A. (1935), *Helv. Chim. Acta 13,* 598.
Shaw, J. E., Kunerth, D. C., and Sherry, J. J. (1973), *Tetrahedron Lett.,* 689.
Shriner, R. L., Fuson, R. C., and Curtin, D. Y. (1964), The Systematic Indentification of Organic Compounds, 5th ed, New York, N.Y., Wiley, p 253.
Silverman, R. B., and Dolphin, D. (1973), *J. Am. Chem. Soc. 95,* 1686.
Silverman, R. B., Dolphin, D. and Babior, B. M. (1972), *J. Am. Chem. Soc. 94,* 4028.
Streitwieser, A., Jr., and Mares, F.)1968), *J. Am. Chem. Soc. 90,* 2444.
Toraya, T., and Fukui, S. (1972), *Biochim. Biophys. Acta 284,* 536.
Valinsky, J. E., Abeles, R. H., and Mildvan, A. S. (1973), *J. Biol. Chem. 249,* 2751.
Wagner, O. W., Lee, H. A., Frey, P. A., and Abeles, R. H. (1966), *J. Biol. Chem. 241,* 1751.