THE MECHANISM OF ACTION OF COENZYME B_{12}

I. MECHANISM OF ACTION OF COENZYME B_{12}. HYDROGEN TRANSFER IN THE ISOMERIZATION OF β-METHYLASPARTATE TO GLUTAMATE.

II. KINETIC STUDIES OF 3-FLUORO-1,2-PROPANEDIOL, A NEW SUBSTRATE FOR DIOLDEHYDRASE.

III. REACTION OF 5′-DEOXYINOSYLCOBALAMIN WITH PROPANEDIOL DEHYDRASE.

IV. ADENOSINE DEAMINASE

Thesis by

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DEDICATION

To my parents

for their guidance,

support, and confidence in me.

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ABSTRACT

PART I

Use of a mixture of non- and tetradeutero-β-methylaspartate with coenzyme B_{12} dependent β-methylaspartate-glutamate mutase has shown that the hydrogen that migrates becomes one of three equivalent hydrogens during the reaction. Kinetic isotope effects suggest that cleavage of the bond in the substrate from carbon to that hydrogen which migrates is an important component of the rate determining step. The evidence also supports the existence of an intermediate which can partition with similar probabilities to β-methylaspartate or to glutamate. Some possible mechanistic implications of these findings are discussed.

PART II

The mechanism and the kinetics of the action of diol dehydrase on 3-fluoro-1,2-propanediol, a new substrate for diol dehydrase, were examined. The results of this study lead to a reexamination of the kinetic behavior of the natural substrate, 1,2-propanediol, with diol dehydrase. The k_{cat} values of (S)-, (RS)-, and (R)-3-fluoro-1,2-propanediol were found to be 340 sec^{-1}, 128 sec^{-1}, and 104 sec^{-1} respectively, compared to 368 sec^{-1}, 250 sec^{-1}, and 191 sec^{-1}, for the corresponding isomers of 1,2-propanediol. A modification of the existing

analytical procedure for the determination of aldehyde was necessary in order to measure the values of K_m for these substrates. K_m values for (S)-, (RS)-, and (R)-3-fluoro-1,2-propanediol were found to be 13.2×10^{-4}M, 3.07×10^{-4}M, and 1.47×10^{-4}M respectively. For (R)-, (RS)-, and (S)-1,2-propanediol, K_m values were measured at 1.1×10^{-4}M, 8.3×10^{-5}M, and 6.5×10^{-5}M respectively.

These results show that the (R)- and (S)- isomers of both substrates exhibit simple competitive behavior for the active site in the racemic mixture, and that both the (R)- and (S)- isomers of the substrate pass through a similar transition state in the enzyme catalyzed conversion to aldehyde.

PART III

The reactivity of 5′-deoxyinosylcobalamin in the propanediol dehydrase reaction was examined. This coenzyme B_{12} analogue showed no coenzyme activity in the diol dehydrase reaction. 5′-Deoxyinosylcobalamin was found to inhibit the activity of coenzyme B_{12} although it bound less efficiently than the natural coenzyme by a factor of 100. Previously reported experiments regarding the release of 5′-deoxyinosine by this system were shown to be inconclusive.

PART IV

The kinetic behavior of 5′-deoxyadenosine in the adenosine deaminase reaction has been examined. The low catalytic rate and

inefficient binding observed for this substrate suggest that the 5′-hydroxyl is very important to both catalysis and binding of ribosyladenines. In spite of the low catalytic rate and the poor binding, adenosine deaminase was used to effectively synthesize 5′-deoxyinosine from 5′-deoxyadenosine.

ABSTRACTS OF THE PROPOSITIONS

PROPOSITION I

A kinetic study of 1,1-dideuterio-3-fluoro-1,2-propanediol in the propanediol dehydrase system is proposed in order to determine the relative contributions of k_1 and k_{cat} to the K_m value for 3-fluoro-1,2-propanediol.

PROPOSITION II

An investigation of the reactivity of γ-fluoroglutamic acid in the glutamate mutase reaction is proposed.

PROPOSITION III

A comparison of some biochemical differences in the blood, hemoglobin, and myoglobin of some diving and non-diving vertebrates is proposed.

PROPOSITION IV

A comparison of the bond polarization caused in binding NADH to the H_4 and M_4 isozymes of lactate dehydrogenase is proposed.

PROPOSITION V

A study of the reactivity of some 3-fluorinated lactic acid analogues with lactate racemase is proposed.

Part I

Mechanism of Action of Coenzyme B_{12}. Hydrogen Transfer in the Isomerization of β-Methylaspartate to Glutamate*

INTRODUCTION

The cobalt containing coenzyme B_{12} (α-dimethylbenzimidazolyl Co-5′-deoxyadenosylcobamide) has been shown to be an obligatory cofactor in several processes in which hydrogen atoms and other groups undergo 1,2-migrations. Some of these are: dehydration of 1,2-propanediol to propionaldehyde (1) (Brownstein and Abeles, 1961; Lee and Abeles, 1963), deamination of ethanolamine to acetaldehyde (2) (Bradbeer, 1965; Babior, 1969), isomerization of methylmalonyl coenzyme A to succinyl coenzyme A (3) (Smith and Monty, 1959; Wood et al., 1964), isomerization of β-methylaspartate to glutamate (4) (Barker et al., 1958; Iodice and Barker, 1963; Barker et al., 1964a), the isomerization of β-lysine to 3,5-diaminohexanoic acid (5) (Stadtman and Renz, 1968), and the isomerization of α-methyleneglutarate to α-methylene-β-methylsuccinate (6) (Kung et al., 1970). These six reactions have several features in common which can be summarized. (eq 7).

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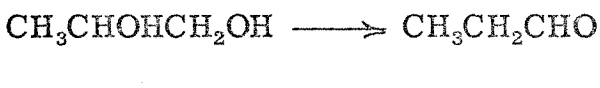

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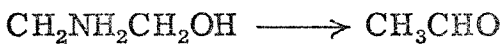

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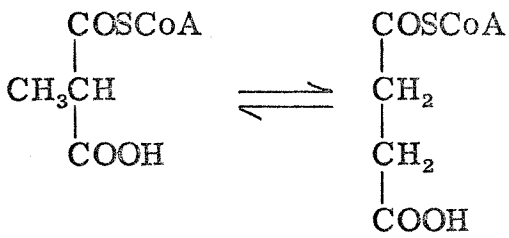

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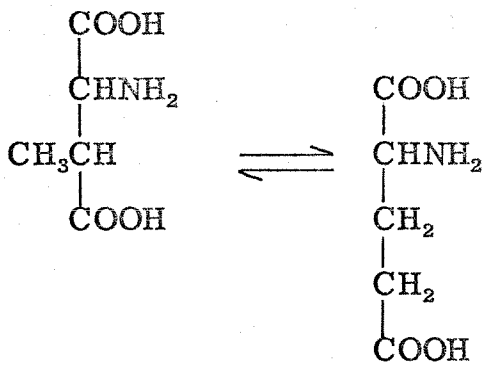

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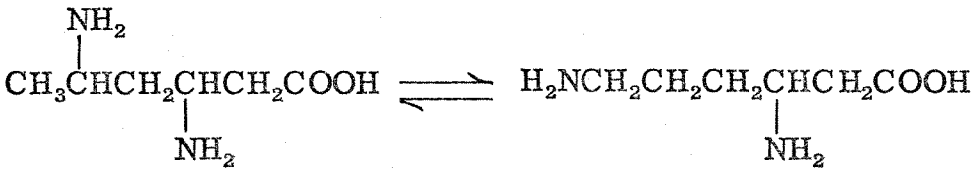

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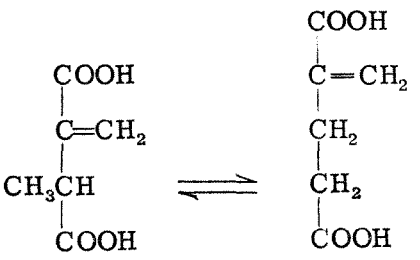

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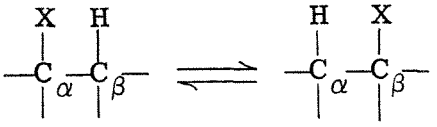

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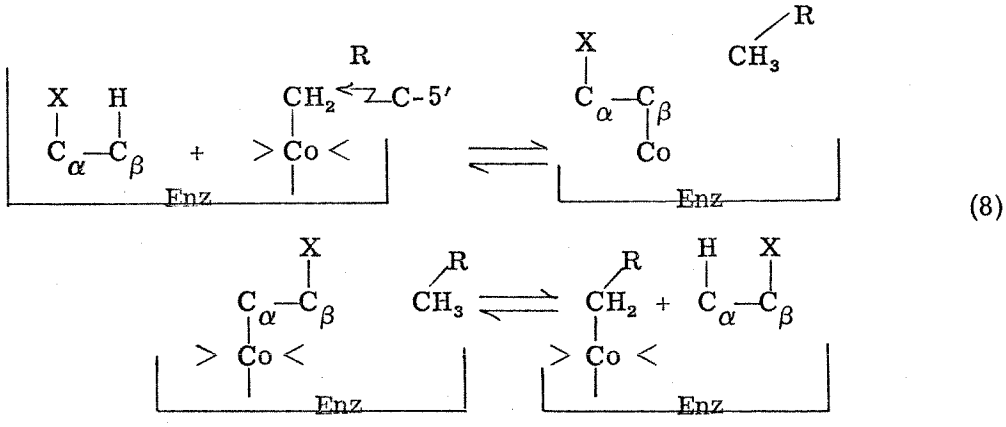
In the isomerizations of both β-methylaspartate (Barker et al., 1964a, and 1964b) and methylmalonyl coenzyme A (Kellermeyer and Wood, 1962; Wood et al., 1964; Phards et al., 1964), the X group (-$CHNH_2COOH$ and -COSCoA, respectively) is known to be transferred intramolecularly. The hydrogen which is transferred does not exchange with the hydrogen of water during the isomerization of β-methylaspartate (Barker et al., 1964a; Iodice and Barker, 1963; Suzuki and Barker, 1966) during the isomerization of methylmalonyl coenzyme A (Overath et al., 1962; Erfle et al., 1964a; and Erfle et al., 1964b) during the dehydration of propanediol (Brownstein and Abeles, 1961) or during the deamination of ethanolamine (Babior, 1969). Exchange of hydrogen between substrate and C-5′ of coenzyme B_{12} is, however, observed for propanediol dehydrase (Abeles and Zagalak, 1966; Riley and Arigoni, 1966; Frey and Abeles, 1966; Abeles and Frey, 1966), for methylmalonyl coenzyme A mutase (Riley and Arigoni, 1966), for ethanolamine deaminase (Babior, 1969), and for glutamate mutase (Switzer et al., 1969). Stereochemical studies of the rearrangements have shown that inversion of configuration is observed at the carbon to which hydrogen migrates in the isomerization of β-methylaspartate to glutamate (Sprecher and Sprinson, 1964) and for dehydration of propanediol (Retey et al., 1966)

(Retey et al., 1966; Zagalak et al., 1966). However, configurational inversion is observed in the rearrangement of methylmalonyl coenzyme A to succinyl coenzyme A (Sprecher et al., 1964).

The hydrogen which migrates has been shown to become one of three equivalent hydrogens in the case of methylmalonyl coenzyme A mutase suggesting that transfer of hydrogen from substrate to C-5′ of the deoxyadenosyl residue of the coenzyme cleaves the cobalt-C-5′ bond and transforms C-5′ to a methyl group with three equivalent hydrogens, one of which is eventually returned to substrate (eq 8) (Miller and Richards, 1969).



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The purpose of the present work was to study in an analogous way the fate of the migrating hydrogen in the isomerization of β-methylaspartate to glutamate to establish the generality of an intermediate with three equivalent hydrogens, one of which is the migrating hydrogen and particularly to see if the difference in stereochemical fate at the carbon to which hydrogen migrates (retention for methylmalonyl coenzyme A mutase, inversion for glutamate isomerase) is reflected in a different mechanism for hydrogen transfer in the two cases.

The method was analogous to that used previously in the study of methylmalonyl coenzyme A isomerization (Miller and Richards, 1969) and briefly, the results are similar to those obtained earlier and support a pathway for hydrogen transfer which involves an intermediate with three equivalent hydrogens.

Results

A mixture consisting of 50% β-methylaspartic acid and 50% β-trideuteromethyl-α-deuteroaspartic acid was partially isomerized to a mixture of variously deuterated glutamic acids by an enzyme preparation of glutamate mutase from Clostridium tetanomorphum in the presence of coenzyme B_{12} under an argon atmosphere. The reaction was stopped by freezing in dry ice and the β-methylaspartic acid and glutamic acid were isolated by ion-exchange chromatography.

Deuterium Analysis. The deuterium compositions of the products were determined by mass spectrometry. Direct analysis of the amino acids themselves was impractical because of the strong tendency for the amino acids to dimerize to their 2,5-diketo piperazine derivatives. This problem was eliminated by conversion of the amino acids to their diethyl esters (Biemann et al., 1961) before mass spectral analysis. The problem of polymerization was further reduced by use of a direct inlet probe, by keeping sample temperature as low as possible (less than 50 °C) and by determining the mass spectra as rapidly as possible after sample introduction (less than 10 mins. in most cases).

Two regions of the spectrum were particularly useful in determining deuterium distribution, one at m/e 84, the other at m/e 130. Table I gives the observed relative intensities in these regions for diethyl β-methylaspartate and the tetradeutero analogue (three deuteriums in the β-methyl group, one

Table I. Relative Intensities for

Diethyl β-Methyl Aspartate

m/e	β-Methyl Aspartate	tetra-^{2}H β-Methyl Aspartate
82	0	0
83	4.6	0
84	100.0	0
85	9.1	0
86	2.0	1.8
87	7.0	9.0
88	0	100.0
89	0	8.3
90	0	1.9
128	0	0
129	4.0	0
130	100.0	0
131	8.2	0
132	0	1.5
133	0	8.5
134	0	100.0
135	0	9.8

deuterium on the α-carbon). Table II records similar data for diethyl glutamate and its pentadeutero analogue (two deuteriums each on the β and γ carbons, one deuterium on C-α). Tables III and IV summarize the mass spectral data for the β-methylaspartate mixture used as substrate and for the glutamate and β-methylaspartate recovered from two reactions after isomerization had proceeded to the extent of 21.2% in one case and 36.4% in the other.

Calculations of Relative Abundances of Variously Deuterated Species. To obtain the relative abundances of non-, mono-, di-, tri-, and tetra-deuterated diethyl β-methylaspartate, one must know the mass spectra of each of these species. In fact, the spectra of only the non- and tetradeutero analogues are available experimentally and spectra for the others must therefore be inferred by interpolation. The absence of a peak at m/e 82 (m/e 84-2) in the spectrum of the nondeuterated species suggests that the peak at m/e 86 in the tetradeuterated analogue comes from loss of a single deuterium and not by loss of two hydrogens from the tetradeutero peak at m/e 86.

Regardless of its exact molecular structure (one possibility is shown), the peak at m/e 84 (or m/e 84 + n, where n is the number of deuteriums) does contain all the hydrogen (or deuterium) atoms of interest -- as for that matter do the peaks in the region m/e 130. If isotope effects played no role, the sum of

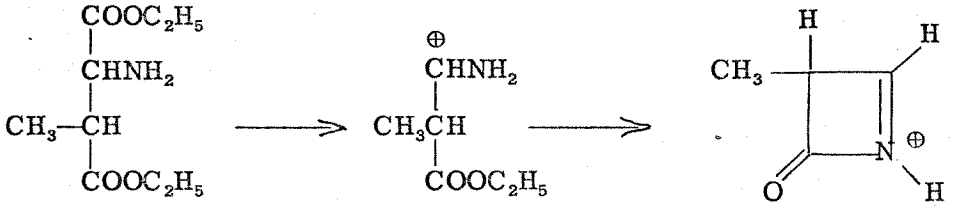


Table II. Relative Intensities for Diethyl Glutamate

m/e	Glutamate	penta-^{2}H-glutamate
82	0	0
83	3.8	0
84	100.0	0
85	5.6	0
86	0.5	0
87	0.5	3.2
88	1.0	0.6
89	0	100.0
90	0	5.6
128	0	0
129	6.0	0
130	100.0	0
131	5.8	0
132	0	0
133	0	5.0
134	5.0	1.0
135	0	100.0
136	0	5.8

Table III. β-Methyl Aspartate Spectra

m/e	β-Methyl Aspartate Before Reaction	β-Methyl Aspartate After 21.2% Reaction	β-Methyl Aspartate After 36.4% Reaction
84	100.0	72.4	50.0
85	7.7	10.3	10.6
86	1.9	4.4	4.3
87	7.1	16.7	18.9
88	96.8	100.0	100.0
130	100.0	67.8	47.4
131	7.9	8.9	9.5
132	0.2	2.9	3.2
133	7.4	12.5	16.3
134	100.0	100.0	100.0

Table IV. Glutamate Spectra

m/e	Glutamate After 21.2% Reaction	Glutamate After 36.4% Reaction
84	100.0	100.0
85	19.1	21.4
86	2.0	2.0
87	9.1	12.0
88	7.3	8.0
130	100.0	100.0
131	18.8	22.6
132	2.6	4.2
133	7.9	11.3
134	10.5	13.1

the m/e-1 and m/e-2 peaks for the tetradeutero diethyl β-methylaspartate (m/e 87 and m/e 86 respectively) should equal m/e-1 for nondeutero diethyl β-methylaspartate (m/e 83). This is not observed, there being in the tetradeutero diethyl β-methylaspartate a peak at m/e 87 with an intensity of 9.0 (relative to the parent peak intensity of 100) and one at m/e 86 of 1.6, whereas the peak at m/e 83 for the nondeuterated analogue has a relative intensity of 4.8. Thus, hydrogen is lost somewhat more readily from the deuterated than from the nondeuterated parent for reasons which are obscure. Interpolation from the experimental spectra of non- and tetradeutero diethyl β-methylaspartate will give an anticipated intensity of the m-1 peak for, say the dideutero species (m-1 = 85) of 4.6 + 2/4 (9.0-4.6) = 6.8. Similarly the m-1 = 86 peak for the trideutero species will be 4.6 + 3/4 (9.0-4.6) = 7.9. The total intensity of the m/e 85 peak will consist of a contribution from m + 1 of nondeutero, m of monodeutero, m-1 of dideutero, m-2 of trideutero, and m-3 of tetradeutero diethyl β-methylaspartate derivatives. These considerations lead to the following set of five simultaneous equations whose solution by a matrix inversion routine using an IBM 360/75 computer and the experimental intensities of the mixture at m/e 84, 85, 86, 87, and 88 yields the relative abundances of β_0, β_1, β_2, β_3, and β_4.

intensity at m/e 84 = $100.0\beta_0 + 5.7\beta_1 + 0.9\beta_2 + 0.0\beta_3 + 0.0\beta_4$

" " " 85 = $9.1\beta_0 + 100.0\beta_1 + 6.8\beta_2 + 1.4\beta_3 + 0.0\beta_4$

" " " 86 = $2.0\beta_0 + 8.9\beta_1 + 100.0\beta_2 + 7.9\beta_3 + 1.8\beta_4$

" " " 87 = $7.0\beta_0 + 2.0\beta_1 + 8.7\beta_2 + 100.0\beta_3 + 9.0\beta_4$

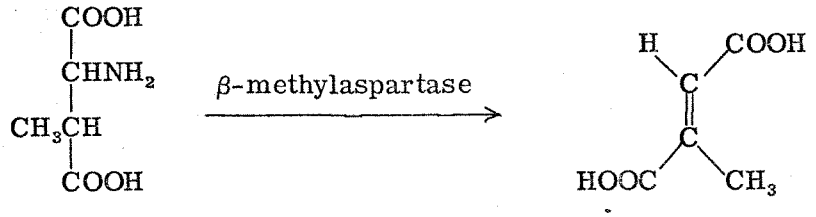
" " " 88 = $0.0\beta_0 + 6.7\beta_1 + 2.0\beta_2 + 8.5\beta_3 + 100.0\beta_4$

Similar procedures were used to calculate the relative amounts of variously deuterated species using the data in the m/e 130 range for diethyl β-methylaspartate and for both the m/e 84 and m/e 130 ranges for diethyl glutamate. The relative intensities experimentally determined for non- and tetradeutero diethyl β-methylaspartate are listed in Table I, for non- and pentadeutero diethyl glutamate in Table II; the interpolated coefficients used in analyzing mixtures of deuterated species are given in Tables V and VI.

Calculations of the type just described lead from the experimentally observed relative mass spectral intensities of Tables III and IV to the relative amounts of variously deuterated species shown in Table VII (for β-methylaspartate before, $\beta_0{}^b$, $\beta_1{}^b$, etc., and after, $\beta_0{}^a$, $\beta_1{}^a$, etc., reaction) and Table VIII (for glutamate after reaction G_0, G_1, etc.)

Discussion

a. Amount of Overall Reaction. One way of determining the amount of β-methylaspartate which has been converted to glutamate is by following the concentration of β-methylaspartate with time. This was done using β-methylaspartase and observing the amount of mesaconate so produced spectrally. This method is fairly inaccurate and was used only to get a rough



idea of the extent of conversion of β-methylaspartate to glutamate so as to quench the reaction at an appropriate time.

Table V. Interpolated Relative Intensities of β_0, β_1, β_2, β_3, and β_4

m/e	β_0 (obsd)	β_1 (calcd)	β_2 (calcd)	β_3 (calcd)	β_4 (obsd)
84	100.0	5.7	0.9	0.0	0.0
85	9.1	100.0	6.8	1.4	0.0
86	2.0	8.9	100.0	7.9	1.8
87	7.0	2.0	8.7	100.0	9.0
88	0.0	6.7	2.0	8.5	100.0
130	100.0	5.1	0.8	0.0	0.0
131	8.2	100.0	6.2	1.2	0.0
132	0.0	8.6	100.0	7.4	1.5
133	0.0	0.0	9.0	100.0	8.5
134	0.0	0.0	0.0	9.4	100.0

Table VI. Interpolated Relative Intensities of G_0, G_1, G_2, G_3, and G_4

m/e	G_0 (obsd)	G_1 (calcd)	G_2 (calcd)	G_3 (calcd)	G_4 (calcd)
84	100.0	3.1	1.2	0.0	0.0
85	5.6	100.0	2.6	1.9	0.0
86	0.5	5.6	100.0	1.9	2.6
87	0.5	0.5	5.6	100.0	1.2
88	1.0	0.5	0.5	5.6	100.0
130	100.0	5.0	2.0	0.0	0.0
131	5.8	100.0	4.0	3.0	0.0
132	0.0	5.8	100.0	3.0	4.0
133	0.0	0.0	5.8	100.0	2.0
134	5.0	0.0	0.0	5.8	100.0

Table VII. Percentages of Variously Deuterated Species Before and After 21.2% Reaction

				From m/e 84 - m/e 86		From m/e 130 - m/e 134	
β_0^b	50.0	β_0^a	38.6	G_0	78.1	G_0	78.5
β_1^b		β_1^a	1.8	G_1	10.6	G_1	10.2
β_2^b		β_2^a	0.5	G_2	0.3	G_2	1.2
β_3^b		β_3^a	2.3	G_3	6.5	G_3	6.1
β_3^b	50.0	β_4^a	56.8	G_4	4.5	G_4	4.0

Table VIII. Percentage of Variously Deuterated Species Before and After 36.4% Reaction

				From m/e 84 - m/e 88		From m/e 130 - m/e 134	
β_0^b	50.0	β_0^a	30.7	G_0	74.8	G_0	72.5
β_1^b		β_1^a	3.7	G_1	11.8	G_1	12.0
β_2^b		β_2^a	0.4	G_2	0.2	G_2	2.0
β_3^b		β_3^a	3.9	G_3	8.5	G_3	8.0
β_4^b	50.0	β_4^a	61.3	G_4	4.7	G_4	5.5

More accurate estimates are possible by determining the concentrations of β-methylaspartate and glutamate after the reaction has been stopped with a Beckman 120C amino acid analyzer. This method showed that in one case, 21.2% of the original β-methylaspartate had been converted to glutamate; in the other reaction, 36.4% of the original β-methylaspartate was converted to glutamate.

Conservation of total deuterium content between reactants and products provides an alternate, though less accurate method, of determining the percent reaction, (i.e., the total deuterium in the starting β-methylaspartate equals the sum of the deuterium in the β-methylaspartate and glutamate isolated after reaction). Two conditions necessary for this calculation are (i) no deuterium be lost to solvent (the absence of exchange between substrate and water has been demonstrated (Barker et al., 1964a; Iodice and Barker, 1963; Suzuki and Barker, 1966)), and (ii) there be only a negligible fraction of the original deuterium that remains in the coenzyme B_{12} after reaction, a condition which is easily met as the amount of substrate so vastly exceeds the amount of coenzyme B_{12} (4×10^{-5} moles of coenzyme B_{12}) that even if all the C-5′ hydrogens of the coenzyme were to be replaced by deuterium, the total deuterium lost in this way would be negligible. If, therefore, X equals the mole fraction of β-methylaspartate converted to glutamate:

$$4\beta_4^{\,b} = X(G_1 + 2G_2 + 3G_3 + 4G_4) + (1-X)(\beta_1^{\,a} + 2\beta_2^{\,a} + 3\beta_3^{\,a} + 4\beta_4^{\,a})$$

From the data in Tables VII and VIII, one calculates that the first reaction went to about 20% conversion and the second to about 30% (Table IX). These numbers are in reasonable agreement with those obtained by amino acid

Table IX. Percentage Overall Reaction Calculated by Conservation of Total Deuterium

21.2% Reaction		36.4% Reaction	
m/e 84 range	m/e 130 range	m/e 84 range	m/e 130 range
19.6	19.4	30.0	30.8

analysis of 21% and 36%, respectively, but because we feel these later values (21% and 36%) to be the most reliable estimates of the amount of overall reaction, they have been used in subsequent computations.

b. Amount of Back Reaction. As the reaction is in fact an equilibration with an equilibrium constant favoring glutamate of 7.69 (Barker, 1961) we need to calculate the amount of glutamate (G) that might have been converted back to β-methylaspartate (β). For the reaction that converted 21% of

$$\text{Keq} = \frac{(\text{glutamate})}{(\beta\text{-methylaspartate})} = \frac{k_{\beta \rightarrow G}}{k_{G \rightarrow \beta}} = 7.69$$

β-methylaspartate to glutamate, the average relative concentration of β-methylaspartate will have been 100-(0.5)(21.2) = 89.4 (relative to a glutamate concentration of 10.6).

Therefore, the relative amount of β-methylaspartate that has been formed as a result of back reaction from glutamate

$$= \frac{(k_{G \rightarrow \beta})(\text{average conc. of G})}{(k_{\beta \rightarrow G})(\text{average conc. of } \beta)} \qquad \begin{pmatrix}\text{amount of G formed} \\ \text{from } \beta \text{ during reaction}\end{pmatrix}$$

Thus, for the reaction which gave 21% glutamate, the amount of β-methylaspartate formed by back reaction from glutamate will be (1/7.69)(10.6)/(89.4)(21.2) = 0.32%. For the reaction which gave 36% glutamate, the amount of β-methylaspartate formed by back reaction from glutamate will be (1/7.69)(18.2)/(81.8)(36.4) = 1.05%. These calculations have been made assuming a constant concentration of both β and G throughout reaction, which is clearly invalid as these concentrations in fact change significantly.

The proper treatment would involve integration of the differential rate equations. The point of importance is, however, that only a negligibly small amount of the β-methylaspartate present after reaction has ever existed as glutamate and in the subsequent discussion we shall assume that the reaction, as studied, involved only the conversion of β-methylaspartate to glutamate and that the reverse reaction did not intervene significantly.

A more subtle question concerns the amount of β-methylaspartate after reaction that may have been converted to some intermediate which partitions to regenerate β-methylaspartate on the one hand and to yield glutamate on the other.

The presence of mono-, di-, and trideutero-β-methylaspartate after reactions requires such a process as these species (β_1, β_2, and β_3) present after reaction must come from such an intermediate. One can calculate the relative amounts of β_0 and β_4 that at some time existed as intermediate by assuming that the intermediate will abstract hydrogen or deuterium from the C-5′ carbon (see subsequent discussion) in the same relative ratios in forming glutamate as in giving back β-methylaspartate. Thus

$$\beta_0^{BR} = \beta_0 \text{ formed by back-reaction from the intermediate as percentage of total } \beta_0^{a}$$
$$= \beta_1^{a} (G_0/G_1)$$

For the reaction that went to 21.2% conversion the data in Table VII lead to

$$\beta_0^{BR} = \beta_1^{a} (G_0/G_1) = 13.3\%$$

and

$$\beta_4^{BR} = \beta_3^{a} (G_4/G_3) = 1.6\% .$$

Taken together with the observed amounts of β_1, β_2, and β_3 (which must have come from back-reaction), these numbers lead to a value of total amount of β-methylaspartate at the end of the reaction which has been converted as far as the intermediate of:

$$\beta_0^{BR} + \beta_1^{a} + \beta_2^{a} + \beta_3^{a} + \beta_4^{BR} = 19.5\%$$

In this particular reaction 78.8% of the total material after reaction is β-methylaspartate. Therefore, (0.788) (19.5%) = 15.4% of the total amount of amino acid after reaction is β-methylaspartate formed by back-reaction from the intermediate. The total amount of the original β-methylaspartate that interacted with enzyme and reached at least an intermediate stage is accordingly (21.2 + 15.4) = 36.6%.

From this intermediate stage the partitioning ratio for formation of glutamate relative to aspartate is 21.5/15.4 = 1.37, that is 58% of the time the intermediate yields glutamate and 42% of the time β-methylaspartate is regenerated.

In the case of the reaction in which 36.4% of the original β-methylaspartate went to glutamate, the partitioning ratio is calculated to be about 1.7 (the values are collected in Table X), i.e., 63% of the time the intermediate partitions to glutamate. Thus the intermediate partitions more readily to glutamate than to β-methylaspartate though this preference is not large.

In analogous studies of the methylmalonyl coenzyme A mutase reaction (Miller and Richards, 1969) the partitioning ratio was found to be 3.7 in favor of succinyl coenzyme A (89% of the time succinyl coenzyme A is formed).

Table X. Partitioning Ratio for Glutamic Acid over β-Methyl Aspartic Acid

	21.2% Reaction		36.4% Reaction	
	m/e 84 range	m/e 130 range	m/e 84 range	m/e 130 range
Partitioning Ratio	1.37	1.35	1.70	1.73

These experimental observations suggest that the relative energies of the transition states from intermediate to reactant or product reflect to some degree the relative ground state energies of reactant and products.

Equilibrium between β-methylaspartate and glutamate favors glutamate by a factor of 7.69:1 (Barker, 1961). Between methylmalonyl coenzyme A and succinyl coenzyme A, the succinyl coenzyme A is favored by a factor of 23:1, almost 3 times larger than for glutamate (Kellermeyer et al., 1964). This difference in equilibrium constant would be anticipated on the basis of the structures of the substrates. In the case of methylmalonyl coenzyme A mutase a skeletal rearrangement occurs that eliminates the condition of having a single carbon bound to two carboxyl groups. Such a marked relief of dipolar opposition does not occur in the rearrangement of β-methylaspartate to glutamate.

Thus for that reaction in which ground state energies favor product over reactant by a larger factor, (methylmalonyl coenzyme A mutase) the intermediate also partitions more completely to product. In line with the tenets of the Hammond postulate (Hammond, 1955), and the expectation that the transition states between intermediate and reactant or product probably lie approximately at the midpoint of the reaction coordinate (because the intermediate on the one hand and coenzyme plus reactant or product on the other are probably of similar energy), the partitioning ratios are much smaller than the equilibrium constant. This is shown diagramatically in Figure 1.

$$\frac{K_{eq}\,\frac{(S)}{(M)}}{K_{eq}\,\frac{(G)}{(\beta)}} > \frac{\Delta G^{\pm}(\text{Intermediate} \rightarrow S)}{\Delta G^{\pm}(\text{Intermediate} \rightarrow M)} \frac{\Delta G^{\pm}(\text{Intermediate} \rightarrow G)}{\Delta G^{\pm}(\text{Intermediate} \rightarrow \beta)}$$

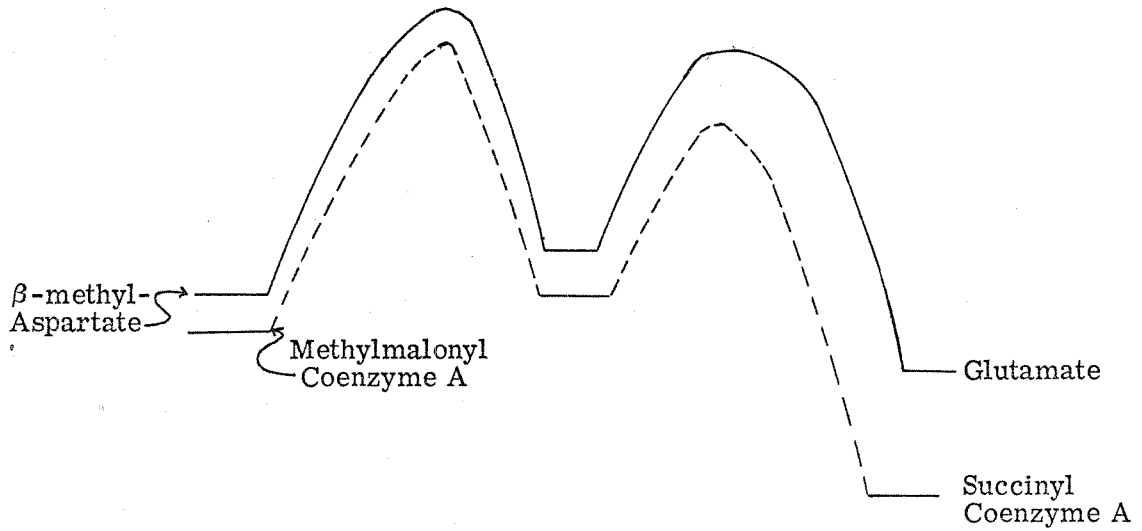


Figure 1

Kinetic Isotope Effect. The experimental results clearly show that formation of protiated glutamate is favored relative to formation of deuterated glutamate. One can use these data to calculate the kinetic isotope effect for the overall reaction averaged over the percent reaction observed.

Consider the following sequence:

$$E + S_H \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S_H \xrightarrow{k_H} P_H + E$$

$$E + S_D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S_D \xrightarrow{k_D} P_D + E$$

We assume that k_1 and k_{-1} are negligibly affected by substitution of deuterium by hydrogen which seems plausible as bonding of substrate to the enzyme-coenzyme complex is due to hydrogen bonding, ionic or van der Waals interactions. Accordingly, replacement of a methyl group by a trideutero methyl group and the α-C-H by deuterium in β-methylaspartate should cause only a slight change in the "effective size" of the substrate. Isotope effects for such changes are known to be small (e. g., $k_H/k_D = 1.030 \pm 0.003$ for a change of CH_3— to CD_3— in the reaction of methyl iodide with 2-methyl pyridine (Brown and McDonald, 1966)). Treatment of such a system by normal Michaelis-Menten kinetics leads to

$$\frac{\frac{dP_H}{dt}}{\frac{dP_D}{dt}} = \frac{k_H(k_{-1} + k_D)[S_H]}{k_D(k_{-1} + k_H)[S_D]}$$

which, on integration, yields

$$\frac{k_H}{k_D} \frac{(k_{-1} + k_D)}{(k_{-1} + k_H)} = \frac{\ln(\beta_0{}^o/\beta_0{}^t}{\ln(\beta_4{}^o/\beta_4{}^t}$$

where $\beta_0{}^t$ = total unreacted β-methylaspartate without any deuterium at time t, $\beta_0{}^o$ = total initial concentration of undeuterated β-methylaspartate and $\beta_4{}^o$ and $\beta_4{}^t$ are analogous values for tetradeuterated substrates. In the subsequent discussion we have assumed that $k_{-1} > k_H$ and k_D, i. e., that dissociation of enzyme-substrate complex is fast relative to chemical conversion of substrate. Though the value of k_H/k_D obtained in this way is not the actual microscopic isotope effect, it is, however, the correct one to compare with the Δ_1 and Δ_2 values of the subsequent discussion.

We now need to determine the composition of the mixture. Before reaction:

$$\beta_0 = 50.0\%$$
$$\beta_4 = 50.0\%$$

After the reaction that went to 21.2% glutamate and using the mass spectral data in the m/e 84 range we can calculate:

$$G_H = (G_0 + G_1 + 1/2\ G_2)\ 0.21$$
$$= [78.1 + 10.6 + 1/2\ (0.3)]\ 0.21 = 18.8\%$$

$$G_D = (G_4 + G_3 + 1/2\ G_2)\ 0.21$$
$$= [4.5 + 6.5 + 1/2\ (0.3)]\ 0.21 = 2.4$$

$$\text{unreacted } \beta_0 = (\beta_0^{\text{total}} - \beta_0^{BR})\ 0.79$$
$$= (38.6 - 13.3)\ 0.79 = 19.9$$

$$\text{unreacted } \beta_4 = (56.8 - 1.6)\ 0.79 = 43.5$$

$$\beta_0^{BR} = (13.3)\ 0.79 = 10.5$$

$$\beta_1^{BR} = (1.8)\ 0.79 = 1.4$$

$$\beta_2^{BR} = (0.5)\ 0.79 = 0.4$$

$$\beta_3^{BR} = (2.3)\ 0.79 = 1.8$$

$$\beta_4^{BR} = (1.6)\ 0.79 = 1.3$$

$$100.0\%$$

Therefore, $k_H/k_D = \frac{\ln(50.0/19.9)}{\ln(50.0/43.5)} = 6.6$

Table XI collects this information for the m/e 130 range for the 21.2% reaction and for the m/e 84 and 130 ranges for the 36.4% reaction.

Table XI. Percentage Composition of the Reaction Mixture after Reaction and the Calculated Isotope Effect

	21.2% Reaction		36.4% Reaction	
	m/e 84range	m/e 130 range	m/e 84 range	m/e 130 range
G_H	18.8	18.9	31.6	31.1
G_D	2.4	2.3	4.8	5.3
β_0 Unreacted	19.9	19.5	4.6	5.3
β_4 Unreacted	43.5	43.6	37.6	37.2
β_0 Back Reaction	10.5	10.9	14.9	14.3
β_1 Back Reaction	1.4	21.4	2.4	2.4
β_2 Back Reaction	0.4	0.4	0.2	0.2
β_3 Back Reaction	1.8	1.8	2.5	2.5
β_4 Back Reaction	1.3	1.2	1.4	1.7
Total %	100.0	100.0	100.0	100.0
k_H/k_D	6.6	6.9	8.4	7.5

The values for the kinetic isotope effects derived by the total conversion of protiated relative to deuterated substrate to product probably have significant associated error. For example, the reaction that went to the greater extent (36.4%) should have a lower average kinetic isotope effect than the reaction that was quenched earlier (21.2%), whereas in fact the experimental values are in the opposite order. We attribute this to experimental inaccuracies and conclude that the average kinetic isotope effect is about 7.5 ± 1.0, which strongly suggests that the rate determining step involves abstraction of a hydrogen from a molecule of substrate with a considerable portion of the carbon-hydrogen vibration of the ground state being frozen out in the transition state of the reaction.

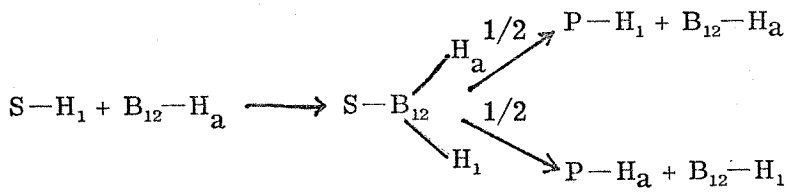
Representative values of kinetic isotope effects for other reactions include: $k_H/k_D \sim 6$ to 10 for abstraction of hydrogen in the keto-enol conversion which is the rate determining step in the bromination of acetone (Reity and Kopp, 1939), $k_H/k_D \sim 12$ for the conversion of D-propanediol 1-d to propionaldehyde catalyzed by propanediol dehydrase and coenzyme B_{12} (Frey et al., 1965) and a value, obtained in a manner similar to that used in this work of $k_H/k_D \sim 3.5$ for the isomerization of methylmalonyl coenzyme A to succinyl catalyzed by the appropriate mutase and coenzyme B_{12} (Miller and Richards, 1969).

Previous discussion has shown that β-methylaspartate is first converted to an intermediate which can partition to give back β-methylaspartate or yield glutamate, this partitioning favoring glutamate by a factor of about 1.5. Thus the energy barrier between intermediate and glutamate is lower than the barrier between β-methylaspartate and intermediate (Fig. I) which makes the abstraction of hydrogen (or deuterium) from the methyl carbon of β-

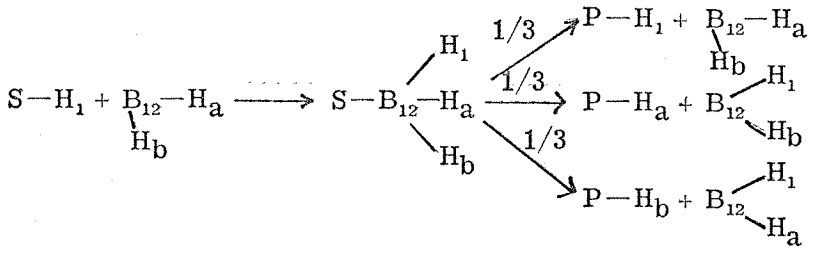
methylaspartate the main rate determining step in the overall reaction.

Models for Hydrogen Transfer: Five model pathways for hydrogen transfer between the two carbons of substrate (from the methyl carbon of β-methylaspartate to C-4 of glutamate) will be considered. One group of these models deals with the number of hydrogens with which the migrating hydrogen becomes equivalent during transfer; another group of merry-go-round mechanisms deals with schemes in which the hydrogen is never returned to the same molecule from which it was abstracted.

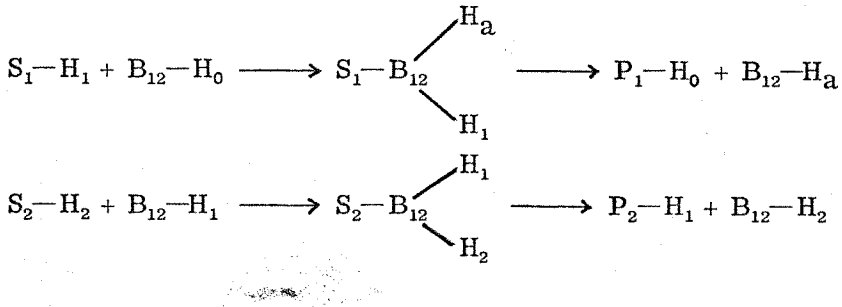
(i) In the "two-equivalent-hydrogen" model, the hydrogen abstracted from substrate becomes equivalent with one additional hydrogen on the coenzyme giving an intermediate with two equivalent hydrogens. Apart from the kinetic isotope effects, either of these two equivalent hydrogens has a statistical probability of 1/2 of being returned to substrate.



(ii) In the "three-equivalent-hydrogen" model, the hydrogen abstracted from substrate becomes equivalent with two additional hydrogens on the coenzyme.

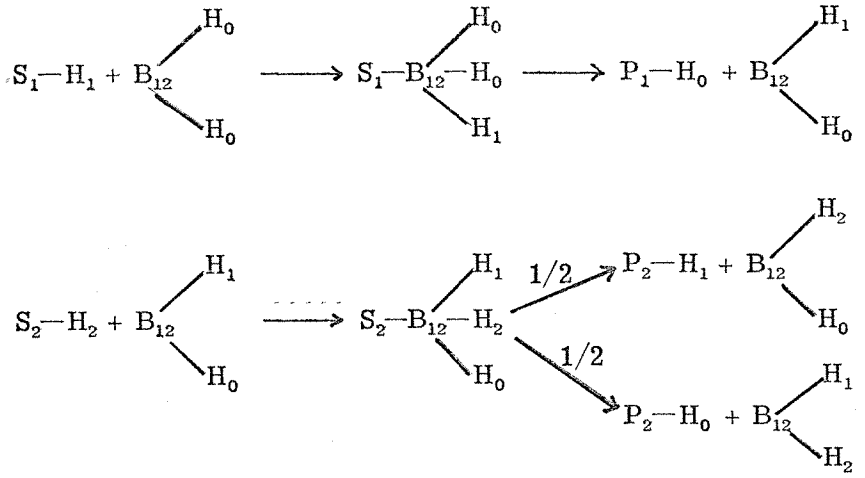


(iii) In the "one-hydrogen-merry-go-round" model, the hydrogen abstracted from substrate is never re-added to the same molecule of substrate. The hydrogen acquired by product comes from the coenzyme, and the hydrogen remaining on the coenzyme is the one just removed from substrate. This hydrogen is, however, transferred to product during the next interaction with substrate.



etc.

(iv) The "two-hydrogen-merry-go-round" model is analogous to the previous case except that two hydrogens are now present on the coenzyme before acquisition of a hydrogen from substrate and either of these two hydrogens -- but not the one just acquired from substrate -- can be released to product.



etc.

(v) The "three-hydrogen-merry-go-round" model is analogous to (iii) and (iv) except that now three hydrogens exist on the coenzyme before any is transferred from substrate.

The equations to calculate the distribution of hydrogen and deuterium to be expected in product as a result of these possible mechanisms have been derived using the following symbols: β_0 = concentration of β-methylaspartate with no deuterium averaged over the percent of reaction observed; β_1 = average concentration of monodeutero-β-methylaspartate with the assumption that all this species comes by transfer of deuterium to an intermediate that was formed from β_0, i. e., $\beta_0 \longrightarrow \beta_1$; β_2 as before with the assumption that half originated from β_1 and half from β_3; β_3 and β_4 having meanings analogous to β_1 and β_0 (i. e., β_3 is formed by back reaction from an intermediate that began as β_4). G_0, G_1, G_2, G_3 and G_4 refer to the concentrations of non-, mono-, di-, tri-, and tetradeuteroglutamate at the end of the reaction. B_0, B_1, B_2, etc. refer

to the average concentration of coenzyme B_{12} with no, one, two, etc. deuteriums. Δ_1 refers to the kinetic isotope effect, expressed as k_H/k_D for transfer of hydrogen or deuterium from β-methylaspartate to coenzyme; Δ_2 refers to k_H/k_D for transfer of hydrogen or deuterium from coenzyme-substrate intermediate to give glutamate.

In order to facilitate preliminary calculations, we assumed the absence of any back reaction. Thus any β_1, β_2, or β_3 was added to β_0 or β_4 depending on which of the two substrates produced them. Therefore,

$$\beta_0 = 1/2\ (\beta_0^{\text{initial}} + \beta_0^{\text{final}}) + 1/2\ \beta_1 + 1/2\ \beta_2$$

$$\beta_4 = 1/2\ (\beta_4^{\text{initial}} + \beta_4^{\text{final}}) + 1/2\ \beta_3 + 1/2\ \beta_2$$

In the calculation for the expected isotope distribution for a mechanism described by model i, the amounts of G_0, G_1, G_2, and G_4 were calculated in the following way:

> G_i = (mole fraction of β_j) x (probability of hydrogen- or deuterium - transfer from β_j to B_k) x (mole fraction of B_k) x (probability of hydrogen - or deuterium - transfer from B_k to give G_i).

The probability of hydrogen, or deuterium, transfer equals the statistical probabilities of transfer of either of these two isotopes times the appropriate kinetic isotope effect, Δ_1 or Δ_2 if hydrogen is transferred and one for transfer of deuterium. As no G_2 can be formed in the absence of β_1 or β_3, which are only formed by back reaction which we are neglecting in these preliminary calculations, the presence of small amounts of G_2 has been ignored.

If we now consider the "two-equivalent-hydrogen" model, G_0 can be produced by reaction of β_0 with B_0:

$$G_0 = \beta_0(\Delta_1)(1)(B_0)(\Delta_2)(1)$$
$$= \beta_0 B_0 \Delta_1 \Delta_2$$

G_0 can also be formed by reaction of β_0 with B_1:

$$G_0 = \beta_0(\Delta_1)(1)(B_1)(\Delta_2)(1/2)$$
$$= 1/2\ \beta_0 B_1 \Delta_1 \Delta_2$$

Similar considerations for G_1, G_3 and G_4 give

$$G_0 = \beta_0 B_0 \Delta_1 \Delta_2 + 1/2\ \beta_0 B_1 \Delta_1 \Delta_2$$

$$G_1 = 1/2\ \beta_0 B_1 \Delta_1$$

$$G_2 = 0$$

$$G_3 = 1/2\ \beta_4 B_0 \Delta_2$$

$$G_4 = \beta_4 B_1 + 1/2\ \beta_4 B_0$$

Application of the steady-station condition based on the assumption that B_0 and B_1 are small and that $dB_0/dt = dB_1/dt = 0$, yield

$$\frac{dB_0}{dt} = 1/2\ \beta_0 B_1 \Delta_1 - 1/2 \beta_4 B_0 \Delta_2 = 0$$

$$\frac{B_0}{B_1} = \frac{\beta_0 \Delta_1}{\beta_4 \Delta_2}$$

Taken together with the condition that $B_0 + B_1 = 1$, these equations give values for B_0 and B_1 in terms of β_0, β_4, Δ_1 and Δ_2 which can in turn be used in the equations for G_i.

Appropriate equations were derived in an analogous manner for each of the other models discussed above. Equations were also derived for the two-equivalent-hydrogen and three-equivalent-hydrogen models which took account of the occurrence of reaction from intermediate back to β-methylaspartate, thus including β_1, β_2, and β_3 taking explicit account of the formation of G_2. These equations were programed for the computer. Where appropriate both Δ_1 and Δ_2 were varied (from 1.0 to 8.0) and comparisons made between the anticipated and observed distributions of G_0, G_1, G_3, and G_4 (and in cases where back reaction was included, G_2). These comparisons between calculated and observed distribution were made using the following equation for absolute error:

$$\text{Error} = 1/n \sum_{i=1}^{n} (G_{i-1}^{\text{observed}} - G_{i-1}^{\text{calculated}})^2$$

where $n = 4$ for calculations neglecting back reaction and $n = 5$ for those cases where back reaction was included. The values of Δ_1 and Δ_2 which give the best agreements and the accuracy with which the various models correlate calculated and observed isotope distributions are summarized in Tables XII to XV.

The data in these tables show that the best model is that in which the migrating hydrogen becomes one of three equivalent hydrogens at some intermediate stage of the isomerization. The agreement is especially good for the three-equivalent-hydrogen model when the occurrence of back

Table XII. Best Fits for Various Models

[For m/e 84 Range of 21.2% Reaction]

Model	Δ_1	Δ_2	Calculated G_0	G_1	G_2	G_3	G_4	Error
i Two equivalent hydrogens	4.6	3.9	80.7	7.0		7.0	5.4	4.96
ib Two equivalent hydrogens with back reaction	5.0	4.1	80.1	7.6	0.4	7.2	4.7	2.81
ii Three equivalent hydrogens	6.7	1.8	78.5	8.5		8.5	4.6	1.89
iib Three equivalent[b] hydrogens with back reaction	7.6	1.5	78.7	8.5	0.4	8.1	4.4	1.40
iii One hydrogen merry-go-round	5.8	a	68.5	14.3		14.3	3.0	41.86
iv Two hydrogen merry-go-round	5.8	a	68.5	14.3		14.3	3.0	41.86
v Three hydrogen	4.3	4.9	63.1	17.6		14.4	4.9	83.71
Observed Distribution			78.1	10.6	0.3	6.5	4.5	

[a] The calculated values for these models are independent of the value of Δ_2.

[b] The equation used to derive these distributions are

Table XII (Continued)

$$G_0 = \beta_0 \Delta_1 \Delta_2 (B_0 + \tfrac{2}{3} B_1 + \tfrac{1}{3} B_2) + \tfrac{1}{3} \beta_1 \Delta_2 (\tfrac{2}{3} B_0 + \tfrac{1}{3} B_1)$$

$$G_1 = \beta_0 \Delta_1 (\tfrac{1}{3} B_1 + \tfrac{2}{3} B_2) + \tfrac{2}{3} \beta_1 \Delta_1 \Delta_2 (B_0 + \tfrac{2}{3} B_1 + \tfrac{1}{3} B_2) + \tfrac{1}{3} \beta_1 (\tfrac{1}{3} B_0 + \tfrac{2}{3} B_1 + B_2) + \tfrac{1}{2} \beta_2 \Delta_2 (\tfrac{2}{3} B_0 + \tfrac{1}{3} B_1)$$

$$G_2 = \tfrac{2}{3} \beta_1 \Delta_1 (\tfrac{1}{3} B_1 + \tfrac{2}{3} B_2) + \tfrac{1}{2} \beta_2 \Delta_1 \Delta_2 (B_0 + \tfrac{2}{3} B_1 + \tfrac{1}{3} B_2) + \tfrac{1}{2} \beta_2 (\tfrac{1}{3} B_0 + \tfrac{2}{3} B_1 + B_2) + \tfrac{2}{3} \beta_3 \Delta_2 (\tfrac{2}{3} B_0 + \tfrac{1}{3} B_1)$$

$$G_3 = \tfrac{1}{2} \beta_2 \Delta_1 (\tfrac{1}{3} B_1 + \tfrac{2}{3} B_2) + \tfrac{1}{3} \beta_3 \Delta_1 \Delta_2 (B_0 + \tfrac{2}{3} B_1 + \tfrac{1}{3} B_2) + \tfrac{2}{3} \beta_3 (\tfrac{1}{3} B_0 + \tfrac{2}{3} B_1 + B_2) + \beta_4 \Delta_2 (\tfrac{2}{3} B_0 + \tfrac{1}{3} B_1)$$

$$G_4 = \tfrac{1}{3} \beta_3 \Delta_1 (\tfrac{1}{3} B_1 + \tfrac{2}{3} B_2) + \beta_4 (\tfrac{1}{3} B_0 + \tfrac{2}{3} B_1 + B_2)$$

$$B_0 = (\tfrac{2}{3} \beta_0 + \tfrac{4}{9} \beta_1 + \tfrac{1}{3} \beta_2 + \tfrac{2}{9} \beta_3)^2 \Delta_1^2$$

$$B_1 = 2(\tfrac{2}{3} \beta_0 + \tfrac{4}{9} \beta_1 + \tfrac{1}{3} \beta_2 + \tfrac{2}{9} \beta_3)(\tfrac{2}{9} \beta_1 + \tfrac{1}{3} \beta_2 + \tfrac{4}{9} \beta_3 + \tfrac{2}{3} \beta_4) \Delta_1 \Delta_2$$

$$B_2 = (\tfrac{2}{9} \beta_1 + \tfrac{1}{3} \beta_2 + \tfrac{4}{9} \beta_3 + \tfrac{2}{3} \beta_4)^2 \Delta_2^2$$

Table XIII. Best Fits for Various Models

[For m/e 130 Range of 21.2% Reaction]

Model	Δ_1	Δ_2	Calculated G_0	G_1	G_2	G_3	G_4	Error
i (same as XII)	4.8	4.0	81.4	6.8		6.8	5.1	5.24
ib	5.0	4.1	80.1	7.6	0.4	7.2	4.7	2.36
ii	6.9	1.9	79.2	8.2		8.2	4.3	2.13
iib[b]	7.8	1.6	78.7	8.6	0.4	8.2	4.1	1.55
iii	5.9	a	69.1	14.0		14.0	2.9	41.92
iv	5.9	a	69.1	14.0		14.0	2.9	41.92
v	4.3	4.9	63.1	17.6		14.4	4.9	90.27
Observed Distribution			78.5	10.2	1.2	6.1	4.0	

Table XIV. Best Fits for Various Models

[For m/e 84 range of 36.4% Reaction]

Model	Δ_1	Δ_2	Calculated G_0	G_1	G_2	G_3	G_4	Error
i (same as XII)	4.9	3.5	79.0	7.4		7.4	6.2	9.89
ib	8.0	2.2	79.5	8.1	0.5	7.2	4.7	7.41
ii	5.3	4.0	75.7	9.7		9.7	4.9	1.59
iib[b]	7.3	2.1	74.8	10.5	0.6	9.7	4.4	0.62
iii	6.0	a	66.4	15.1		15.1	3.4	31.88
iv	6.0	a	66.4	15.1		15.1	3.4	31.88
v	4.9	4.9	63.1	17.6		14.4	4.9	51.34
Observed Distribution			74.8	11.8	0.2	8.5	4.7	

Table XV. Best Fits for Various Models

[For m/e 130 Range of 36.4% Reaction]

Model	Δ_1	Δ_2	Calculated G_0	G_1	G_2	G_3	G_4	Error
i (as in XII)	4.4	3.2	76.7	8.0		8.0	7.4	9.40
ib	4.6	3.3	74.1	9.6	0.6	8.9	6.8	2.61
ii	4.4	4.5	73.3	10.4		10.4	6.0	2.07
iib[b]	7.0	1.5	72.9	10.8	0.7	10.1	5.6	1.51
iii	5.4	a	63.7	16.1		16.1	4.1	39.66
iv	5.3	a	63.2	16.3		16.3	4.2	42.84
v	4.9	4.9	63.1	17.6		17.6	4.9	51.19
Observed Distribution			72.5	12.0	2.0	8.0	5.5	

reaction is included in the calculations. Moreover, the values of Δ_1 that give the best agreement between calculated and observed isotope distributions are in good agreement with k_H/k_D calculated for the rate-determining hydrogen abstraction from β-methylaspartate. In the region near the minima, the variation of calculated distribution with changes in Δ_1 and Δ_2 is small and as a result the combination of isotope effects, Δ_1 and Δ_2, is defined only within relative broad limits, e.g., $\Delta_1 \sim 6 \pm 1.5$, $\Delta_2 \sim 2 \pm 0.5$ for the three-equivalent-hydrogen model.

Also distinguishing between a three-equivalent-hydrogen model and one with four-, or five-equivalent hydrogens is difficult because the change from a model with three-equivalent-hydrogens to a model with four-equivalent hydrogens does not drastically alter the calculated isotopic distributions. Thus, though in general the experimental results fit better a model with three-equivalent hydrogens, the elimination of a model with four-equivalent hydrogens is not unambiguously possible on the basis of these data alone.

In addition to the poor agreement between the calculated and observed isotope distribution for merry-go-round models, another objection can be raised. These models fail to provide a clear pathway for formation of β-methylaspartate containing one, two or three deuteriums (β_1, β_2, β_3). For example, if deuterium is removed from β_4, this same deuterium should be transferred back if the intermediate reverted to β-methylaspartate.

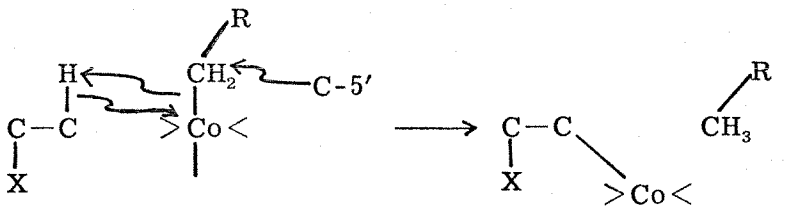
The isotope effect for transfer of hydrogen or deuterium from intermediate to give glutamate (Δ_2) requires comment. If the glutamate, once formed, does not interact again to reform an enzyme intermediate,

Δ_2 would be the kinetic isotope effect in this second transfer averaged over the extent of reaction that has occurred. The occurrence of back reaction from glutamate will cause Δ_2 to approach a value which will represent the differences in energy of the intermediate relative to free glutamate with hydrogen or deuterium bonded to the appropriate atom. Such an equilibrium type isotope effect would probably be near one. The small amount of back reaction of glutamate under the conditions of this work suggests that this possibility is only a negligible perturbation to the contention that Δ_2 represents the kinetic isotope effect in the second transfer of hydrogen from intermediate to glutamate.

One might anticipate that Δ_1 and Δ_2 would have similar values (as they did in the earlier work on methylmalonyl coenzyme A mutase (Miller and Richards, 1969)) but Δ_2 is computed to be significantly smaller than Δ_1, which might indicate that the differences in the ground state vibrational energies of C-H vs C-D are much smaller in the intermediate than in the substrates, glutamate or β-methylaspartate.

The result of this experiment and the foregoing discussion indicate that, as in the case of methylmalonyl coenzyme A mutase, so in the present case of β-methylaspartate mutase, transfer of hydrogen is intermolecular and involves an intermediate stage where the migrating hydrogen has become one of three equivalent hydrogens -- probably involving the conversion of the C-5′ methylene carbon of the deoxyadenosyl moiety of coenzyme B_{12} to a methyl group in the intermediate.

Mechanism: The experimental results of importance for our discussion of mechanism can be summarized: (i) isotope exchange occurs between the migrating hydrogen of a number of substrates and the hydrogens attached to C-5′ of the deoxyadenosyl moiety of coenzyme B_{12}, (ii) primary kinetic isotope effects in the range $k_H/k_D \sim 2$ to 8 are observed when the migrating hydrogen is replaced by deuterium, and (iii) in the case of methylmalonyl coenzyme A mutase and the present work on glutamate mutase, the migrating hydrogen has been shown to become one of three equivalent hydrogens. These observations lead us to support a mechanism which features, as its first step, cleavage of the C-5′ carbon-cobalt bond in the coenzyme B_{12}, cleavage of the bond between the migrating hydrogen and the carbon to which it was attached, and formation of new bonds between cobalt and the carbon of substrate from which hydrogen was removed and between C-5′ and the migrating hydrogen.



This interchange of groups has several perplexing features not the least of which is the umprecedented nature of the postulated bond shifts. Though we presently lack a significant body of known chemical changes to support these changes, we can speculate on possible mechanisms for them.

Two points are especially relevant. The structure of coenzyme B_{12} has the corrin ring somewhat puckered up toward the deoxyadenosyl moiety so that approach of substrate to allow a concerted bond rearrangement

is sterically forbidden. Secondly, recent findings of epr signals characteristic of B_{12r} in ethanolamine deaminase (Babior, 1970) and ribonucleotide reductase (Hamilton et al., 1971) suggest that homolytic scission of the carbon-cobalt bond may be important. These considerations lead to the detailed mechanistic possibility of Scheme I. This produces a Co^{II} species which has the advantage that the binding of the dimethylbenzimidazole group would be significantly weakened relative to its attachment to a Co^{III} or Co^{I} species (Brodie, 1969) thus allowing relaxation of the groups around cobalt and making it more accessible to substrate.

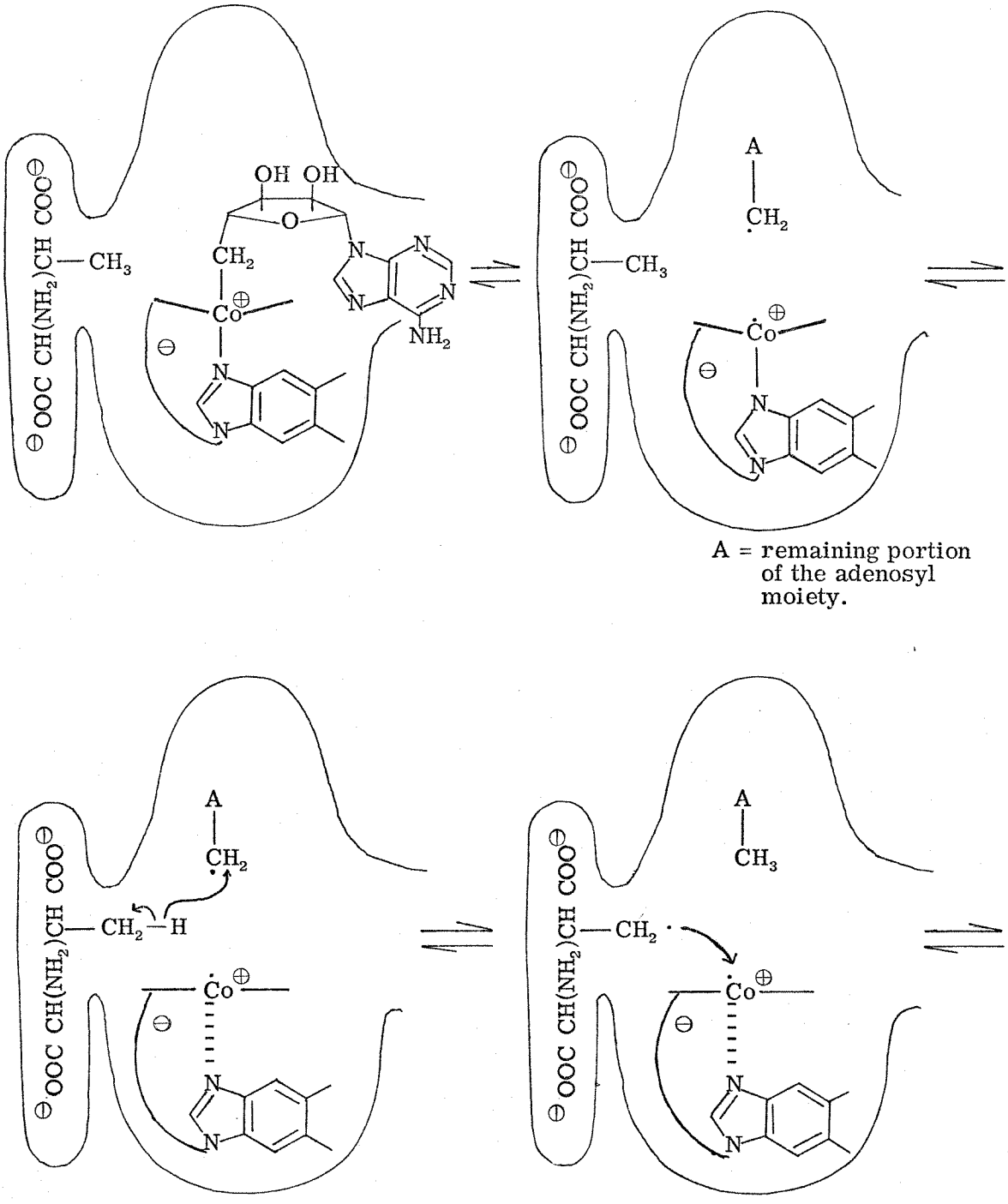
This process also generates 5′-deoxyadenosine which is presumably held near the active site by groups on the enzyme until a reverse hydrogen transfer releases product and reforms the cobalt-C-5′ bond. Some evidence for the intermediate presence of the 5′-deoxynucleoside comes from the observation that the use of 5′-deoxyinosylcobamide with propanediol and propanediol dehydrase releases 5′-deoxyinosine to solution (Jayme and Richards, 1971). (In the absence of any one of the three components -- coenzyme, enzyme, substrate -- no 5′-deoxyinosine is released.)

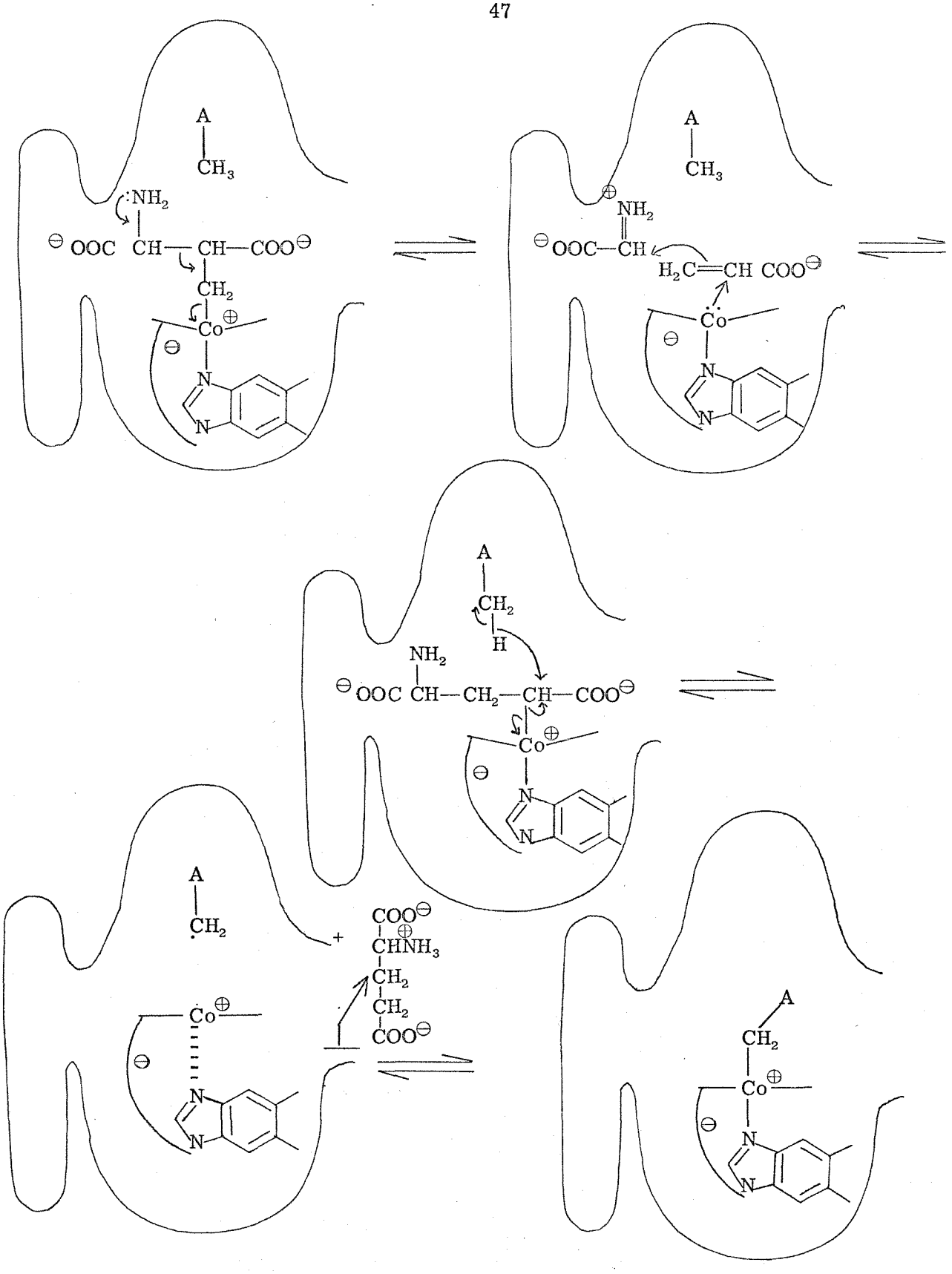
The observed kinetic isotope effect is also in the region of some reported for radical induced hydrogen abstractions. For example, k_H/k_D = 4.5 has been observed for the reaction of phenyl radicals with toluene (Bridger and Russell, 1963) and k_H/k_D = 7 for reaction of methyl radicals with toluene (Wilen and Eliel, 1958).

An earlier version of an analogous mechanism (Miller and Richards, 1969) utilized the ether oxygen of the ribose ring in the interaction between coenzyme and substrate, a possibility which now seems eliminated by the unanticipated finding that an analogue of coenzyme B_{12} with a methylene group in place of this ether oxygen (Kerwar et al., 1970).

Scheme I

Mechanistic Scheme



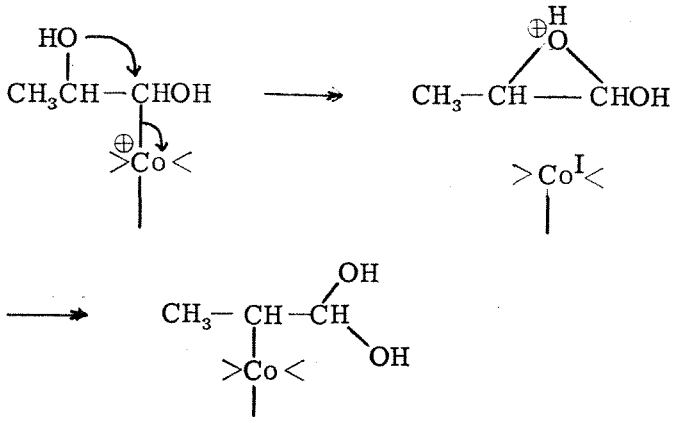


The importance of cobalt in rearrangement processes has been demonstrated in interconversion of model substances, for example, α- and β-substituted ethylcobaloximes (Schrauzer and Windgassen, 1967). The importance of a Co^{I} nucleophile in rearrangement processes has also been shown, for example, in propanediol dehydrase which is significantly inhibited in the presence of nitrous oxide (Schrauzer, 1971). (Oxidation states of cobalt, other than I are not subject to such inhibition.) Under similar conditions, no inhibition was observed for methylmalonyl coenzyme A mutase (Schrauzer, 1971). The subsequent intermediates in the rearrangement sequence of Scheme I could explain such a result as the relative instability of the carbonyl coenzyme A (or glycyl) migrating group might limit the completeness of acquisition of the electron pair by cobalt and therefore its character as a Co^{I} nucleophile and, in turn, its susceptibility to attack by nitrous oxide. However, in the case of propanediol dehydrase, the intermediate should have sufficient Co^{I} character to react with nitrous oxide and account, thereby, for the partial inhibition observed.

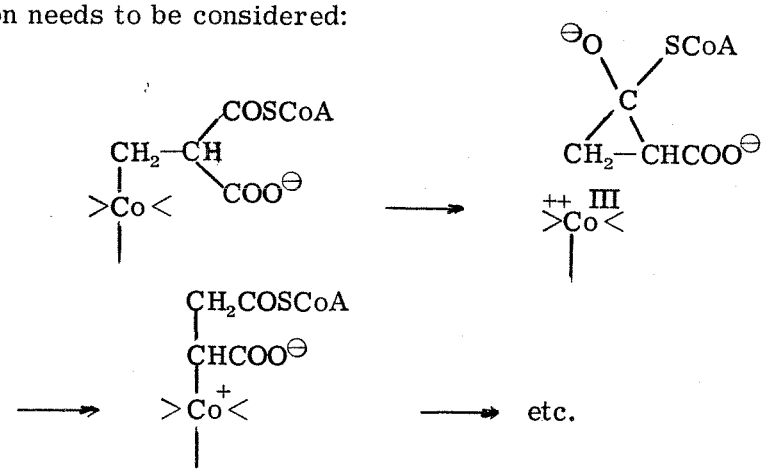
The state of ionization of the amino group of β-methylaspartate at the active site assumes importance for a mechanism such as that of Scheme I as only the neutral form will be able to supply electrons to facilitate rearrangement of a carbonium ion skeleton.

This aspect of the B_{12} mechanism essentially involves a flow of electrons from the substrate moiety to cobalt. The migrating group assumes positive character and the cobalt is "reduced" to Co^{I}. In the case of propanediol dehydrase, for example, the electron flow could lead

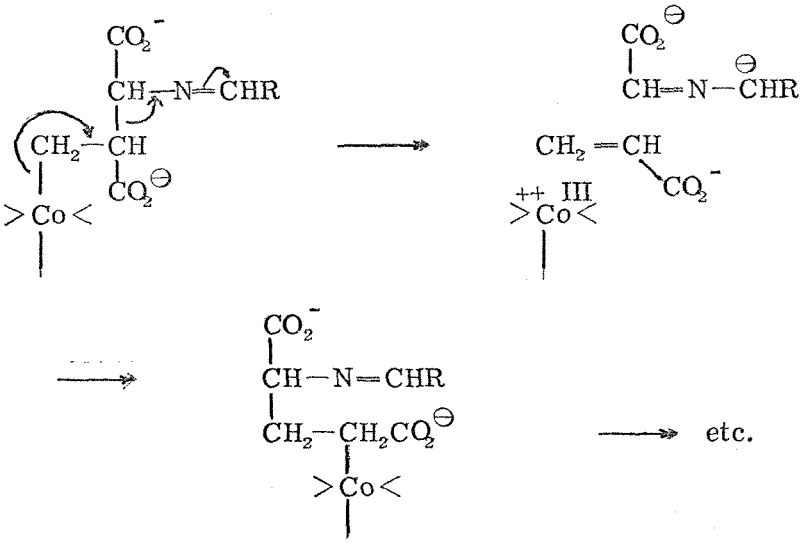
to migration by way of the oxide (with or without an attached proton).



Too little is known about the chemistry of these systems however to require electron flow of this type and release of electrons from the cobalt (which becomes thereby Co^{III}) and rearrangement of a carbon ion skeleton needs to be considered:



In the case of the rearrangement of β-methylaspartate, such migration might be facilitated by interaction of the amino acid fragment with pyridoxal phosphate.



Finally, rearrangement of radicals themselves cannot be eliminated.

In the foregoing discussion, we have tacitly assumed that the rearrangement reactions and dehydrase reactions in which coenzyme B_{12} is required, all share a common mechanistic scheme, at least in broad outline. Though evidence demanding such a simplifying assumption is not presently available, the scheme involving formation of a bond from cobalt to substrate and rearrangement of the substrate carbon skeleton as a carbonium ion with an adjacent cobalt I can rationalize all the known facts of coenzyme B_{12} dependent reactions.

Conclusion: This work demonstrates that in the isomerization of β-methylaspartate to glutamate, the hydrogen which migrates becomes equivalent with two other hydrogens of the coenzyme. The mechanistic implications of this have been discussed already. Though no specific role has been assigned to the enzyme itself, we do not suggest that the protein is unimportant, but that our present state of knowledge of these reactions allows only the general observations that the protein correctly positions

a particular substrate and coenzyme for reaction. Two more well-defined roles for the protein are to keep the 5′-deoxyadenosine bound to the complex throughout the reaction and possibly to provide some electron source to accept the migrating group, if in fact the isomerization involves a flow of electrons toward cobalt as shown in Scheme I.

Experimental: 3-Tri-^{2}H-Methyl 2-^{2}H-Aspartic Acid. The deuterated amino acid was made by an analogous scheme as the reported preparation of the triated compound (Switzer et al., 1969). Itaconic acid was converted to deuterated mesaconate by three treatments with deuterated water and sodium deuteroxide. Treatment with dilute ammonium hydroxide (pH 9) yielded the diammonium salt of the deuterated mesaconate. The nmr and mass spectrum of this compound showed less than 0.5% hydrogen in the methyl or the vinyl positions. The deuterated diammonium mesaconate (1.48 g, 8.8 m mole) was converted to L-threo-3-tri-$^2H_2$-methyl 2-^{2}H-aspartic acid in a reaction mixture containing 40 mM potassium phosphate buffer, pH 8.2, 1 mM magnesium chloride, and 2400 units of β-methylaspartase in a total volume of 8 ml at pH 8.2. After incubation at 35°C for 45 min, the decrease in 240 mμ absorbance of the reaction mixture indicated that the reaction was complete. The solution was deproteinized by chilling, adding glacial acetic acid to pH 5.0 and heating at 90-95°C for 3 min. After filtering, conc hydrochloric acid was added to pH 3.1. Absolute ethanol (9 ml) was added, and the β-methylaspartate was allowed to crystallize with 95% ethanol and ethyl ether. Recrystallization from a minimal amount of water yielded 0.82 g (61.9%) 3-tri-^{2}H-methyl 2-^{2}H-aspartic acid. The deuterated β-methylaspartate was shown to have no

erythro isomer and less than 1.2% (none detected) mesaconate by electrophoresis. Absorbance at 240 mμ showed 0.92% mesaconate.

Labeled β-methyl Aspartic Acid Mixture. Equal weights of tetradeutero- and non-deutero-β-methylaspartic acid were mixed and crystallized from water. Absorbance at 240 mμ showed less than 0.25% mesaconate. A 0.2M solution of this mixture was prepared by dissolving 59.6 mg in 0.4 ml 1 N sodium hydroxide and diluting to 2 ml.

Isomerization of the Above Mixture. The reaction mixture (.24 ml) contained 40 mM tris-Cl buffer, pH 8.2, 20 mM mercaptoethanol, 20 mM calcium chloride, 0.5 ml of the above mixture (40 mM), 1×10^{-3} M cyanocobinamide, 3.2×10^{-5}M deoxyadenosylcobalamin, 4.08 units Component S, and 0.84 units Component E. The mixture was prepared by the method of Barker and incubated in a 5 ml syringe in the dark at 37°C. (Switzer et al., 1969). The reaction was followed by removing 2 μl portions and following the amount of mesaconate formed from a reaction with β-methylaspartase. After approximately 1/3 reaction, the reaction was stopped by rapid freezing. The solution was deproteinized by thawing the solution and adding 1 M hydrochloric acid until the color had changed from red to brown. The solution was heated at 100°C for 2 min, centrifuged, and the solution decanted. The deproteinized reaction mixture was acidified to pH 1-2 with 2 drops of 4N hydrochloric acid. A small portion of the reaction mixture was then adsorbed to a 0.9 cm by 53 cm column of AA-15 custom spherical resin (Dowex 50) of a Beckman 120 C amino acid analyzer and eluted at a rate of 68 ml/hr with a sodium citrate buffer (without detergents), pH 3.22. These analytical runs showed one reaction to have

proceeded 21.2% and another 36.4%. The remaining solution was then split into two portions and separated on the same column. The amino acid portions were located by spotting 5 μl portions on paper and staining with ninhydrin. Salts and other buffer constituents were removed according to the procedure of Dréze et al. (Dréze et al., 1954) on column 0.9 cm by 12 cm of Bio Rad AG2-X8 resin. Before use, the columns were washed with 25 ml of 1 M hydrochloric acid in 50% ethanol followed by 25 ml of water. The columns were charged with 100 ml 2 N sodium hydroxide and washed until pH 7 with water. After loading the samples, 5 ml of water was passed through the column and the salts were eluted with 1 N acetic acid until the light colored band was within 1 cm of the bottom. At this point, an additional 25 ml of the acetic acid was used to elute the amino acid. The solutions were evaporated to dryness with a rotary evaporator.

The amino acids were converted to the diethyl esters by the method of Bieman (Bieman et al., 1961) before introduction into the mass spectrometer.

Mass Spectra. All mass spectra were taken with an AEI MS-9 mass spectrometer. The mass spectra of the diethyl esters of the β-methylaspartate and the glutamate samples were taken by introducing the samples in direct inlet probe without external heating. All spectra were taken at 70 eV and as rapidly as possible (approximately 10 minutes). The molar intensities of both β-methylaspartate and deutero β-methylaspartate and both glutamate and deutero glutamate were shown to be the same within experimental error.

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Part II

Kinetic Studies of 3-fluoro-1,2-propanediol, a New Substrate for Dioldehydrase

Introduction

Dioldehydrase (D, L-1,2-propanediol hydro-lyase; EC 4.2.1.28) from Klebsiella pneumoniae (ATCC 8724) catalyzes the conversion of (R)- and (S)-1,2-propanediol to propionaldehyde and of ethylene glycol to acetaldehyde and requires coenzyme B_{12} [α-(5,6-dimethylbenzimidazolyl)-Co-5′-deoxyadenosylcobamide] as an obligatory cofactor (Lee and Abeles, 1963). The rearrangement has been shown to involve the abstraction of a hydrogen atom from C—1 of the propanediol and subsequent rearrangement to propionaldehyde (Browstein and Abeles, 1961). Hydrogen labeling experiments with dioldehydrase (Abeles and Zagalak, 1966; Frey and Abeles, 1966; Frey et al., 1967) and other coenzyme B_{12} dependent enzymes [methylmalonyl CoA mutase (Riley and Arigoni, 1966), ethanolamine deaminase (Babior, 1969), and glutamate mutase (Switzer et al., 1969)] have shown that the C—5′ of the coenzyme B_{12} acts as an intermediate hydrogen atom carrier, and considerable evidence suggests that the two C—5′ hydrogens and the hydrogen abstracted from the substrate are chemically equivalent (Miller and Richards, 1969; Essenberg et al., 1971; Eagar et al., 1972).

Whereas other B_{12} dependent enzymes such as glutamate mutase (Barker et al., 1964) and methylmalonyl CoA mutase (Cannata et al., 1965) show strong stereoselectivity toward substrate binding and

activity, dioldehydrase acts upon both enantiomers of 1,2-propanediol (Lee and Abeles, 1963). Nevertheless, dioldehydrase exhibits remarkable substrate specificity. Small modifications in substrate structure cause large decreases in both binding and activity (Lee and Abeles, 1963; Toraya and Fukui, 1972). Although the enzyme has been shown to catalyze the rearrangement of (R)-1,2-propanediol nearly two times as fast as (S)-1,2-propanediol, the binding affinities of the two isomers have received little attention due to the limitations of the assay method (Lee and Abeles, 1963; Zagalak et al., 1966).

K_m values for 1,2-propanediol have appeared previously in the literature on two separate occasions. The first study reported $K_m = 8.3 \times 10^{-4}$M for both isomers of 1,2-propanediol as well as the racemic mixture (Yamane et al., 1966). In a subsequent paper from the same laboratory, a different K_m was reported. This time only the (RS)-mixture was considered, and the K_m reported was 8.14×10^{-5}M (Toraya and Fukui, 1972). In both cases, K_m values were obtained by measuring the amount of propionaldehyde produced in a given time period at varying initial substrate concentrations. In the first study, the amount of propionaldehyde produced in the given time interval for each initial substrate concentration correlated rather well with the total amount of substrate present initially. In the second report the amount of substrate used during the reaction ranged between 40% and 100%. Thus neither of these values is a reliable one. Furthermore, the observed rate for racemic mixtures of 1,2-propanediol cannot be explained if the K_m values for the two enantiomers are identical. Lee

and Abeles (1963) have reported that maximal velocities are obtained at concentrations of 5×10^{-4}M (RS)-1,2-propanediol and that the analytical procedures used do not allow meaningful measurements of initial velocities at substrate concentrations below this level. The same analytical procedures were employed in the K_m determinations discussed above.

The purpose of the work described in this paper was to investigate the nature of the catalysis and binding for dioldehydrase and indirectly to examine the nature of the carbon-cobalt bond cleavage in the coenzyme moiety. (R)- and (S)-3-fluoro-1,2-propanediol were examined to determine the effect of a non-bulky electron withdrawing substituent on the catalytic rates of rearrangement; and due to an increase in the assay sensitivity for the aldehyde product from their rearrangement (acrolein), the difference in K_m values for the two enantiomers of 3-fluoro-1,2-propanediol could be determined. These K_m values were then compared to approximate values obtained for the two isomers of 1,2-propanediol in order to determine possible binding mechanisms for the two isomers.

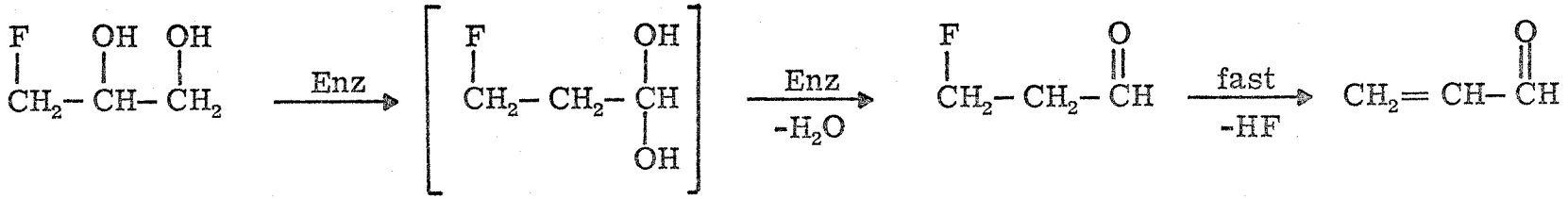
Results

Mechanism of reaction of 3-fluoro-1,2-propanediol. The rate of conversion of 3-fluoro-1,2-propanediol to aldehyde by dioldehydrase is linear for at least thirty minutes. The product aldehyde was identified as acrolein by an acrolein specific assay (Circle et al., 1945) and by nmr of the 2,4-dinitrophenylhydrozine (DNP) adduct. The progress of the reaction was also followed by ^{19}F nmr. The only change that

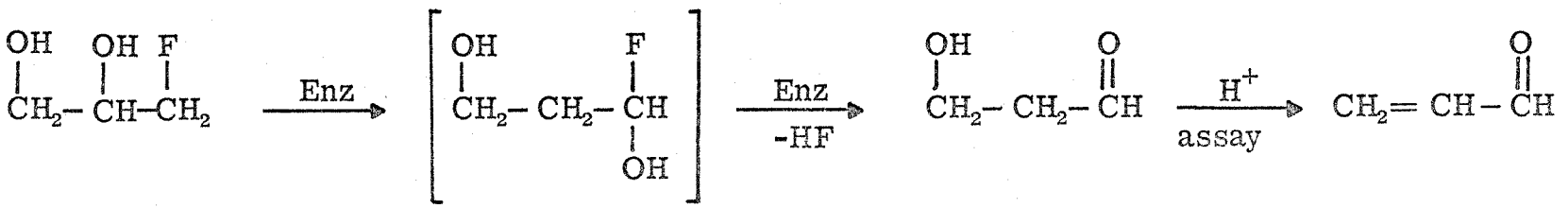
could be detected in the ^{19}F nmr spectrum was due to the production of inorganic fluoride. If β-fluoropropionaldehyde is formed, its lifetime is very short at 35° (nmr probe temperature).

The possibility that the presence of fluorine on C—3 could provide an alternate pathway for the enzymic reaction was considered. Paralleling the regular mechanism, the initial abstraction of hydrogen could be visualized as taking place from C—3 followed by the enzymatic transfer of the hydroxyl at C—2 to C—3. The elimination of HF would result in the formation of β-hydroxypropionaldehyde. This product would, of course, also be identified as acrolein since it would eliminate water under the conditions of the methods used to identify the products (Fig. 1). In order to determine if Pathway B is followed to any extent, both (R)- and (S)-3-fluoro-3,3-dideuterio-1,2-propanediol were examined for reactivity and binding. The dideuterio compounds in both cases behaved identically to the corresponding non-deuterated substrates. In addition the reaction product in each case was isolated as the DNP adduct. The ^{1}H nmr of both adducts indicated only the presence of 3,3-dideuterioacrolein. We conclude that pathway B is not a viable alternate pathway.

Comparative Rate Determinations. A comparison of the reactivities of (R)- (S)-, and (RS)-3-fluoro-1,2-propanediol is shown in Fig. 2. The corresponding rate comparisons for (R)-, (S)-, and (RS)-1,2-propanediol is shown in Fig. 3. The k_{cat} values obtained



Pathway A



Pathway B

Fig. 1. Possible reaction pathways for 3-fluoro-1,2-propanediol.

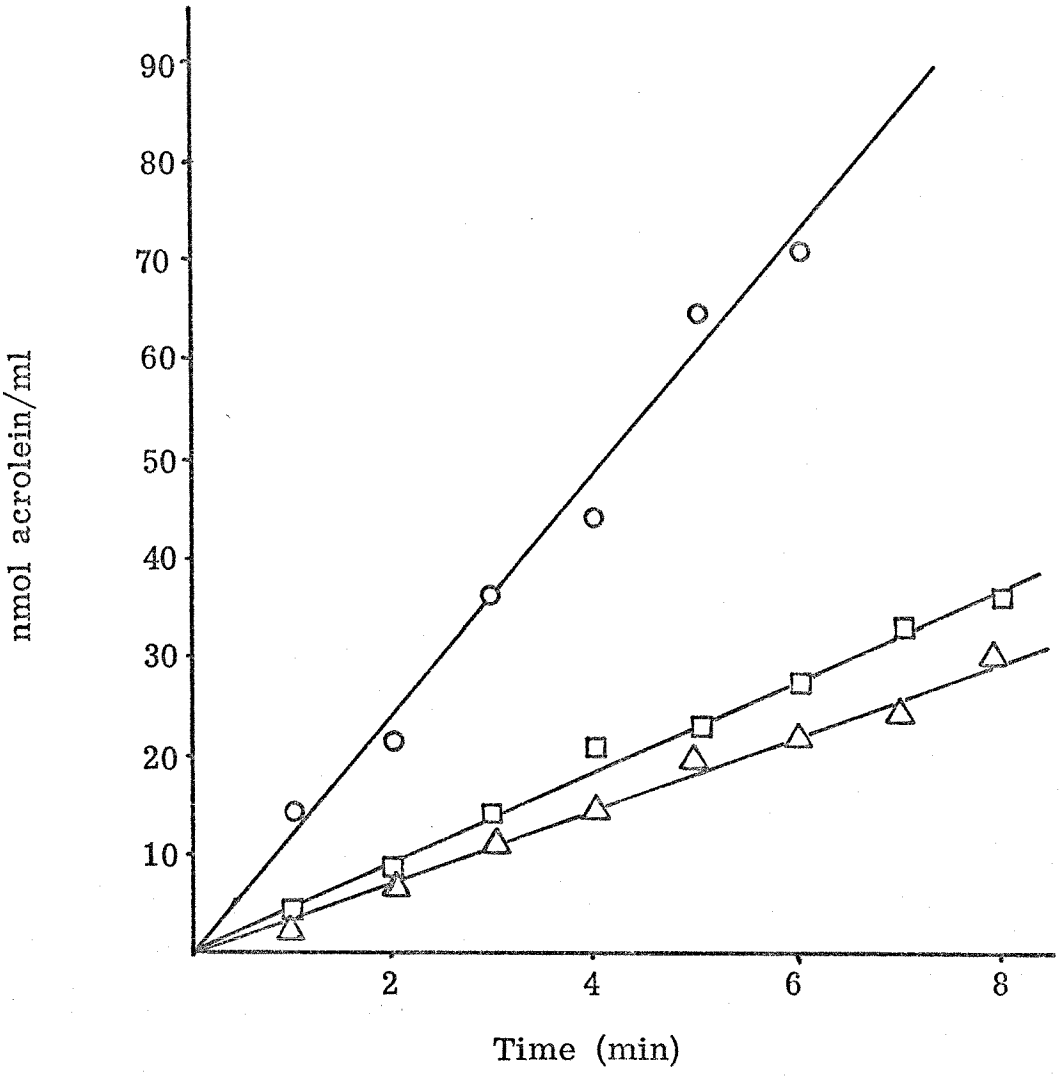


Fig. 2. Relative rates of (S)-, (RS)-, and (R)-3-fluoro-1,2-propanediol. Reaction mixtures consisted of the following: diol-dehydrase, .009 units; potassium phosphate buffer, pH 8.0, 80 μmoles; DBC coenzyme, 40γ; substrate, 100μmoles; BSA, .02mg. Total volume, 2 ml, 37°. O, (S)-3-fluoro-1,2-propanediol;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

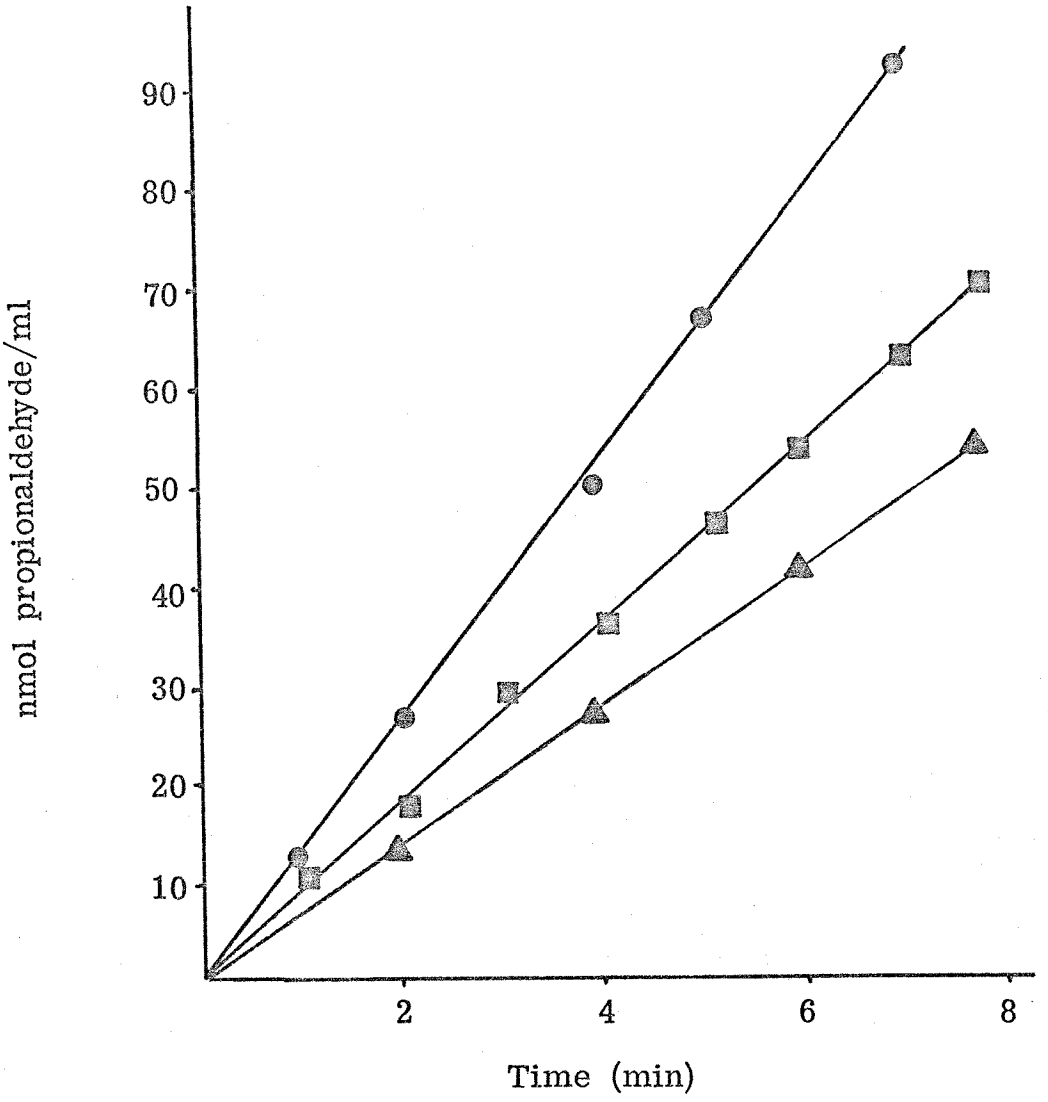


Fig. 3. Relative rates of (S)-, (RS)-, and (R)-1,2-propanediol. Reaction mixtures consisted of the following: dioldehydrase, 0.009 units; potassium phosphate buffer, pH 8.0, 80 μmoles; DBC coenzyme, 40 γ; substrate, 100 μmoles; BSA, 0.02 mg. Total volume, 2 ml, 37°. ●, (R)-1,2-propanediol; ■, (RS)-1,2-propanediol;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

from these plots are summarized in Table I. The convention used for designating the configurations may be the source of some confusion in comparing the fluorodiols to the propanediols. For example, the faster reacting fluorodiol is correctly labeled (S)-fluorodiol although it is the 3-fluoro substituted analogue of (R)-propanediol. Intriguingly the substitution of a fluorine in the 3 position of 1,2-propanediol results in almost no change in k_{cat} for the (R)-isomer whereas the rate of the (S)-isomer is decreased by almost one half. Another curious feature evident in both plots is that the rate of the (RS)- mixture is not equal to the average of the rates of the two isomers, but is more nearly equal to that of the slow isomer. The effect is not as pronounced for 1,2-propanediol as it is for 3-fluoro-1,2-propnaediol but is nevertheless obvious. This indicates that there may be a difference in K_m values between the (R)- and (S)-isomers for both 3-fluoro-1,2-propanediol and 1,2-propanediol.

K_m Determination of 3-Fluoro-1,2-Propanediol. The acrolein produced by the action of dioldehydrase on 3-fluoro-1,2-propanediol can be measured with the same DNP assay used for propionaldehyde with the advantage that the color resulting from acrolein is 2.75 times as intense. The increased sensitivity of the acrolein assay together with a modification of the DNP assay procedure enabled us to measure meaningful initial velocities of 3-fluoro-1,2-propanediol at concentrations as low as 5×10^{-5}M. This permitted determinations

Table I

Substrate†	Vmax (nmol/min)	k_{cat}* (sec^{-1})	K_m X 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	1.1 ±0.33
(RS)-H-diol	8.90 ±0.34	250 ±10	0.83 ±0.25
(S)-H-diol	6.82 ±0.17	191 ± 5	0.65 ±0.20

† F-diol = 3-fluoro-1,2-propanediol; H-diol = 1,2-propanediol.

* Based on a M.W. of 250,000 and a specific activity of 60 units/mg. (Essenberg et al., 1971).

to be made of the rate dependence on substrate concentration for (R)-, (S)-, and (RS)-3-fluoro-1,2-propanediol. The results obtained are presented as the Lineweaver-Burk plots in Figs. 4, 5 and 6, and the K_m values are summarized in Table I.

The K_m value for (S)-3-fluoro-1,2-propanediol was found to be considerably larger than that of the (R)-isomer and (RS)-mixture. This suggested that a determination of the K_m for the (R)-isomer of 1,2-propanediol may be possible.

K_m Determination of 1,2-Propanediol. The modified DNP assay for propionaldehyde permitted meaningful determinations of initial velocities to be made only at initial propanediol concentrations at or above 1.4×10^{-4}M. This was sufficient, however, to observe subsaturation behavior for (R)-1,2-propanediol (Fig. 7) and ascertain that both the (S)-isomer and the (RS)-mixture were nearly saturated at 1.4×10^{-4}M. Approximate K_m values were obtained for the (S)-isomer and the (RS)-mixture by defining the reaction curve; and estimating, by tangent fitting, the point on the curve where the rate became equal to 1/2 V_{max} (Figs. 8 and 9). This is admittedly not a very precise method. The values obtained are however, the best available at this time, and they at least clearly indicate that the K_m values for the two isomers are not the same.

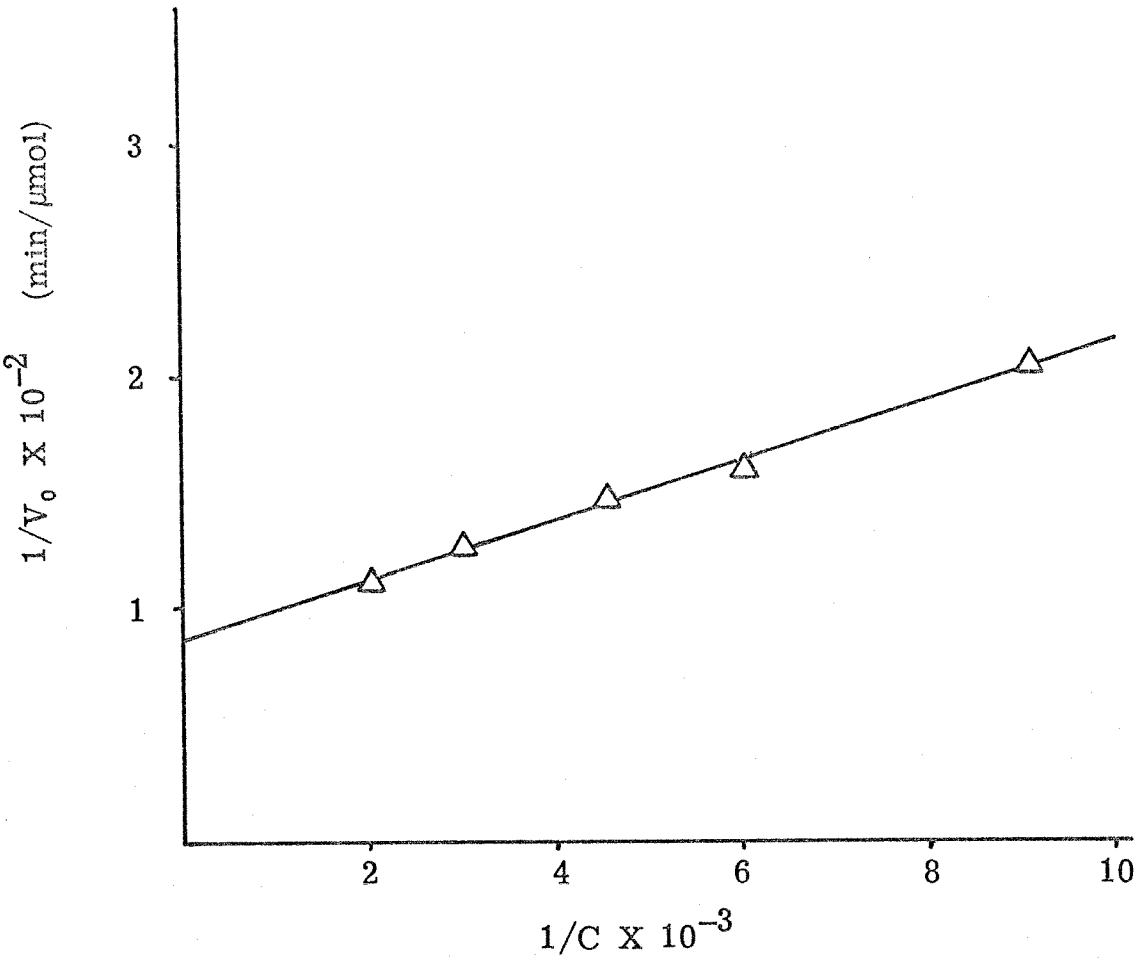


Fig. 4. The Lineweaver-Burk plot for (R)-3-fluoro-1,2-propanediol. Reaction mixtures consisted of the following; apoenzyme, 0.028 units; potassium phosphate buffer, pH 8.0, 80 μmoles; DBC coenzyme, 40 γ; BSA, 0.02 mg; varying amounts of substrate. Total volume, 2 ml, 37°. The production of acrolein was 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.

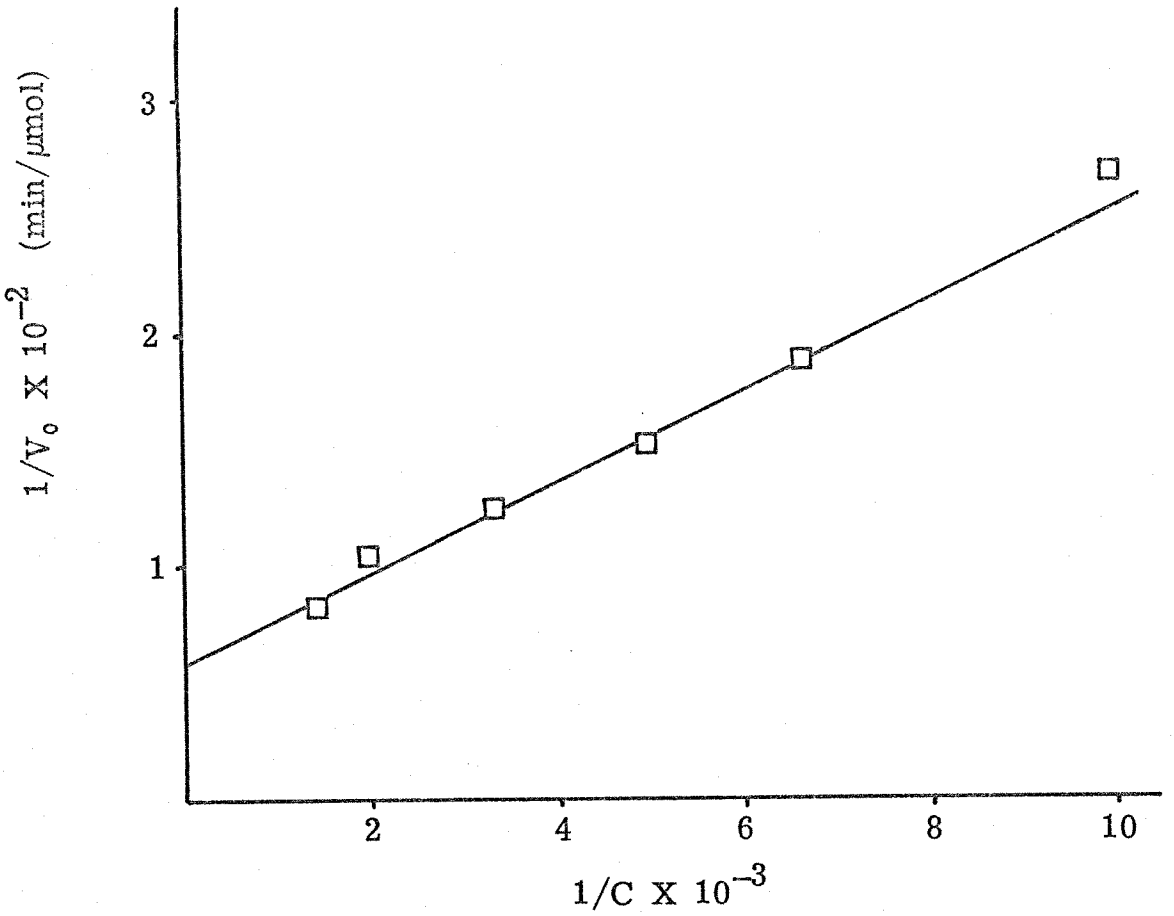


Fig. 5. The Lineweaver-Burk plot for (RS)-3-fluoro-1,2-propanediol. Reaction mixtures consisted of the following; apoenzyme, 0.032 units; potassium phosphate buffer, pH 8.0, 80 μmoles; DBC coenzyme, 40 γ; BSA, 0.02 mg; varying amounts of substrate. Total volume, 2 ml, 37°. The production of acrolein 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.

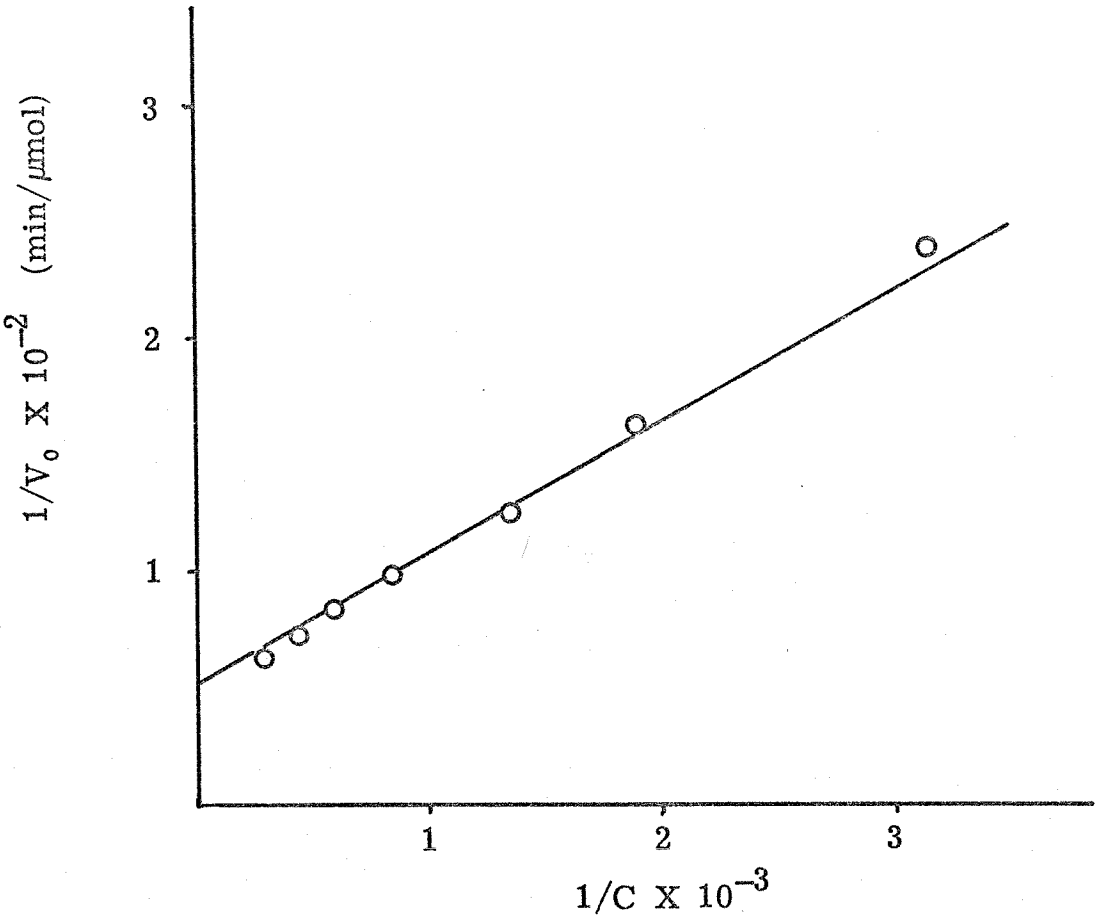


Fig. 6. The Lineweaver-Burk plot for (S)-3-fluoro-1,2-propanediol. Reaction mixtures consisted of the following: apoenzyme, 0.016 units; potassiun phosphate buffer, pH 8.0, 80 μmoles; DBC coenzyme, 40 γ; BSA, 0.02 mg; varying amounts of substrate. Total volume, 2 ml, 37°. The production of acrolein was 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.

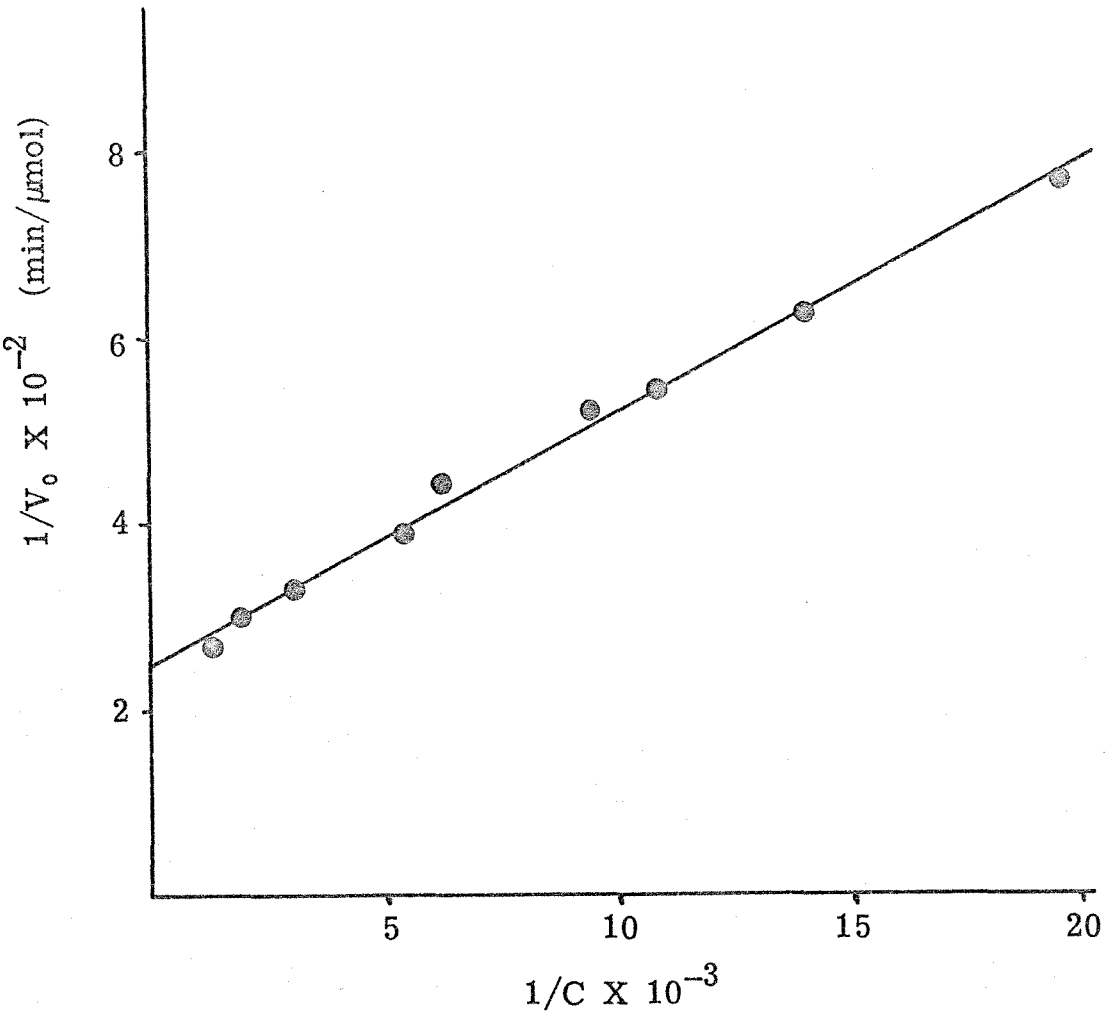


Fig. 7. The Lineweaver-Burk plot for (R)-1,2-propanediol. Reaction mixtures consisted of the following; apoenzyme, 0.028 units; potassium phosphate buffer, pH 8.0, 80 μmoles; DBC coenzyme, 40 γ; BSA, 0.02 mg; varying amounts of substrate. Total volume, 2 ml, 37°. The production of propionaldehyde was determined at thirty second intervals for each substrate concentration. In all cases the rate was linear for four minutes. Each initial velocity plotted above represents the slope of the least squares line.

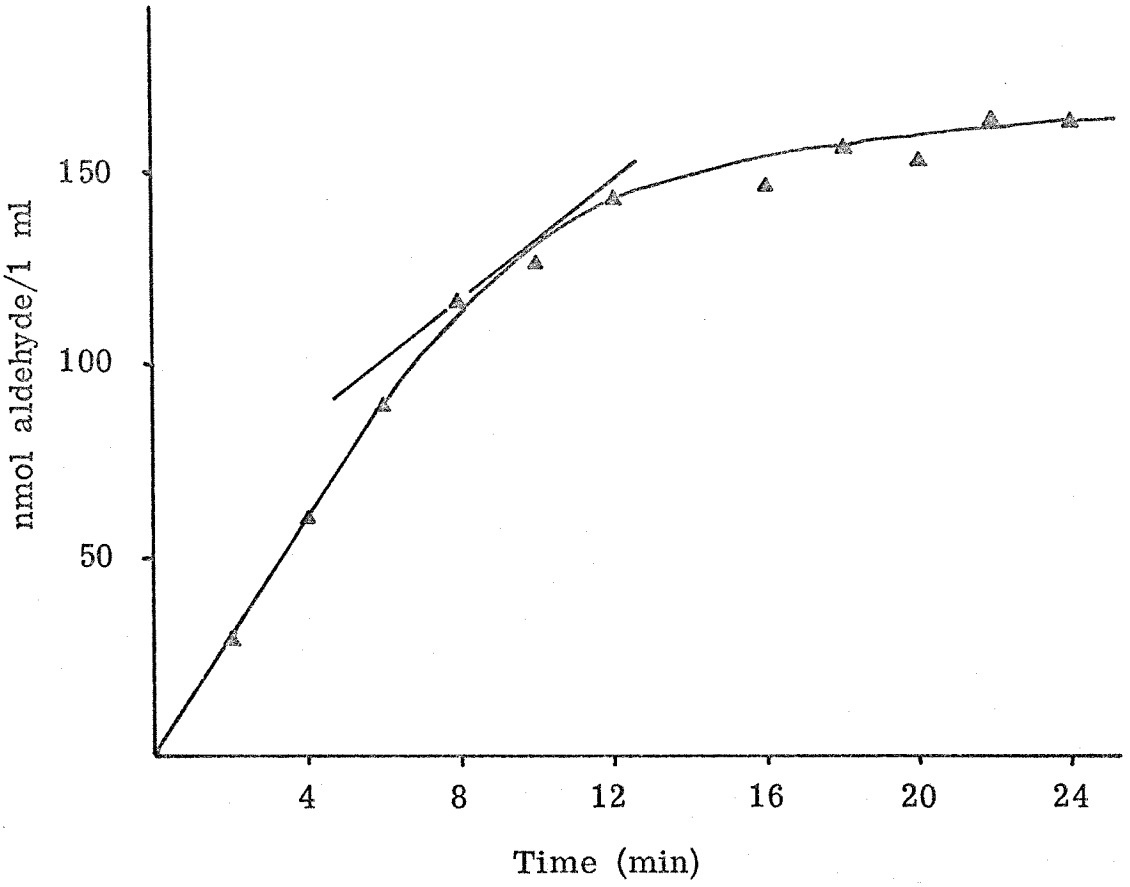


Fig. 8. Reaction profile of (S)-1,2-Propanediol. Reaction mixtures contained: apoenzyme, 0.016 units; potassium phosphate buffer, pH 8.0, 80 μmoles; DBC coenzyme, 40 γ; BSA, 0.02 mg; substrate initially present, 390 nmoles; Total volume, 2 ml; 37°.

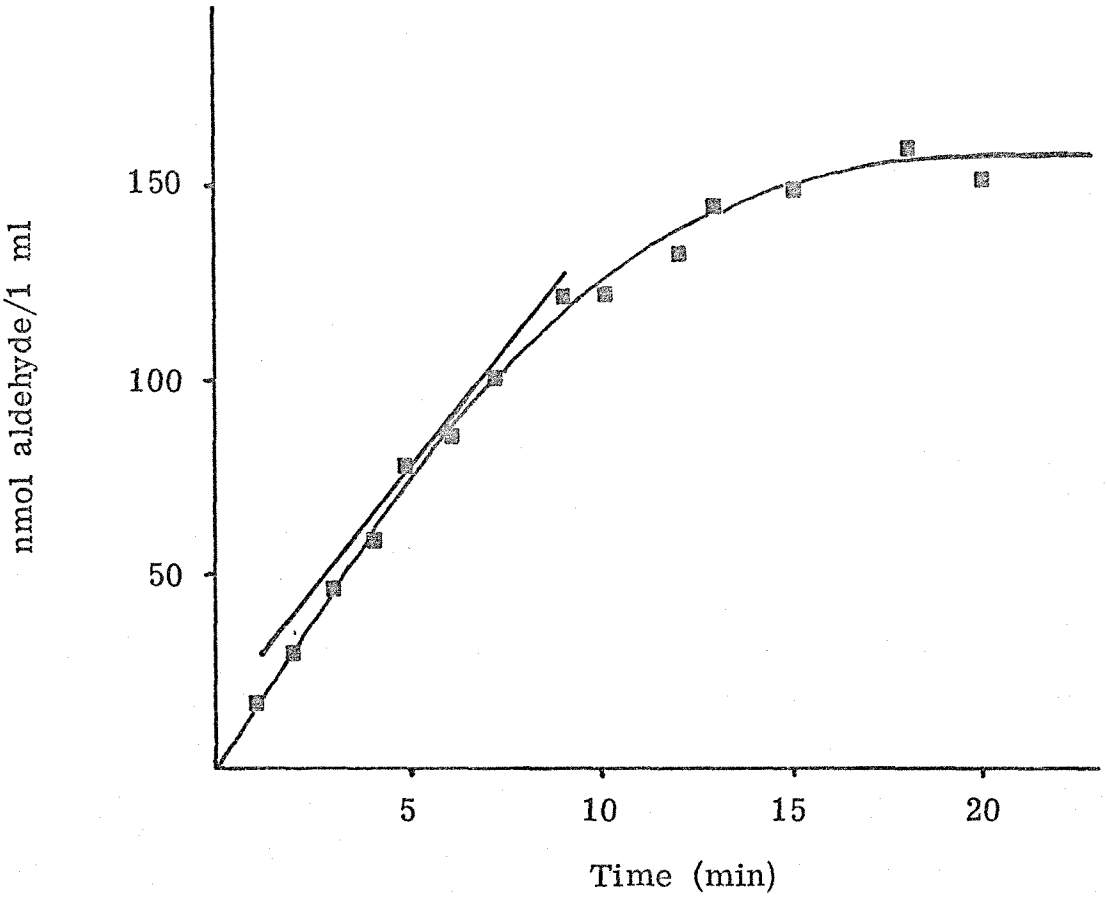


Fig. 9. Reaction profile of (RS)-1,2-Propanediol. Reaction mixtures contained: apoenzyme, 0.025 units; potassium phosphate buffer, pH 8.0, 80 μmoles; DBC coenzyme, 40 γ; BSA, 0.02 mg; substrate initially present, 320 nmoles; Total volume, 2 ml; 37°.

Discussion

Competition for One Site. The experimentally obtained values of K_m and k_{cat} for both substrates indicate that there is competition between the (R)- and (S)-isomers for the same active site. For two competing substrates the equations are:

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

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

Making the usual steady state approximations and solving for [ES] and [ER]:

where

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

$$[ER] = \overline{K}_R[E][S] \qquad \overline{K}_R = \frac{k_4}{k_5 + k_6}$$

and

where

$$E = \frac{E_0}{1 + K_S[S] + K_R[R]}$$

E = free enzyme concentration

E_0 = total enzyme concentration

The rate of the reaction of the (RS)-mixture is given by:

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

where k_{cat} is that observed for the RS mixture.

Thus:

$$k_{cat}\left\{\frac{\overline{K}_S[E_0][S]+\overline{K}_R[E_0][R]}{1+\overline{K}_S[S]+\overline{K}_R[R]}\right\} = \frac{k_3\overline{K}_S[E_0][S]}{1+\overline{K}_S[S]+\overline{K}_R[R]} + \frac{k_6\overline{K}_R[E_0][R]}{1+\overline{K}_S[S]+\overline{K}_R[R]}$$

In a racemic mixture, [R] = [S] and thus the equation reduces to:

$$k_{cat} = \frac{k_3\overline{K}_S + k_6\overline{K}_R}{\overline{K}_S + \overline{K}_R}$$

Using the experimentally determined values of K_m and k_{cat} from Table 1 for the (R)- and (S)-isomers, a k_{cat} for the racemic mixture can be calculated. For 1,2-propanediol the calculated value is 257 sec^{-1}, less than 3% different from the experimentally observed value. For 3-fluoro-1,2-propanediol the calculated k_{cat} is 128 sec^{-1}, exactly what is observed experimentally. Thus the (R)- and (S)-isomers of both substrates exhibit simple competitive behavior for the active site.

Reaction Mechanism. The introduction of a single fluorine substituent on the methyl group of 1,2-propanediol should cause a larger change in the electronic properties than in the steric properties of the molecule. The fluoro-substituent is not much larger than a proton; (van der Waals radii fluorine = 1.35A, hydrogen = 1.2A) but considerably more electronegative (Pauling's Electronegativity scale,

fluorine = 4.0; hydrogen = 2.1). Almost no decrease is seen in the reactivity in the case of one isomer and only a 50% decrease in the other. This provides further support for a mechanism that does not involve the development of charge for the enzymic reaction (Finlay et al., 1973). An ionic mechanism should be affected to much larger degree by the electronegative fluorine substituent (Nigh and Richards, 1969). In addition, the electronic effect of fluorine should be the same for both isomers. Sterically, however, the effect of the fluoro substituent on the enzymatic reaction need not be the same for both isomers. Thus the larger depression in rate seen for one isomer could be entirely due to steric reasons.

Binding. The ratio of K_m values; (R)-/(RS)-/(S)-1,2-propanediol is 1.7/1.27/1.0. This is nearly the same as the corresponding ratio of k_{cat} values which is 1.9/1.25/1.0. This indicates that k_{cat} may be large compared to k_2

where,

$$K_m = \frac{k_2 + k_3}{k_1} \qquad k_3 = k_{cat}$$

If this is true a value for k_2 and k_1 can be calculated by assuming k_2 and k_1 are the same for both the (R)- and the (S)-isomers. The calculated values are $k_2 = 65\ sec^{-1}$, $k_1 = 3.93 \times 10^6\ moles^{-1}sec^{-1}$. The assumptions that k_2 and k_1 are the same for both isomers, of course, may not be valid. In any case a limiting value for k_1 can be obtained. If $k_2 = 0$, the values for k_1 will be $2.9 \times 10^6 moles^{-1}sec^{-1}$ for the (S)-

isomer and 3.3×10^6 moles^{-1}sec^{-1} for the (R)-isomer. These represent the smallest possible values for k_1 since if $k_2 > 0$, k_1 must be larger than 2.9×10^6 moles^{-1}sec^{-1}. Thus, k_1 must be diffusion controlled for both isomers (Eigen and Hammes, 1963).

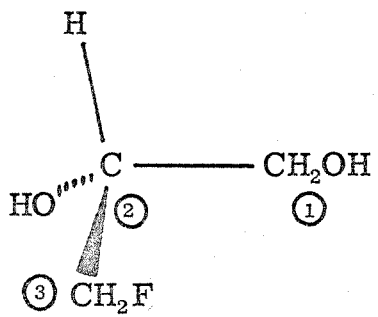
In the case of 3-fluoro-1,2-propanediol, the ratio of K_m values (S)-/(RS)-/(R)-3-fluoro-1,2-propanediol is 8.98/2.04/1.0. No assumption about the relative magnitudes of k_2 and k_3 is possible. However, the binding equilibrium, $K_S = k_2/k_1$, cannot be the same for both the (R)- and (S)-isomers. Either k_1, k_2 or both must be different for each isomer. Conceivably, the observed correspondence in the ratios of K_m and k_{cat} values for 1,2-propanediol was purely fortuitous. An experimental determination of the equilibrium k_2/k_1, for each isomer will be necessary before a final conclusion can be reached.

Non-productive binding was considered as a possible explanation for the seemingly anomalous behavior of 3-fluoro-1,2-propanediol. Figure 10 depicts the productive and possible non-productive binding modes for 3-fluoro-1,2-propanediol. The circled numbers represent sites on the enzyme. For non-productive binding:

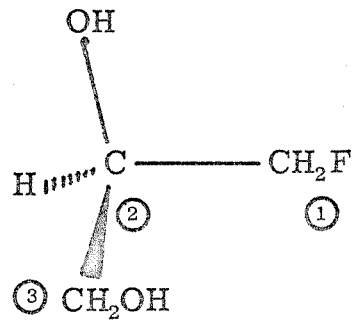
$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \overset{k_3}{\rightarrow} E + P$$

$$E + S \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EB \overset{k_6}{\not\rightarrow} E + P$$

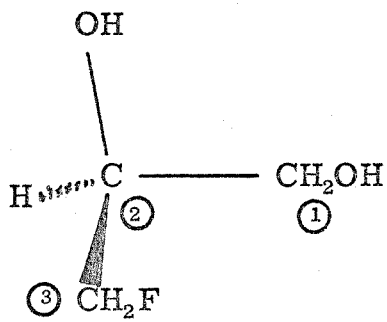
Earlier, k_6 was shown to be ≈ 0. The form of the rate equation used to describe this type of behavior is:



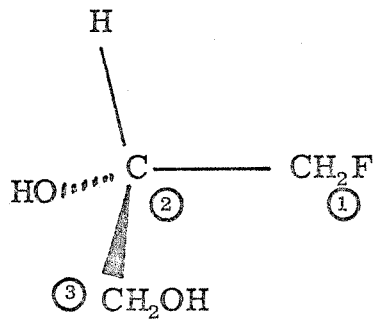
(R)-Productive



(R)-Non-Productive



(S)-Productive



(S)-Non-Productive

Fig. 10. Possible productive and non-productive binding modes for (R)-, and (S)-3-fluoro-1,2-propanediol.

$$V = \frac{k_O[E_0][S]}{K_O + S} \quad \text{where;} \quad k_O = \frac{k_3 K_I}{K_m + K_I}; \quad K_O = \frac{K_m K_I}{K_m + K_I}$$

Thus, if non-productive binding is important the measured k_{cat} is k_O and the measured K_m is K_O (Hamilton, et al., 1966). We are left with two equations in three unknowns. Making the assumption that the decrease in k_{cat} observed for the 3-fluoro-1,2-propanediol over that for the corresponding isomer of 1,2-propanediol is due solely to non-productive binding, a K_m and K_I can be calculated. The resulting values for (S)-3-fluoro-1,2-propanediol are; $K_m = 1.43 \times 10^{-3}$M and $K_I = 1.74 \times 10^{-2}$M, and for (R)-3-fluoro-1,2-propanediol; $K_m = 2.7 \times 10^{-4}$M, and $K_I = 3.24 \times 10^{-4}$M. These results do not offer a ready explanation for the anomalous behavior exhibited by 3-fluoro-1,2-propanediol. However, they were based on an assumption that is almost certainly not correct. There are undoubtedly other factors contributing both to the changes in binding and rates of catalysis of the 3-fluoropropanediols. Non-productive binding cannot be excluded as a contributing factor, and further experiments will be necessary to determine what role, if any, non-productive binding plays in the system.

Conclusions. The rearrangement of 1,2-propanediol occurs with considerable stereospecificity. Both hydrogen atom abstraction and hydroxyl loss differ for the two enantiomers of the substrate. For

(R)-1,2-propanediol, the pro-(R) C—1 hydrogen is abstracted; whereas, for (S)-1,2-propanediol, the pro-(S) C—1 hydrogen is abstracted (Zagalak et al., 1966; Karabatsos et al., 1966). During the dehydration step of the (R)-isomer, the hydroxyl originally at C—1 is lost to solution, however, for the (S)-isomer, the hydroxyl originally at C—2 is lost to solution (Retey et al., 1966). The reaction of both isomers proceeds with inversion at C—2 (Zagalak et al., 1966; Retey et al., 1966).

We have shown earlier, that the two enantiomers of the substrate compete for the same binding site on the enzyme. As Abeles has previously pointed out, the steric course of the rearrangement suggests that both enantiomers of the substrate react by an identical mechanism and that both substrate isomers interact through three identical groups with three geometrically rigidly fixed binding sites of the enzyme. Assuming that the positions of C—1 and C—2 are fixed by hydrogen bonding with the enzyme and that the pro-(R) hydrogen on C—1 of the (R)-enatiomer occupies the same relative position as the pro-(S) hydrogen on C—1 of the (S)-enantiomer, we see that the methyl groups of the two isomers occupy slightly different sites. This slight difference could easily account for the observed difference in binding affinities for the two isomers.

If the previous argument is correct and the reaction of each isomer results in a similar transition state, the rate difference for the two isomers may be solely a reflection of the difference in ground

state energy of the bound substrates. By assuming $K_m = K_S$, i.e., k_{cat} is small relative to k_{off}, the difference in ground state energy of the two bound substrates may be calculated.

Thus

$$\Delta G_{(R)\text{-isomer}} = -RT\ln(1.1 \times 10^{-4}) = 5.59 \text{ K cal/mole}$$

$$\Delta G_{(S)\text{-isomer}} = -RT\ln(0.65 \times 10^{-4}) = 5.92 \text{ Kcal/mole}$$

$$\Delta\Delta G = 0.33 \text{ K cal/mole}$$

Using the rate data for the two isomers, the free energy of activation may be calculated:

$$k_{cat} = \frac{kT}{h} e^{-\Delta G^{\ddagger}/RT}$$

or $$\Delta G^{\ddagger} = -RT\ln \frac{k_{cat} h}{kT}$$

$$\Delta G^{\ddagger}_{(R)\text{-isomer}} = -RT\ln \frac{368 \text{ sec}^{-1} \text{ h}}{kT} = 14.48 \text{ K cal/mole}$$

$$\Delta G^{\ddagger}_{(S)\text{-isomer}} = -RT\ln \frac{191 \text{ sec}^{-1} \text{h}}{kT} = 14.88 \text{ K cal/mole}$$

$$\Delta\Delta G^{\ddagger} = 0.40 \text{ K cal/mole}$$

These calculations show that the difference in binding energy for the two isomers may indeed account for their rate differences.

Similar calculations for the two isomers of 3-fluoro-1,2-propanediol show:

$$\Delta G_{(S)\text{-isomer}} = 4.07 \text{ K cal/mole}$$

$$\Delta G_{(R)\text{-isomer}} = 5.42 \text{ K cal/mole}$$

$$\Delta\Delta G = 1.35 \text{ K cal/mole}$$

$$\Delta G^{\ddagger}_{(S)\text{-isomer}} = 14.53 \text{ K cal/mole}$$

$$\Delta G^{\ddagger}_{(R)\text{-isomer}} = 15.25 \text{ K cal/mole}$$

$$\Delta\Delta G^{\ddagger} = 0.72 \text{ K cal/mole}$$

For both cases the energy differences between $\Delta\Delta G$ and $\Delta\Delta G^{\ddagger}$ are small. Thus, we conclude that for each substrate the transition state for both isomers must be similar.

Experimental

Enzyme Preparation. Dioldehydrase was obtained from Klebsiella pneumonia (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 five fold. In general, 2 ml of aldehyde containing solution was assayed by adding 0.1 ml 2 N hydrochloric acid and 0.1 ml 2,4-dinitrophenylhydrazine solution (prepared by dilution of 100 mg 2,4-dinitrophenylhydrazine plus 0.4 ml concentrated hydrochloric acid to 25 ml with carbonyl free methanol). After standing for 30 min, 0.5 ml spectroquality pyridine (Matheson, Coleman and Bell) and 0.1 ml methanolic potassium hydroxide (prepared by dilution of 10 g potassium hydroxide dissolved in 10 ml distilled water to 50 ml with carbonyl free methol) were added; and the resulting mixture was allowed to stand for 6 min and then centrifuged. Absorbance was determined at 475 mμ and at 440 mμ.

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., 1943). The resulting (R)-1,2-propanediol had $[\alpha]_D^{25}$ - 18.4° (7.5% w/w in water).**

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

(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., 1943). The resulting (S)-1,2-propanediol had $[\alpha]_D^{25}$ + 19.5° (7.5% w/w in water).**

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 $[\alpha]_D^{25}$ of -19.5° and +19.5° (7.5% w/w in water)** 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 $[\alpha]_D^{25}$ + 20.8 (7.5% w/w in water).**

(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 $[\alpha]_D^{25}$ - 14.9° (13% w/w in absolute

ethanol) as opposed to the value $[\alpha]_D^{25}$ - 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)-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). Due to the higher rotation of the (R)-isomer the (S)-3-fluoro-1,2-propanediol showed $[\alpha]_D^{25}$ + 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 mmoles) was dissolved in 50 ml hexamethylphosphoramide which contained 5.1 ml water and potassium hydroxide (1.7 g, 30 mmoles). The mixture was stirred for 30 min and methyl iodide (11.3 g, 80 mmoles) 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 water and extracted twice with 75 ml portions of ethyl ether. The

combined ether extracts were washed twice with 25 ml portions of water, once with 10 ml 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 mmoles) was dissooved in 50 ml anhydrous ethyl ether and added dropwise to a solution of lithium aluminum deuteride (1.0 g, 24 mmoles) (99% ^{2}H from Stohler Isotope Chemicals) in 150 ml ice cold anhydrous ether with stirring. After 3 hr, the reaction was quenched by adding 1 ml water, 1 ml 15% sodium hydroxide, and 3 ml 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, $[\alpha]_D^{25}$ - 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 nmr.

(S)-3-fluoro-3,3-dideuterio-1,2-propanediol. (S)-3-fluoro-3,3-dideuterio-1,2-propanediol, $[\alpha]_D^{25}$ + 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.

Acrolein 2,4-dinitrophenylhydrazone. Acrolein 2,4-dinitrophenylhydrazone was prepared as described previously (Shriner et al., 1964). The ^{1}H 100 mega Hz nmr 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 (Fig. 11). 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 dinitrophenyl hydrazones 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.

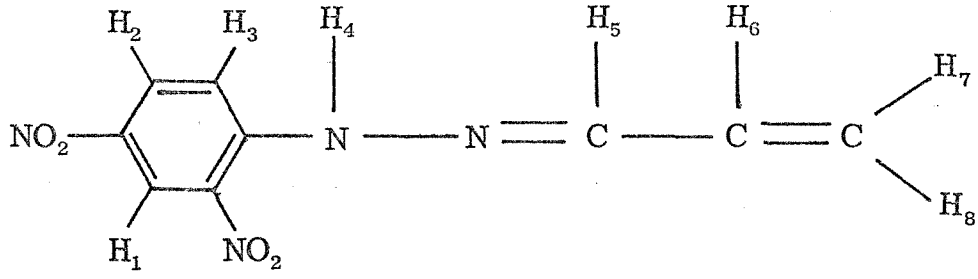


Figure 11. NMR data for Acrolein 2, 4-dinitrophenylhydrazone

H		
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	

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PART III

Reaction of 5′-deoxyinosylcobalamin with Propanediol Dehydrase

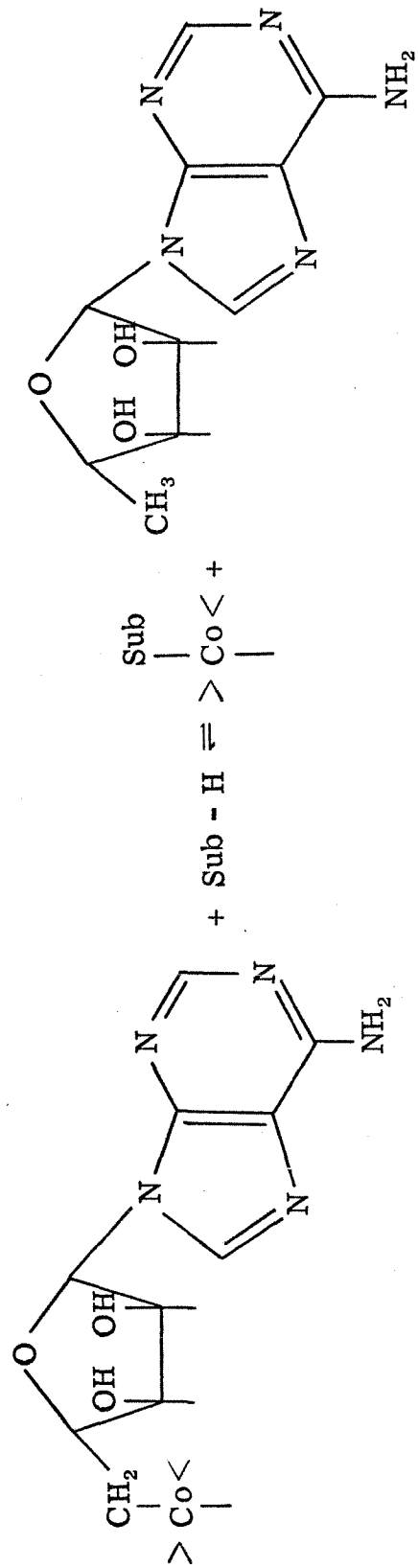
INTRODUCTION

Coenzyme B_{12} has been shown to be a cofactor in several processes which involve the transfer of a hydrogen atom between adjacent carbon atoms of the substrate (Stadtman, 1971). During this reaction, the C-5′ of the coenzyme acts as an intermediate hydrogen carrier. The hydrogen which migrates has been shown to become one of three equivalent hydrogens in both the methylmalonyl coenzyme A mutase (Miller and Richards, 1969) and the glutamate mutase (Eagar et al., 1972) systems. Accordingly the intermediate formation of 5′-deoxyadenosine has been proposed as a central part of the mechanism of action of coenzyme B_{12} (Scheme I).

Some evidence for the formation of 5′-deoxyadenosine in the propanediol dehydrase system has been provided by studies with the inhibitors glycoaldehyde (Wagner et al., 1966) and chloroacetaldehyde (Finlay et al., 1972). Although both of these inhibitors formed covalent bonds with the enzyme, in a faster competitive process, they caused the release of 5′-deoxyadenosine from the enzyme-coenzyme complex. In similar studies with ethanolamine deaminase, the addition of ethylene glycol (a very poor substrate which undergoes hydrogen exchange with the coenzyme) was found to cause the quantitative release of 5′-deoxyadenosine from enzyme bound coenzyme B_{12} (Babior, 1970).

Although experiments with inhibitors show that the carbon-cobalt bond of the coenzyme is cleaved to form 5′-deoxyadenosine, they do not prove that formation of 5′-deoxyadenosine is part of the

Scheme I



catalytic process. In order to demonstrate the formation of 5′-deoxynucleoside as a result of the reaction with the natural substrate, the reactivity of a modified coenzyme (5′-deoxyinosylcobalamin) was examined in the propanediol dehydrase system (Jayme and Richards, 1971). These experiments claimed that as a result of reaction with 1,2-propanediol (the natural substrate) 5′-deoxyinosine was released from the enzyme-coenzyme analogue complex. The release was believed to result from the inverted sequence of hydrogen bond acceptor and donor groups in the 5′-deoxyinosine relative to 5′-deoxyadenosine.

In order to determine if the release of 5′-deoxyinosine was a result of the normal catalytic pathway, the ability of the coenzyme analogue to abstract a hydrogen atom from the substrate was to be examined. These experiments have established, however, that the previously reported results were an artifact of the isolation procedure used. As a result of this isolation procedure and the lack of sufficient sensitivity in the experiments, the previously reported results may not be used to conclusively establish the release of 5′-deoxyinosine from 5′-deoxyinosylcobalamin in the propanediol dehydrase system.

RESULTS

Inhibition of propanediol dehydrase by 5′-deoxyinosylcobalamin. The coenzyme B_{12} analogue, 5′-deoxyinosylcobalamin, has been reported to be catalytically inactive in the methylmalonyl coenzyme A mutase system (Overath et al., 1962; Müller and Bernhauer, 1964).

This coenzyme analogue also proved to be catalytically inactive in the propanediol dehydrase system. In addition, 5′-deoxyinosylcobalamin acts as an inhibitor to coenzyme B_{12} binding. Similar inhibition experiments with aquocobalamin and cyanocobalamin gave results identical to those previously reported (Lee and Abeles, 1963). The results of these inhibition experiments are summarized in figures 1 and 2. Although these experiments suggest that the apparent K_m for 5′-deoxyinosylcobalamin is approximately 5×10^{-5} M, the mechanistic significance of this value is uncertain (Kerwan et al., 1970).

Inhibition by 5′-deoxynucleosides. Neither 5′-deoxyinosine nor 5′-deoxyadenosine showed any inhibition of propanediol dehydrase at concentrations up to 8×10^{-3} M. The limited solubilities of these nucleosides made studies at higher concentrations impossible.

Reaction of 5′-deoxyinosylcobalamin with propanediol dehydrase. Although a previous report claimed that 5′-deoxyinosine was released from the enzyme-5′-deoxyinosylcobalamin complex when in the presence of substrate (Jayme and Richards, 1971), a careful examination of the experimental evidence did not completely confirm this conclusion (Jayme, 1970). The amount of 5′-deoxyinosine released did not vary directly with either the enzyme concentration or the reaction time.

In all these previously reported experiments, the enzymatic reactions were quenched by adding 2N hydrochloric acid. Previous

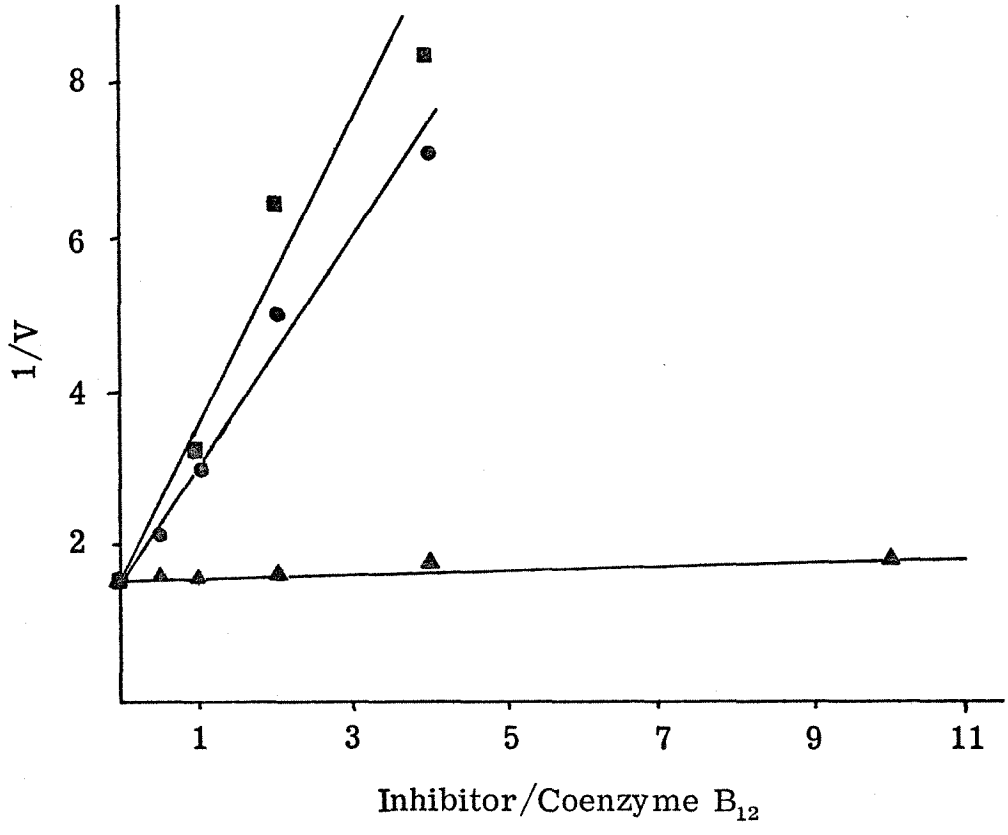


Figure 1.

Inhibition of propanediol dehydrase by cyano- (●), hydroxo- (■), and 5′-deoxyinosylcobalamin (▲). Each reaction mixture contained: potassium phosphate buffer, 0.04 M, pH 8.0; 1,2-propanediol, 0.1 M; enzyme, 0.03 units/ml; coenzyme B_{12}, 6.3×10^{-6} M; and inhibitor as indicated. Inhibitor and coenzyme were added simultaneously. V = μmole aldehyde formed after 20 min. at 37°C.

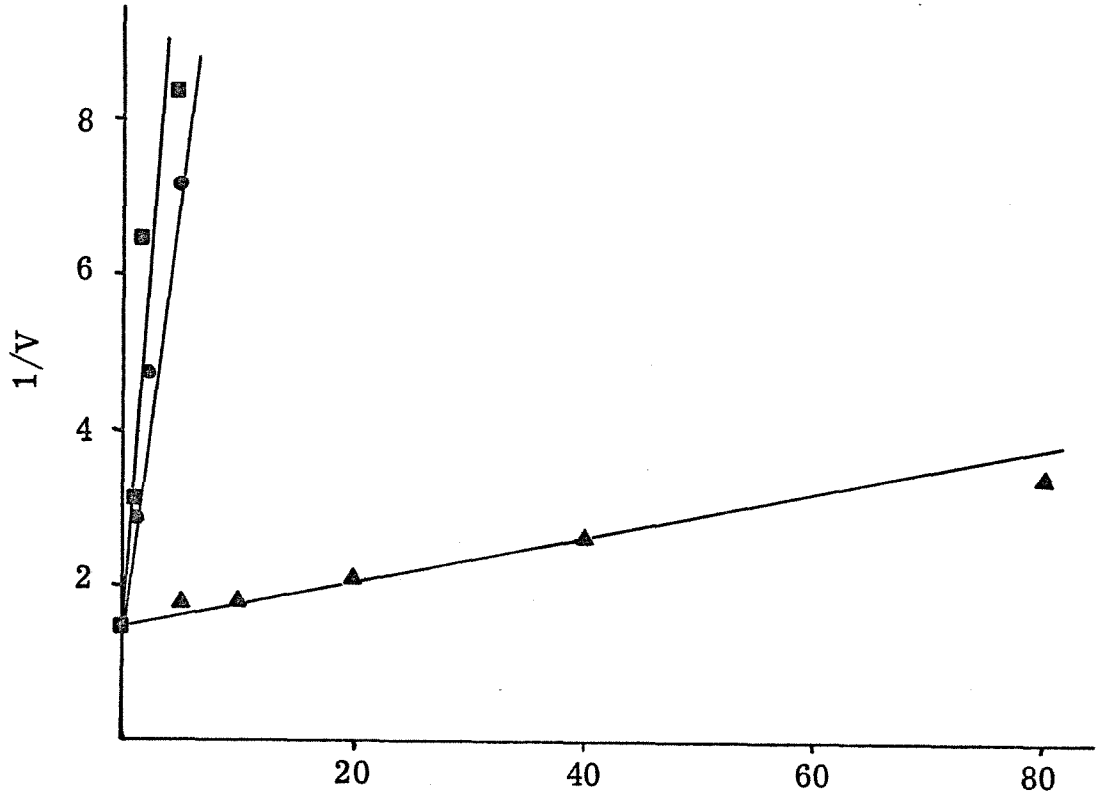


Figure 2

Inhibition of propanediol dehydrase by cyano- (●), hydroxo- (■), and 5′-deoxyinosylcobalamin (▲). Each reaction mixture contained: potassium phosphate buffer, 0.04 M, pH 8.0; 1,2-propanediol, 0.1 M; enzyme, 0.03 units/ml; coenzyme B_{12}, 6.3×10^{-6} M; and inhibitor as indicated. Inhibitor and coenzyme were added simultaneously. V = μmole aldehyde formed after 20 min. at 37°C.

acid hydrolysis reactions with 2′, 3′-isopropylidene-5′-deoxyadenosylcobalamin suggested that acid would cleave the ribose-adenine bond to some extent. Thus the observed release of ^{14}C could have resulted from the acid cleavage of the coenzyme analogue to yield 8-^{14}C-hypoxanthine (Scheme II). Preliminary experiments suggested that the ^{14}C release was indeed random and appeared to depend on the length of reaction with hydrochloric acid. On careful tlc separation on silica gel plates, both 5′-deoxyinosine carrier and hypoxanthine were identified. All the released ^{14}C appeared in the hypoxanthine fraction. These results are summarized in Table I.

Similar reactions were carried out that were quenched by freezing at -80°C. These results summarized in Table II clearly show that under the conditions employed the detection of released 5′-deoxyinosine was not possible.

The ability of 5′-deoxyadenosine to exchange with enzyme bound 5′-deoxyinosylcobalamin. If 5′-deoxyadenosine could exchange with 5′-deoxyinosylcobalamin that was bound to the enzyme, an active enzyme-coenzyme B_{12} complex would be formed. Under a wide variety of conditions and concentrations no such exchange was observed.

The ability of 5′-deoxyadenosine to exchange with enzyme bound coenzyme B_{12}. Although 5′-deoxyadenosine does not inhibit the propanediol dehydrase reaction, the possible exchange of enzyme bound 5′-deoxyadenosine from the coenzyme B_{12} with free

Scheme II

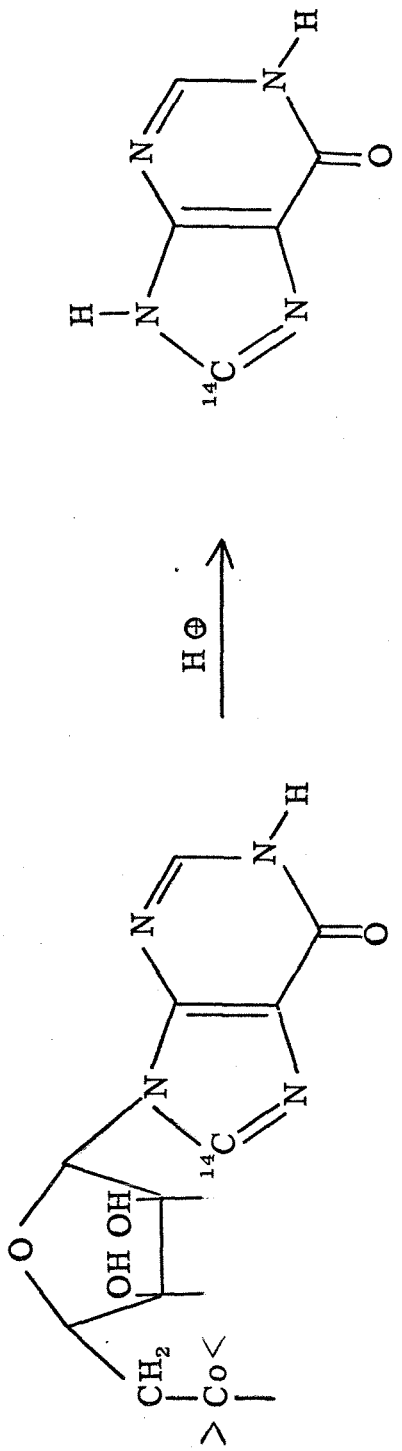


TABLE I

Enzyme (units)	1, 2-propanediol (M)	8-^{14}C-5′-deoxyinosylcobalamin (M)	dpm	hypoxanthine recovered mg	dpm	5′-deoxyinosine recovered mg	dpm
9.75	1×10^{-2}	1.8×10^{-4}	7,000	0.61	1,164	0.87	-0-
14.0	1×10^{-2}	1.8×10^{-4}	7,000	0.29	1,534	1.28	-0-
21.0	1×10^{-2}	3.6×10^{-4}	14,000	0.32	2,895	1.38	300
9.75	0	1.8×10^{-4}	7,000	0.86	1,741	0.50	150

All reaction mixtures contained potassium phosphate buffer, pH 8.0, 0.04 M, 1, 2-propanediol, enzyme, and 8-^{14}C-5′-deoxyinosylcobalamin as indicated above in a total volume of 1.0 ml. After incubation at 37°C for 30 min, the reactions were quenched by adding 0.1 ml 2N HCl, and 2 mg 5′-deoxyinosine was added as a carrier.

TABLE II

Enzyme (units)	1, 2-propanediol (M)	8-^{14}C-5′-deoxyinosyl cobalamin (M)	dpm	5′-deoxyinosine recovered mg	dpm	reaction time min
12	1×10^{-2}	3.6×10^{-4}	14,000	1.83	-0-	30
13.7	0	3.6×10^{-4}	14,000	1.80	-0-	30
30	1×10^{-2}	1.4×10^{-3}	56,000	0.99	317	60
30	0	1.4×10^{-3}	56,000	1.03	373	60

All reaction mixtures contained potassium phosphate buffer pH 8.0, 0.04 M, 1, 2-propanediol, enzyme, and 8-^{14}C-5′-deoxyinosylcobalamin as indicated above in a total volume of 1.0 ml. After incubation at 37°C for the time noted above, 2 mg 5′-deoxyinosine was added as a carrier, and the reactions were quenched by freezing at -80°C.

5′-deoxyadenosine was investigated. No such exchange was observed under a wide variety of conditions. The results are summarized in Table III.

DISCUSSION

The poor binding and lack of reactivity observed for 5′-deoxyinosylcobalamin indicate that the adenine moiety of the coenzyme is essential for reactivity. Thus by inverting the sequence of hydrogen bond acceptor and donor groups on the nucleoside base, the coenzyme does not interact favorably with the enzyme. Minor changes in the ribose function do not result in a drastic loss of binding ability or reactivity (Hogenkamp and Oikawa, 1964; Kerwar et al., 1970). The results of experiments with some coenzyme analogues are summarized in Table IV.

The lack of reactivity of 5′-deoxyinosylcobalamin plus the failure to observe exchange between 5′-deoxyadenosine and enzyme bound coenzyme B_{12} suggest that the adenosyl moiety of the coenzyme is tightly held by the enzyme. This result is not entirely unexpected since the enzyme may not be dialized free of coenzyme B_{12} or vitamin B_{12} (Lee and Abeles, 1963; Kerwar et al., 1970).

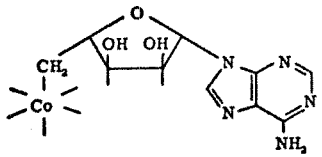
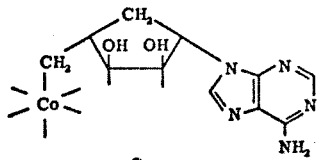
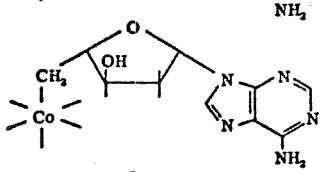
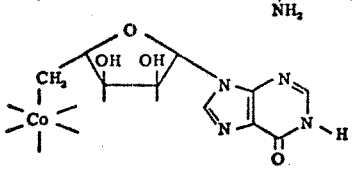
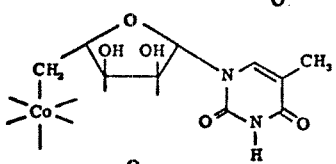
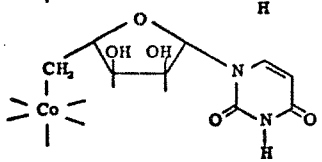
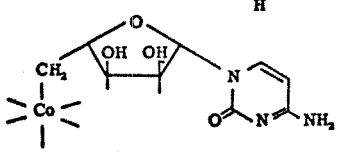
The 5′-deoxyinosylcobalamin release experiments confirm that the previously reported results (Jayme and Richards, 1971) were an artifact of the isolation procedure. Although some ^{14}C was detected in the 5′-deoxyinosine recovered from these reactions, the small amounts of label recovered could have easily resulted

TABLE III

Enzyme (units)	1, 2-propanediol (M)	8-^{14}C-5′-deoxyadenosylcobalamin (M)	dpm	2 mg 5′-deoxyadenosine added before or after reaction	5′-deoxyadenosine recovered mg	dpm	reaction time min
10	1×10^{-2}	6.3×10^{-4}	11,000	before	1.52	-0-	
10	1×10^{-2}	6.3×10^{-4}	11,000	before	1.70	120	30
10	1×10^{-2}	6.3×10^{-4}	11,000	after	1.85	202	30
10	0	6.3×10^{-4}	11,000	before	1.41	88	30
25	1×10^{-2}	6.3×10^{-4}	11,000	before	1.14	449	15
25	1×10^{-2}	6.3×10^{-4}	11,000	before	1.20	97	30

All reaction mixtures contained potassium phosphate buffer, pH 8.0, 0.04 M, 1, 2-propanediol, enzyme, 8-^{14}C-coenzyme B_{12}, and 5′-deoxyadenosine as indicated above in a total volume of 1.0 ml. The mixtures were preincubated 15 min at 37°C before adding coenzyme to initiate the reaction. The reactions were quenched by freezing at -80°C.

Table IV

Compound	Structure	% activity for diol dehydrase or glycerol dehydrase	Reference
5′-deoxyadenosyl-cobalamin		100%	Lee and Abeles, 1963 Kerwar et al., 1970
carbocyclic 5′-deoxyadenosyl-cobalamin		33%	Kerwar et al., 1970
2′, 5′-dideoxyadenosyl-cobalamin		13%	Hogenkamp and Oikawa, 1964
5′-deoxyinosyl-cobalamin		0%	
5′-deoxythymidyl-cobalamin		0%	Hogenkamp and Oikawa, 1964
5′-deoxyuricyl-cobalamin		0%	Zagalak and Pawelkiewicz, 1964
5′-deoxycytidyl-cobalamin		0%	Zagalak and Pawelkiewicz, 1964

from a slight photodecomposition of the 5′-deoxyinosylcobalamin during the isolation procedure. The amount of ^{14}C recovered was not reproducible and did not depend on either the amount of enzyme used or the reaction time.

Although these experiments disprove the previous results, they may not be used to completely reject the theory that the inactivity of 5′-deoxyinosylcobalamin is due to 5′-deoxyinosine leaving the ternary enzyme-coenzyme-substrate complex during an intermediate stage of the reaction. Recent reevaluation of both the purity of the enzyme and its molecular weight suggest that the specific activity of the 5′-deoxyinosylcobalamin used in these experiments was not sufficient to detect the release of 5′-deoxyinosine if only one molecule of coenzyme analogue reacts per molecule of enzyme (Finlay et al., 1972).

Since the enzyme was saturated with 5′-deoxyinosylcobalamin during these reactions, the expected release of ^{14}C may be calculated by simply assuming a 1:1 molecular ratio for reaction. Using the current values of a specific activity of 60 units/mg† and a molecular weight of 250, 000, the molar amount of enzyme used for a typical reaction may be calculated

$$60 \text{ units/mg} \times 250,000 \text{ mg/mmole} = 1.5 \times 107 \text{ units/mmole}$$

Thus for a reaction using 30 units of enzyme, the molar amount of

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† One unit = 1 μmole aldehyde produced/min (Lee and Abeles, 1963).

enzyme used was

$$\frac{30 \text{ units}}{1.5 \times 10^{7} \text{ units/mmole}} = 2 \times 10^{-6} \text{ mmoles}$$

Thus only 2×10^{-6} mmoles of 5′-deoxyinosylcobalamin would be expected to be released on reaction. The specific activity of the coenzyme analogue was 4×10^{7} dpm/mmole. A release of 2×10^{-6} mmoles of 5′-deoxyinosine would contain only 80 dpm. Considering the problem of photodecomposition during the isolation procedure, these experiments lacked sufficient sensitivity to detect a reasonable release of 5′-deoxyinosine.

SUMMARY

These experiments establish that 5′-deoxyinosylcobalamin is not reactive in the propanediol dehydrase system. This lack of reactivity is not necessarily due to the inability of this coenzyme analogue to bind to the enzyme. Although the previous experiments searching for the release of 5′-deoxyinosine by this system have been shown to be inconclusive, the intermediate release of 5′-deoxyinosine may indeed cause the inactivity of 5′-deoxyinosylcobalamin. In order to truly test this hypothesis, 8-^{14}C-5′-deoxyinosylcobalamin with a significantly higher specific activity than that described here must be used.

EXPERIMENTAL

Enzyme Preparation. Propanediol dehydrase 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 described (Frey et al., 1967).

Coenzyme B_{12} and (R, S)-1, 2-propanediol. Coenzyme B_{12} was purchased from Sigma Chemical Company. (R, S)-1, 2-propanediol was purchased from Matheson, Coleman, and Bell.

Assays and Reaction Conditions. All assays were carried out in the dark at 37°C, and the aldehyde products were determined as previously described (Lee and Abeles, 1963).

Synthesis of 5′-deoxynucleosides:

5′-deoxyadenosine. 5′-deoxyadenosine was prepared by the intermediate synthesis of 5′-indo-2′, 3′-isopropylidene-6-N-acetyladenosine and 5′-deoxy-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 from both methods were identical.

5′-deoxyinosine. Attempts to prepare 5′-deoxyinosine by

analogous procedures resulted in very low yields. As a result of these low yields, 5′-deoxyinosine was prepared enzymatically from 5′-deoxyadenosine using adenosine deaminase (Type I, calf intestinal mucosa from Sigma Chemical Company). A stock solution of enzyme was prepared that contained 0.65 mg enzyme/ml in sodium citrate buffer, 0.005 M, pH 8.5. The specific activity of the enzyme was 239 units/mg. 5′-deoxyadenosine (67 mg, 0.27 mmoles) was mixed with 1.2 ml of the enzyme stock solution in 11.2 ml water and allowed to stand at room temperature for 3 days. Another 0.8 ml of enzyme stock was added, and the reaction was allowed to stand for an additional 5 days. The reaction progress was estimated by tlc on silica gel plates using water saturated n-butanol plus 1% concentrated ammonium hydroxide as an eluting solvent (R_f 5′-deoxyadenosine = 0.35; R_f 5′-deoxyinosine = 0.20), and by cellulose tlc using water saturated n-butanol as an eluting solvent (R_f 5′-deoxyadenosine = 0.52; R_f 5′-deoxyinosine = 0.27). When the reaction was complete, the protein was removed using an Amicon Diaflow apparatus equipped with a UM-2 membrane; and the eluate was evaporated to dryness. The residue was washed several times with hot absolute ethanol, filtered, and the filtrate was evaporated to give 60 mg (88%) 5′-deoxyinosine, mp 156-159°C (uncorrected), UV $\lambda_{max}^{H_2O}$ = 149 (ϵ = 14,626) (Levine and Tipson, 1935). ^{1}H nmr in d_6-dimethylsulfoxide was identical to inosine with the exception of the disappearance of the 5′-hydroxyl resonance at 5.36 δ and the increase in intensity of the 5′-hydrogen doublet which shifted to 1.42 δ.

Synthesis of coenzyme analogues:

The previously described syntheses of coenzyme B_{12} and its analogues (Müller and Müller, 1962; Johnson et al., 1963; Hogenkamp and Pailes, 1968) all suffer because in the last step the acid hydrolysis of the isopropylidene ketal also leads to considerable decomposition of the nucleoside. Thus a new procedure which avoids the acid hydrolysis was employed.

8-^{14}C-5′-deoxyadenosylcobalamin. 8-^{14}C-adenosine (Calatomic, 100 μcuries) and unlabeled adenosine (200 mg, 0.75 mmoles) were dissolved in 1.1 ml N, N-dimethylformamide and heated to 150°C under an atmosphere of argon. Phenol (68 mg) and diphenylcarbonate (240 mg) were added, and after 30 min the mixture was poured into 60 ml of ethyl ether. The resulting precipitate was dissolved in ethanol, filtered, and the filtrate was evaporated to yield crude 8-^{14}C-adenosine-2′, 3′-cyclic carbonate (203 mg, 0.69 mmole). The material was identical to the previously described compound (Law et al., 1971).

This crude material was dissolved in 1.1 ml cold anhydrous pyridine and mixed with 182 mg tosyl chloride. After standing overnight at 5°C, the mixture was poured into cold ethyl ether. The resulting precipitate was dissolved in absolute ethanol and reprecipitated with cold ethyl ether to yield 179 mg crude 5′-tosyl-8-^{14}C-adenosine-2′, 3′-cyclic carbonate (Law et al., 1971). Cellulose tlc using n-butanol-acetic acid-water (5:2:3) as an eluting solvent indicated that this material was only 50% pure.

This crude hygroscopic precipitate was dissolved in 5 ml water-pyridine (4:1), and the pH was adjusted to 9.0. After 12 hrs. at room temperature, cellulose tlc indicated that the majority of the mixture contained 5′-tosyl-8-^{14}C-adenosine (Law et al., 1971). Without further purification, this material was reacted with reduced aquocobalamin as described by Hogenkamp and Pailes (1968). The resulting 8-^{14}C-deoxyadenosylcobalamin (10 mg, 17,500 dpm/mmole) was fully active in the propanediol dehydrase system.

5′-deoxyinosylcobalamin and 8-^{14}C-5′-deoxyinosylcobalamin. Both 8-^{14}C- and unlabeled 5′-deoxyinosylcobalamin were prepared by a procedure analogous to that just described. These materials were identical to that described previously (Overath et al., 1962; Müller and Bernhauer, 1964). The specific activity of the 8-^{14}C-5′-deoxyinosylcobalamin was 4×10^7 dpm/mmole.

Reactivity of 5′-deoxyinosylcobalamin. No aldehyde was formed by the propanediol dehydrase system when 5′-deoxyinosylcobalamin was substituted for coenzyme B_{12}. Up to 30 units of enzyme were allowed to react for up to 30 minutes during these tests. Thus if 5′-deoxyinosylcobalamin reacts, it reacts at least 10,000 times more slowly than the natural coenzyme. Under the same conditions, no exchange between 5′-deoxyadenosine and 5′-deoxyinosylcobalamin to form active coenzyme was detected.

Chromatographic procedures used to isolate 5′-deoxyadenosine and 5′-deoxyinosine. All isolation procedures were performed

in either dim red light or in total darkness. After quenching the enzyme reactions and adding 2 mg of carrier (either 5′-deoxyinosine or 5′-deoxyadenosine), the reaction mixtures were lyophylized and streaked on 2 mm silica gel F-254 preparative tlc plates (Brinkman). The plates were developed in 2-butanol-water ammonium hydroxide (100:36:14). Often the plates were developed twice to gain complete separation of the bands. The bands of interest were removed from the plates and extracted with methanol. The identity of each band was confirmed by an analytical tlc (R_f 5′-deoxyadenosine = 0.27; R_f 5′-deoxyinosine = 0.16; R_f hypoxanthine = 0.23). The amount of each compound was determined by their ultraviolet extinction.

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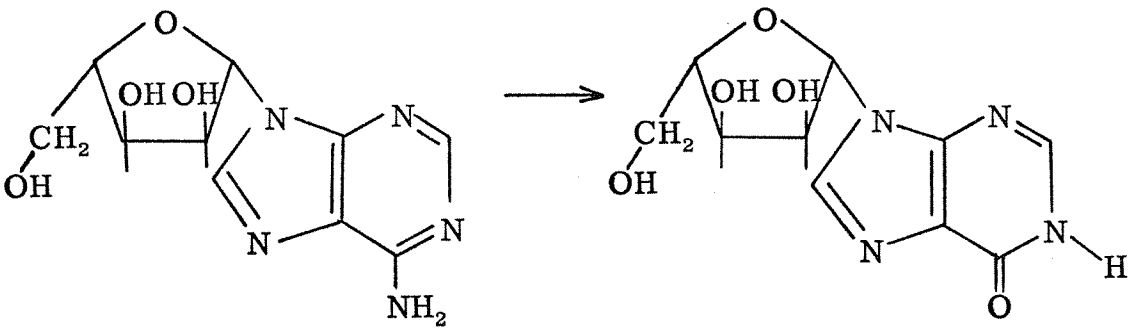
PART IV

Kinetic Studies of the Reaction of 5′-deoxyadenosine with Adenosine Deaminase

INTRODUCTION

Adenosine deaminase (adenosine aminohydrolase EC 3.5.4.4) converts adenosine to inosine (eq. 1) (Conway and Cooke, 1939).



eq. 1

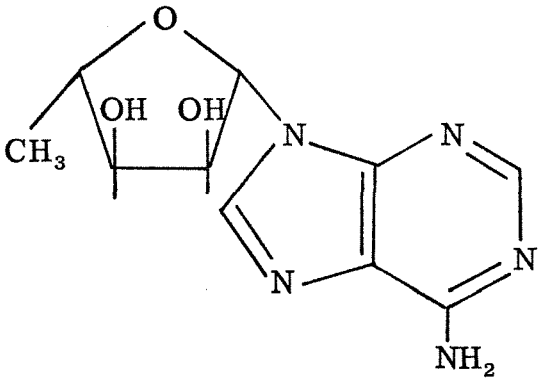
This relatively monospecific class of isozymes is also capable of catalyzing the hydrolytic cleavage of nitrogen, halogen, oxygen, and sulfur from purine ribonucleosides (Cory and Suhadolnik, 1965; Wolfenden, 1966; Baer and Drummond, 1966; Chassy and Suhadolnik, 1967; Wolfenden and Kirsch, 1968). Primary interest in this enzyme stems from its vast medical importance.

Adenosine relaxes bronchiolar (Bennet and Drury, 1931), intestinal (Sehorn and Borowitz, 1971), and certain vascular (Drury and Szent-Gyorgyi, 1929) smooth muscles, whereas inosine is inactive. Thus adenosine deaminase has been implicated as having a regulatory role in coronary blood flow (Berne, 1964). High levels of adenosine deaminase activity have been found in patients with certain severe infections (Litnansky and Seelich, 1958a and 1958b), lymphatic leukemia (Litnansky and Seelich, 1958a and 1958b), tumors (Straub et al., 1957), cancer (Schwartz and Bodansky, 1959), and severe immunodeficiency diseases (Knudsen and

Dissing, 1973). Adenosine deaminase is also capable of inactivating many chemotherapeutic agents which possess a nucleoside structure by deamination (Hubert-Habart and Cohen, 1962; Simon et al., 1970). Assays for the various isozymes of adenosine deaminase are also useful in detecting parentage (Lefevre et al., 1972).

Although many substrate analogues for adenosine deaminase have been examined for activity and inhibition, no clear structure-binding correlation has been made. The structural positioning of the hydroxyl groups of the adenosyl moiety certainly play an important role in the binding mechanism. In fact, one report claimed that the 5′-hydroxyl was necessary for both binding and activity since 5′-deoxyadenosine (I) was found to be neither a substrate nor an inhibitor of the adenosine deaminase reaction (Block et al., 1967). Other, conflicting reports showed that although the 5′-hydroxyl was important for both binding and activity, it was not essential (York and LePage, 1966). These reports showed that 5′-deoxyarabinosyladenine (II) and 5′-deoxyxylosyladenine (III) were both substrates for adenosine deaminase.

In order to clarify the importance of the 5′-hydroxyl for both binding and activity, the reaction of 5′-deoxyadenosine with adenosine deaminase has been reexamined. Although the removal of the 5′-hydroxyl greatly reduces the binding efficiency and the catalytic conversion rate, adenosine deaminase was in fact found to deaminate 5′-deoxyadenosine. This system was used to synthetically prepare 5′-deoxyinosine from 5′-deoxyadenosine in high yield.



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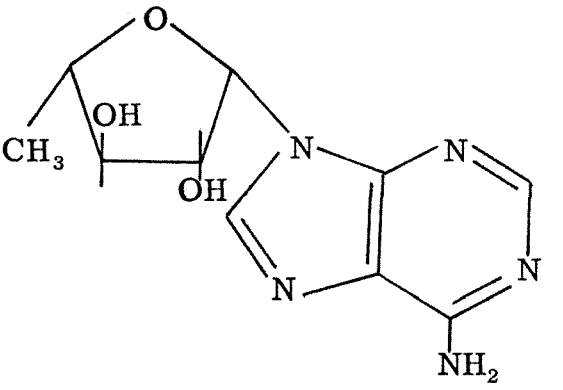
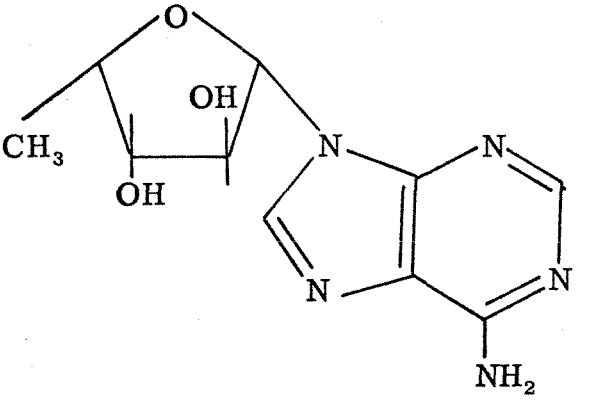


II



III

RESULTS AND DISCUSSION

Adenosine deaminase was found to quantitatively deaminate 5′-deoxyadenosine to 5′-deoxyinosine. The binding and rate data comparing the deamination of 5′-deoxyadenosine with adenosine is shown in Table I. The values were determined from Lineweaver-Burk plots using a least squares fit of the data. A typical plot for 5′-deoxyadenosine at pH 7.5 is shown in figure 1. In the range of pH 6-9 both substrates showed little change in either K_m or V_{max} values. Although the removal of the 5′-hydroxyl resulted in an increase of only 10^2 in K_m, the catalytic rate is reduced by over 10^3.

Thus we conclude that reports showing 5′-deoxyadenosine not to be a substrate for adenosine deaminase are quantitatively in error. These errors resulted primarily from insufficient assay sensitivity. However, as previously postulated the 5′-hydroxyl is very important for both binding and efficient catalysis.

A compilation of K_m and V_{max} data is given in Table II for some analogues of adenosine. Of these compounds, 5′-deoxyadenosine has the lowest V_{max} value as well as the largest K_m. Although 5′-deoxyarabinosyladenine shows similar binding and catalytic properties, 5′-deoxyxylosyladenine binds nearly the same as adenosine; however its V_{max} is less than adenosine by a factor of 15. The singular loss of either a 2′- or a 3′-hydroxyl causes only slight changes in K_m and V_{max}. Thus while the 5′-hydroxyl appears to be of great importance to both binding and catalysis, no

Table I. Kinetic constants for enzymatic deamination of 5′-deoxyadenosine vs. pH (A), and adenosine vs. pH (B).

A	pH	K_m (M)	V_{max} (M/min)	k^*_{cat} (min^{-1})
	5.1	1.36×10^{-3}	9.9×10^{-6}	7.5
	6.1	1.24×10^{-3}	1.48×10^{-5}	11.1
	7.0	1.33×10^{-3}	1.65×10^{-5}	12.4
	7.0	1.00×10^{-3}	1.39×10^{-5}	10.4
	7.5	7.4×10^{-4}	1.24×10^{-5}	9.3
	7.5	1.01×10^{-3}	1.37×10^{-5}	10.3
	7.6	1.14×10^{-3}	1.45×10^{-5}	10.9
	8.0	1.67×10^{-3}	1.78×10^{-5}	13.4
	8.0	1.15×10^{-3}	1.46×10^{-5}	11.0
	9.0	9.2×10^{-4}	1.23×10^{-5}	9.2

B	pH	K_m (M)	V_{max} (M/sec)	k^*_{cat} (sec^{-1})	k^*_{cat} (min^{-1})
	6.0	3.15×10^{-5}	1.45×10^{-7}	227	13,620
	7.0	3.90×10^{-5}	1.81×10^{-7}	283	16,980
	7.0	3.84×10^{-5}	1.80×10^{-7}	280	16,800
	7.5	2.26×10^{-5}	1.55×10^{-7}	243	14,580
	7.5	3.09×10^{-5}	1.64×10^{-7}	256	15,360
	7.5	4.15×10^{-5}	1.40×10^{-7}	219	13,140
	8.0	5.21×10^{-5}	1.97×10^{-7}	308	18,480
	9.0	3.07×10^{-5}	9.51×10^{-8}	149	8,940

* Assumes a molecular weight of 52,000 (Wolfenden et al., 1968).

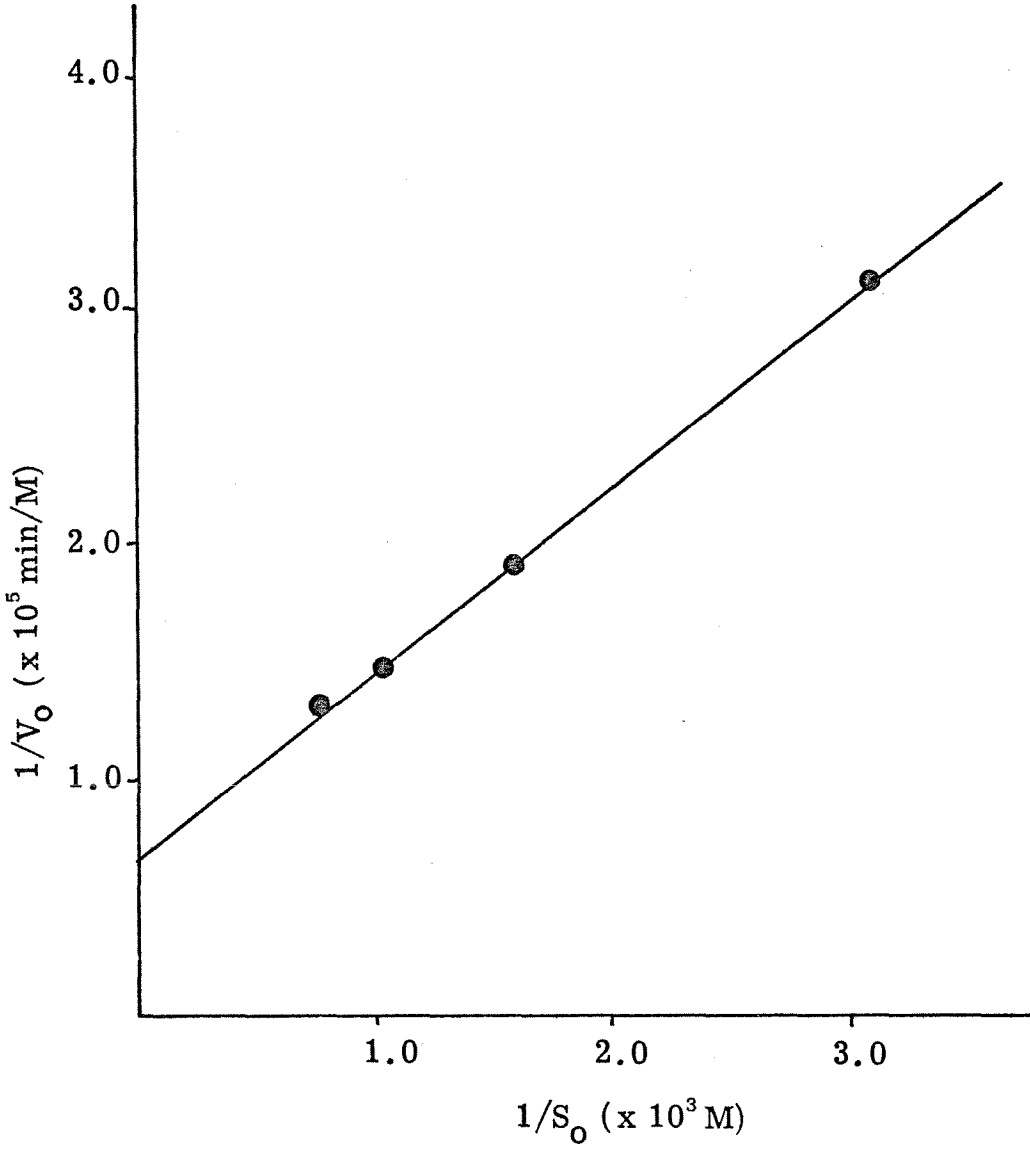


Figure 1. Lineweaver-Burk plot for 5′-deoxyadenosine. Typical plot for pH 7.5, conditions are given in the experimental section.

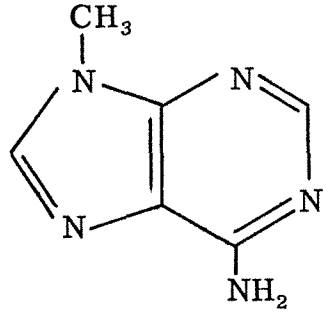
Table II. Kinetic Values for Various Adenosine Analogues at pH 7.5, 25°C

Compound	K_m (M)	V_{max}^{rel} (V_{max} Adenosine/V_{max} Analogue)	Reference
Adenosine	3.0×10^{-5}	1	York and Le Page, 1966
	4.4×10^{-5}	1	
5′-deoxyadenosine	1.1×10^{-3}	1,380	
2′-deoxyadenosine	2.2×10^{-5}	1.1	Fredericksen, 1966
2′,3′-dideoxy-adenosine	8.7×10^{-5}	0.97	Fredericksen, 1966
	7.7×10^{-5}	1.2	York and Le Page, 1966
3′-deoxyadenosine	5.2×10^{-5}	1.8	York and Le Page, 1966
	3.1×10^{-5}	2.0	Fredericksen, 1966
5′-deoxyxylosyl-adenosine	8.0×10^{-5}	15.5	York and Le Page, 1966
5′-deoxyarabimosyl-adenine	poor substrate		York and Le Page, 1966

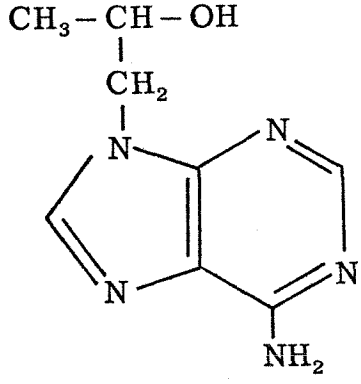
clear cut role for each hydrogel grouping in the ribosyl moiety is evident.

Although the 5′-hydroxyl appears to be very important in the binding of ribosyl analogues, N-9 open chain substituted adenines show a different hydroxyl dependence. Inhibitor studies suggest that a 2′-hydroxyl is of great significance for binding these N-9 open chain analogues. These studies suggest that in the absence of other hydroxyl groups, the 2′-hydroxyl may greatly enhance the binding properties of the inhibitor (see structures IV, V, and VI). The inhibitor showed 50% inhibition at the following inhibitor/substrate ratios: IV = 7.3; V = 0.15; VI = 0.00029 (Schaeffer, 1970). Thus while hydroxyl groups in the N-9 substituent of the adenine certainly play an important role in the binding process, the exact role is dependent on the structure of the N-9 substituent. The catalytic changes that occur when the structure of the N-9 grouping is altered are probably due to the resulting positioning of the C-6 group in the catalytic site.

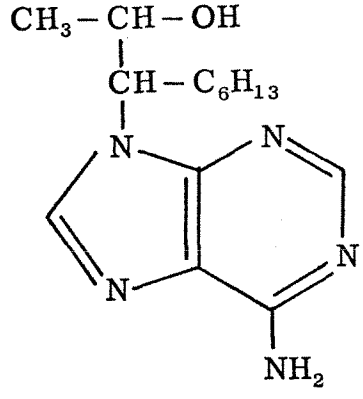
Summary. Although 5′-deoxyadenosine is a substrate for adenosine deaminase, the 5′-hydroxyl is very important for both binding and efficient catalysis of adenosine. The exact contribution to binding and catalysis of each hydroxyl grouping on the N-9 substituent of adenine is highly dependent on the structure of the substrate analogue or inhibitor. In spite of the low catalytic rate and the poor binding, adenosine deaminase may be used to synthetically prepare 5′-deoxyinosine from 5′-deoxyadenosine.



IV



V



VI

EXPERIMENTAL

Enzyme. Adenosine deaminase from calf intestinal mucosa was crystalline, Type I, from Sigma Chemical Company. The specific activity was 237 units/mg.

Kinetics. Reactions of adenosine were carried out in 1 cm cuvettes at 25°C, and ΔOD at 265 nm was recorded versus time. Each cuvette contained 1 ml 0.15 M sodium phosphate buffer at the pH desired; 10, 50, 100, or 150 μl of substrate 80 mg/100 ml; enzyme, 6.4×10^{-10} M; and water to a final volume of 3.0 ml.

Reactions of 5′-deoxyadenosine were carried out in 0.1 cm cuvettes at 25°C and Δ OD at 265 nm was recorded versus time. Each cuvette contained 100 μl 0.15 M sodium phosphate buffer at the pH desired; 5, 10, 15, or 20 μl 5′-deoxyadenosine (4.9 mg/ml); enzyme (1.33×10^{-6} M); and water to a final volume of 0.3 ml.

Synthesis of 5′-deoxyadenosine. 5′-deoxyadenosine was prepared from 5′-iodo-5′-deoxyadenosine (Aldrich Chemical Company) by catalytic hydrogenation using previously described conditions (McCarthy et al., 1968). The material was identical to that previously described (Jahn, 1965; McCarthy et al., 1968).

Synthesis of 5′-deoxyinosine. 5′-deoxyadenosine (100 mg, 0.4 mmole) was dissolved in 10 ml water and deaminated for 8 days with barium nitrite (470 mg) (K and K Laboratories) by the general procedure of Levine and Tipson (1935). The barium nitrite was

neutralized with 3N sulfuric acid, and the resulting suspension was centrifuged to remove the barium salts. The supernatant was chromatographed on an AG 11 A8 ion-exchange column to remove the last traces of barium salts. Evaporation of the resulting solution yielded 25 mg (25%) 5′-deoxyinosine, m.p. 156-159° (uncorrected, UV $\lambda_{max}^{H_2O}$ = 249 (ϵ = 14,626) (Levine and Tipson, 1935). ^{1}H nmr in d_6-N, N-dimethyl-formamide was identical to inosine with the exception of the disappearance of the 5′-hydroxyl resonance at 5.36 δ and the increase in intensity of the 5′-hydrogen doublet which shifted to 1.42 δ.

Enzymatic preparation of 5′-deoxyinosine. A stock solution of adenosine deaminase containing 0.65 mg/enzyme/ml in sodium citrate buffer, 0.005 M, pH 8.5 was prepared. 5′-deoxyadenosine (67 mg, 0.27 mmoles) was mixed with 1.2 ml of the enzyme stock solution in 11.2 ml water and allowed to stand at room temperature for 3 days. Another 0.8 ml of enzyme stock solution was added, and the reaction was allowed to stand for an additional 5 days. The reaction progress was estimated by tlc on silica gel plates using water saturated n-butanol plus 1% concentrated ammonium hydroxide as an eluting solvent (R_f 5′-deoxyadenosine = 0.35; R_f 5′-deoxyinosine = 0.20), and by cellulose tlc using water saturated n-butanol as an eluting solvent (R_f 5′-deoxyadenosine = 0.52; R_f 5′-deoxyinosine = 0.27). When the reaction was complete, the protein was removed using an Amicon Diaflow apparatus equipped with a UM-2 membrane; and the eluate was evaporated to dryness. The residue

was washed several times with hot absolute ethanol, filtered, and the filtrate was evaporated to give 60 mg (88%) 5′-deoxyinosine. This 5′-deoxyinosine was identical to that previously described.

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PROPOSITION I

Recently experimental improvements have allowed the measurement of meaningful K_m values for the coenzyme B_{12} dependent propanediol dehydrase system (eq. 1) (Eagar, 1974). The values

$$CH_3-\underset{\displaystyle OH}{\underset{|}{CH}}-\underset{\displaystyle OH}{\underset{|}{CH_2}} \xrightarrow[\text{coenzyme } B_{12}]{\text{Propanediol dehydrase}} CH_3-CH_2-\underset{\displaystyle OH}{\underset{|}{CH}} \qquad \text{eq. 1}$$

obtained for 1, 2-propanediol and for 3-fluoro-1, 2-propanediol are summarized in Table I. Considering the Michaelis-Menton hypothesis, the propanediol dehydrase may be summarized as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S \underset{k_4}{\overset{k_{cat}}{\rightleftharpoons}} E + P$$

Inhibition experiments coupled with labeling experiments have shown that k_4 is negligible in the reaction sequence (Lee and Abeles, 1963; Frey et al., 1967).

Therefore, this system exhibits the simple kinetic situation where if $k_{-1} \cong k_{cat}$,

$$K_m = \frac{k_{-1} + k_{cat}}{k_1}$$

In order to more accurately assess the mechanistic implications of the measured K_m values, the relative contribution of k_{-1}, and k_{cat} must be determined. If $k_{-1} \gg k_{cat}$,

Table I

Substrate†	Vmax (nmol/min)	k_{cat}* (sec^{-1})	K_m X 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	1.1 ±0.33
(RS)-H-diol	8.90 ±0.34	250 ±10	0.83 ±0.25
(S)-H-diol	6.82 ±0.17	191 ± 5	0.65 ±0.20

† F-diol = 3-fluoro-1,2-propanediol; H-diol = 1,2-propanediol.

* Based on a M.W. of 250,000 and a specific activity of 60 units/mg. (Essenberg et al., 1971).

$$K_m \cong \frac{k_{-1}}{k_1} = K_s$$

And if $k_{cat} \gg k_{-1}$

$$K_m \cong \frac{k_{cat}}{k_1}$$

Although equilibrium dialysis experiments could be used to ascertain the value of K_s in the absence of coenzyme B_{12}, these values could not be reliably used to determine the contribution of k_{-1} to the observed K_m values. The K_s value for the binary enzyme-substrate complex is probably significantly different from the K_s value for the ternary enzyme-substrate-coenzyme B_{12} complex. In addition, equilibrium dialysis experiments would require a large amount of enzyme. Consequently, a kinetic method of evaluating the contributions of k_{-1} and k_{cat} to K_m would be desirable.

Abeles has shown that the reaction of 1, 2-propanediol proceeds via an abstraction of a C-1 hydrogen atom by the coenzyme moiety (Frey et al., 1967). The kinetic isotope effect for the reaction of 1, 1-dideuterio-1, 2-propanediol is approximately 10 (Zagalak et al., 1966; Kerwar et al., 1970). The rearrangement of 3-fluoro-1, 2-propanediol also proceeds via an abstraction of a C-1 hydrogen atom (Eagar, 1974).

A comparison of the k_{cat} and K_m values for the substrate and the 1, 1-dideuterio analogue of the substrate could be used to determine the relative contribution of k_{cat} to the K_m value. Since

the accuracy for measuring K_m is better for 3-fluoro-1, 2-propanediol, the k_{cat} and K_m values for this system could be used for a more meaningful interpretation of the K_m value.

As the two enantiomers of 3-fluoro-1, 2-propanediol exhibit different kinetic properties, the k_{cat} and K_m values for (R)-, (S)-, as well as (R, S)-1, 1-dideuterio-3-fluoro-1, 2-propanediol should be compared to their non-deuterated analogues. The racemic 1, 1-dideuterio-3-fluoro-1, 2-propanediol can be synthesized by a lithium aluminum deuteride reduction of β-fluorolactic acid. A stereospecific synthesis of the (R)- and the (S)-isomers presents a more difficult problem for a chemical synthesis. An enzymatic synthesis may be used to overcome this difficulty. The M_4 isozyme of lactate dehydrogenase will synthesize (R)-3-fluorolactic acid from β-fluoropyruvic acid (Eisman et al., 1965; Grassetti et al., 1966). Lithium aluminum deuteride reduction of (R)-3-fluorolactate will yield (R)-1, 1-dideuterio-3-fluoro-1, 2-propanediol. The (S)-enantiomer may be prepared from the (R)-enantiomer as previously described (Lloyd and Harrison, 1971).

The possible contribution of non-productive binding must also be evaluated in order to fairly assess the meaning of the K_m values for 3-fluoro-1, 2-propanediol. A determination of the K_I values for such non-productive binding analogues as 1-fluoro-2-propanol and 1, 3-difluoro-2-propanol would accomplish this goal.

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PROPOSITION II

Coenzyme B_{12} catalyzes a rearrangement process in which a hydrogen atom and another group undergo a 1, 2-migration. These reactions are summarized by eq. 1. The coenzyme B_{12} has been

$$-\overset{X}{\underset{|}{C}}_{\alpha}-\overset{H}{\underset{|}{C}}_{\beta}- \rightleftharpoons -\overset{H}{\underset{|}{C}}_{\alpha}-\overset{X}{\underset{|}{C}}_{\beta}- \qquad \text{eq. 1}$$

shown to act as an intermediate hydrogen atom carrier along the reaction, and the intermediate production of a 5′-deoxyadenosyl moiety has been postulated (Eagar, 1974a). Consequently, the reaction may be more explicitly summarized by Fig. 1.

This mechanism requires the cleavage of the 5′-carbon-cobalt bond of the coenzyme moiety during the reaction. The nature of this cleavage has received considerable attention (Babior and Gould, 1969; Schrauzer et al., 1971; Finlay et al., 1972; Cockle et al., 1972; Finlay et al., 1973). The observation of esr signals from the ternary complex of enzyme-substrate-coenzyme B_{12} for ethanolamine deaminase (eq. 2) (Babior and Gould, 1969), glycerol dehydrase (eq. 3) (Cockle et al., 1972), and propanediol dehydrase (eq. 4) (Finlay et al., 1973) suggests the cleavage occurs via a homolytic process. The homolytic cleavage process is further supported by kinetic data. When significant changes in the electronic nature of the substrate are made, no significant alterations in the reaction rates are observed for the reactions of ethanolamine deaminase (Law and Wood, 1973) and propanediol dehydrase

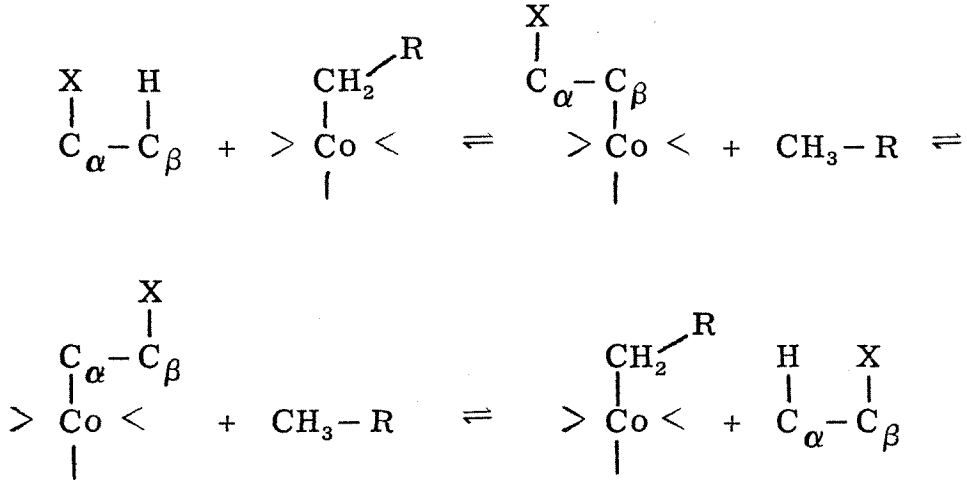

Figure 1

(Eagar, 1974b).

As the mechanisms of the B_{12} dependent reactions appear to be similar, the glutamate mutase and the methylmalonyl CoA mutase reactions would be expected to proceed by a homolytic cleavage of the 5′-carbon-cobalt bond of the coenzyme. An examination of the catalytic rates for substrate analogues with changes in their electronic nature would assist in determining if these mutase reactions proceed as the dehydrase reactions.

Therefore, a comparison of the catalytic rates of glutamic acid with γ-fluoroglutamic acid in the glutamate mutase system would be of interest. The natural substrate for glutamate mutase is L-glutamic acid which is converted to L-threo-β-methylaspartic acid during the reaction (Barker et al., 1964). L-γ-fluoroglutamic acid may be separated from its racemic mixture (Ukeless and Goldman, 1971). Although no difference in reactivity between L-erythro- and L-threo-γ-fluoroglutamic acid is expected, these isomers may be resolved (Ukeless and Goldman, 1971) and tested. A steric requirement of some intermediate step in the reaction sequence may cause the erythro- and threo- isomers to react differently.

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PROPOSITION III

The ability of diving vertebrates to survive extended periods of hypoxia has been of interest to scientists for over 100 years (Bert, 1870). The physiological adaptations which permit this survival have been extensively investigated (Irving, 1939; Andersen, 1966). However, these adaptations do not completely explain the tolerance of diving vertebrates to hypoxia. Accordingly, some studies of possible biochemical adaptations have been undertaken (Simon et al., 1974).

Many diving vertebrates exhibit respiratory adaptations suited for diving (Andersen, 1966). While the lung volume/body weight of most vertebrates is similar, divers have considerably higher tidal volumes. Some vertebrate divers nearly empty their lungs during expiration (Scholander and Irving, 1941). Consequently, these animals rid their lungs of large volumes of carbon dioxide more easily than non-divers.

The diving animals are generally less responsive to alveolar carbon dioxide pressure than non-divers (Andersen, 1966). While a rise of about 0.02 atmosphere of carbon dioxide causes the respiratory rate of man to double, the increase in rate for some diving mammals is negligible up to 0.1 atmosphere of carbon dioxide. Through a type of carbon dioxide storage mechanism, these animals are able to keep the carbon dioxide level in the lungs less than 0.1 atmosphere. This phenomenon even occurs during prolonged dives where up to 95% of their available oxygen has been consumed.

Due to exceptionally large blood volumes, many diving vertebrates are able to store a large quantity of oxygen although many show a fairly low blood oxygen affinity (Andersen, 1966). Many diving mammals also have a large muscular store of myoglobin. This myoglobin is capable of storing large amounts of oxygen (up to 47% of the total available during a dive).

However, quantitative comparisons show that these animals can dive up to four times longer than their total oxygen supply would allow. Thus other metabolic changes occur during the prolonged diving process (Andersen, 1966). In general, these animals employ anerobic metabolism during a dive to provide the necessary energy. Some animals even show a decreased metabolic rate during a dive (Scholander et al., 1942). Reduction of their heart rate (bradycardia) also aids these animals in conserving energy.

The increased anerobic metabolism in addition to the efficient aerobic metabolism causes a large buildup of acidic products. During the dive, the lactic acid produced by anerobic metabolism is stored in the muscles and is not released to the blood until emersion. The lactate is not released during the dive because the majority of divers are capable of preventing a significant portion of the blood flow to the muscles during the dive. At the same time, normal blood flow occurs between the heart and the brain. Aerobic metabolism in these organs results in a large buildup of carbon dioxide which increases the acidity of the blood. Some animals may control this acidity by having efficient carbon dioxide storage mechanisms,

while others may simply have a greater buffer capacity in the blood. In one of the few cases studied, seals appear to have a lower blood buffer capacity and a more efficient carbon dioxide storage mechanism than humans (Irving et al., 1935; Robin et al., 1963).

Considering these physiological problems and adaptations, a study of some biochemical differences in the blood, hemoglobin, and myoglobin of some diving and non-diving vertebrates is proposed. First, differences in the buffer capacity of the blood should be examined in order to compare the ability of the blood from various vertebrates to tolerate acid increase. While a higher buffer capacity may enable the animal to withstand large amounts of acid, less tolerance may be useful in obtaining more efficient utilization of the existing oxygen supply. This phenomenon has already been noted in some diving reptiles. The hemoglobin of alligators and crocodiles shows a significantly larger Bohr effect than the hemoglobin of their non-diving relatives, the chuckwalla and the gila monster (Dill and Edwards, 1931; Edwards and Dill, 1935; Dill et al., 1935; Dill and Edwards, 1935). Hence, a comparison of the Bohr effects for both hemoglobin and myoglobin of diving and non-diving vertebrates would be of interest.

Although the oxygen affinity of the blood of diving vertebrates has received considerable examination, the carbon dioxide affinity has received little attention. Considering the differences observed in oxygen affinities, the carbon dioxide affinities of blood from diving vertebrates may be different from that of non-diving

vertebrates. Much of the carbon dioxide transported by blood is carried in the carbamate form with the α-amino groups of hemoglobin (Roughton, 1970; Kilmartin and Rossi-Bernardi, 1971). These carbamino derivatives are formed from CO_2 and the basic form of the amine group (Morrow et al., 1973). Therefore, diving vertebrates may differ from non-divers in both carbon dioxide affinity and pK of the α-amino groups of their hemoglobin. Similar arguments also hold for myoglobin.

Possible differences in α-amino group pK and carbon dioxide affinity of both hemoglobin and myoglobin may be studied by ^{13}C nmr using $^{13}CO_2$ (Morrow et al., 1973). This technique would allow both pH and affinity studies to be carried out with hemoglobin, myoglobin, and whole blood.

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PROPOSITION IV

Nicotinamide adenine dinucleotide ($NAD^{\oplus}$) was one of the first coenzymes to be discovered. $NAD^{\oplus}$ and NADH, the reduced form of the coenzyme (Fig. 1) are involved in a large number of biological oxidation-reduction reactions catalyzed by dehydrogenases. One reaction of this type is catalyzed by L-lactate dehydrogenase (LDH) as shown in eq. 1. The reaction catalyzed by LDH formally

$$CH_3\underset{\displaystyle OH}{\underset{|}{C}}H\,COO^{\ominus} + NAD^{+} \rightleftharpoons CH_3\overset{\displaystyle O}{\overset{\|}{C}}\,COO^{\ominus} + NADH + H^{\oplus} \qquad \text{eq. 1}$$

involves the transfer of a hydride ion ($H^{\ominus}$) between substrate and C-4 of the pyridinium ring of the coenzyme moiety (Loewus et al., 1953; Pullman et al., 1954). The transfer is highly stereospecific, and the hydrogen transferred from the coenzyme is in the α configuration (Fig. 2) (Kaplan, 1960).

A considerable amount of evidence suggests that the reaction catalyzed by LDH obeys a Theorell-Chance mechanism (eqs. 2-6).

$$E + NAD^{\oplus} \rightleftharpoons E \cdot NAD^{\oplus} \qquad \text{eq. 2}$$

$$E \cdot NAD^{\oplus} + Lac \rightleftharpoons E \cdot NAD^{\oplus} \cdot Lac \qquad \text{eq. 3}$$

$$E \cdot NAD^{\oplus} \cdot Lac \rightleftharpoons E \cdot NADH \cdot Pyr + H^{\oplus} \qquad \text{eq. 4}$$

$$E \cdot NADH \cdot Pyr \rightleftharpoons E \cdot NADH + Pyr \qquad \text{eq. 5}$$

$$E \cdot NADH \rightleftharpoons E \cdot NADH \qquad \text{eq. 6}$$

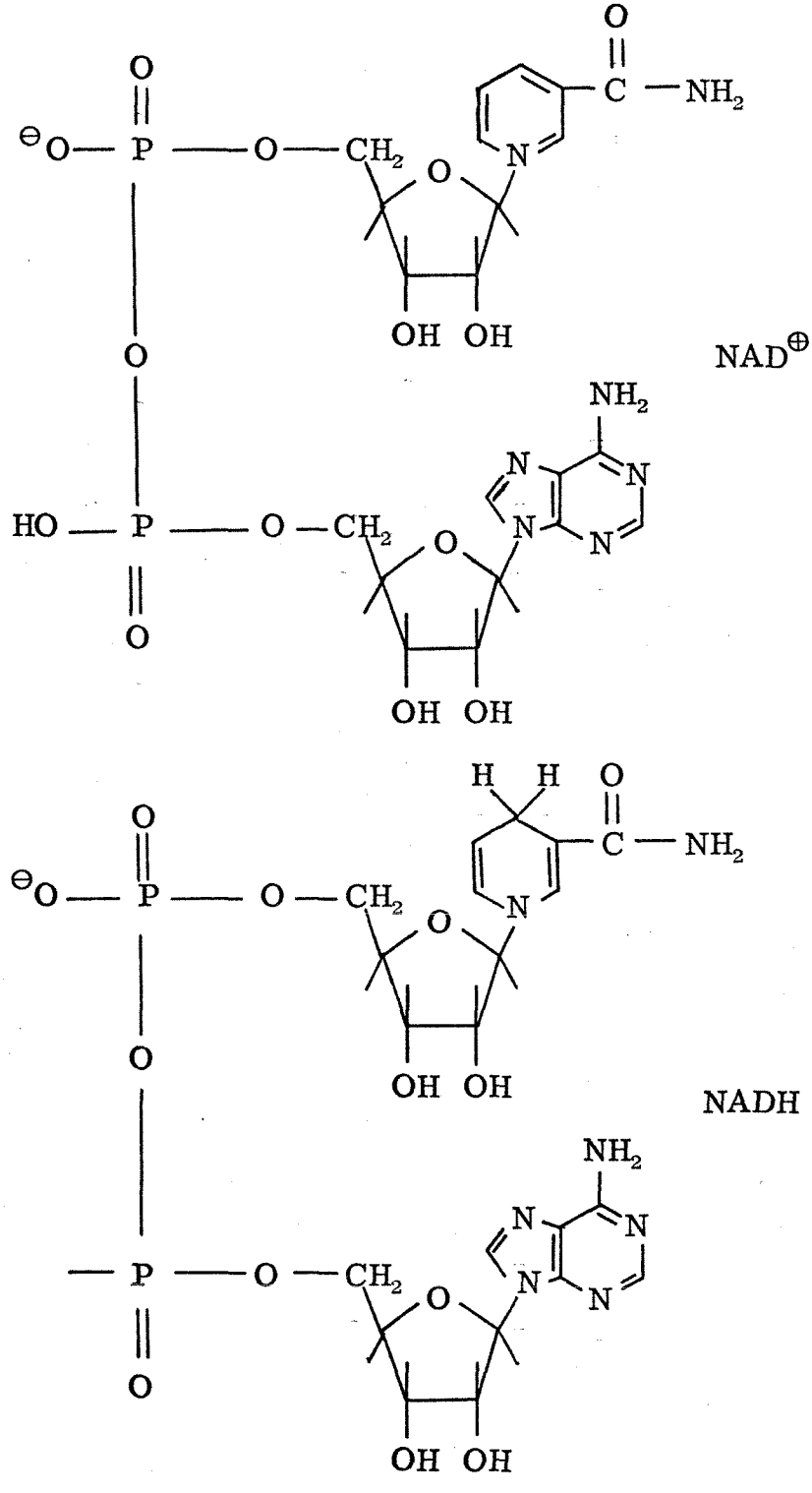
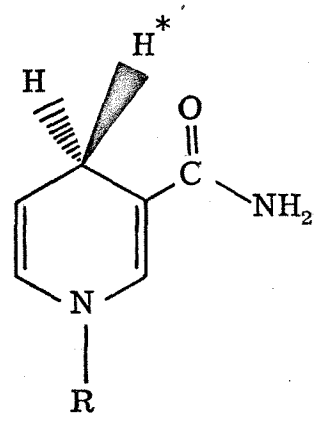


Figure 1. The Structure of NAD⊕ and NADH.



H^* = C—4 α Hydrogen

Figure 2. The Stereochemistry of the pyridinium ring.

The reaction is characterized by a compulsory sequence in which the coenzyme must bind before the substrate can bind (Takenaka and Gilbert, 1956; Novoa and Schwert, 1961; Silverstein and Boyer, 1964). Although the complex kinetic data from this system make the identification of a rate limiting step difficult, the reactive ternary complex surely plays a significant role in the reaction rate (Vestling, 1969).

Binding of a substrate to an enzyme may result in the polarization of the chemical bonds in the substrate, thus activating the substrate for reaction. The activity of NADH bound to LDH may result from such a structural change caused by the binding process. Such structural changes are suggested by spectral shifts which occur when NADH binds to LDH (Chance and Neilands, 1952), by fluorescence spectral shifts which occur when NADH binds to LDH (Winer et al., 1959), by fluorescence polarization experiments (White et al., 1968), and by X-ray crystallographic studies (Chandrasekhar et al., 1973). Recent observations that radiation induced oxidation of NADH by $O_2^{\ominus}$ radicals is greatly enhanced when the coenzyme is bound to LDH, further suggest that the binding process plays a significant role in activating the C-4 hydrogens for abstraction (Bielski and Chan, 1973).

LDH is a tetramer composed of four subunits of molecular weight 35, 000. Two type subunits occur in animals (Fondy and Kaplan, 1965). One, designated M, exists primarily in muscle, whereas the other, designated H, exists primarily in the heart.

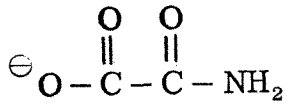
Accordingly, most muscle LDH is a M_4 tetramer, and most heart LDH is a H_4 tetramer. However, all possible combinations (M_3H_1, M_2H_2, and M_1H_3) have been found. Since pyruvate readily inhibits LDH-H_4, the latter is more suited to an aerobic organ which removes lactate from circulation. Conversely, LDH-M_4 readily converts pyruvate to lactate, and hence is useful in aerobic glycolysis.

Although the binding of coenzyme to LDH is fairly independent of isozyme, the non-productive binding of pyruvate to the enzyme-NAD$\oplus$ binary complex is markedly increased from LDH-M_4 to LDH-H_4 (Winer, 1969). However, some coenzyme analogues do not display similar binding properties with the various isozymes (Fondy and Kaplan, 1965). As a result of the differences in the M and H subunits, V_{max} for pyruvate is greater for LDH-M_4 than for LDH-H_4 by a factor of 1.5. LDH-M_4 also requires higher concentrations of substrate for reaction. Therefore, the differences in kinetic behavior between LDH-H_4 and LDH-M_4 may result from either slight structural changes in the coenzyme binding site or from differences in the reactive ternary complex.

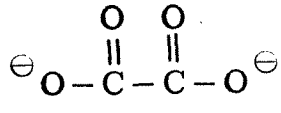
In order to distinguish between these possibilities, a comparison of the bond polarization caused in binding NADH to LDH-H_4 and to LDH-M_4 is proposed. The previously reported method of radiation induced hydrogen abstraction from C-4 of the coenzyme moiety is ideally suited to such a study (Bielski and Chan, 1973). In addition to comparing the differences in NADH activation in

the binary complex for various isozymes, this method would also be useful in examining the ternary complexes formed with the inhibitors oxalate and oxamate (Fig. 3) (Novoa et al., 1959; Winer and Schwert, 1959).

Oxamate inhibits the reaction of pyruvate, whereas oxalate inhibits the reaction of lactate (Novoa et al., 1959). In addition, oxamate quenches the fluorescence of the LDH-NADH complex, while oxalate enhances this fluorescence (Winer and Schwert, 1959). Winer and Schwert proposed that oxamate reduced the quinoid character of the coenzyme by binding to one of the negatively polarized C-4 hydrogens (Fig. 4). In contrast, the negative carbonyl of the oxalate repelled the C-4 hydrogen thereby increasing the quinoid character. If the changes in the fluorescence spectrum of these ternary complexes result from interactions with the C-4 hydrogens of the coenzyme, the ease of hydrogen abstraction should be different for these two ternary complexes. An examination of these ternary complexes with LDH-H_4 and LDH-M_4 could also be used to detect structural differences in the ternary complexes of the two isozymes.



Oxamate



Oxalate

Figure 3. Structures of oxamate and oxalate

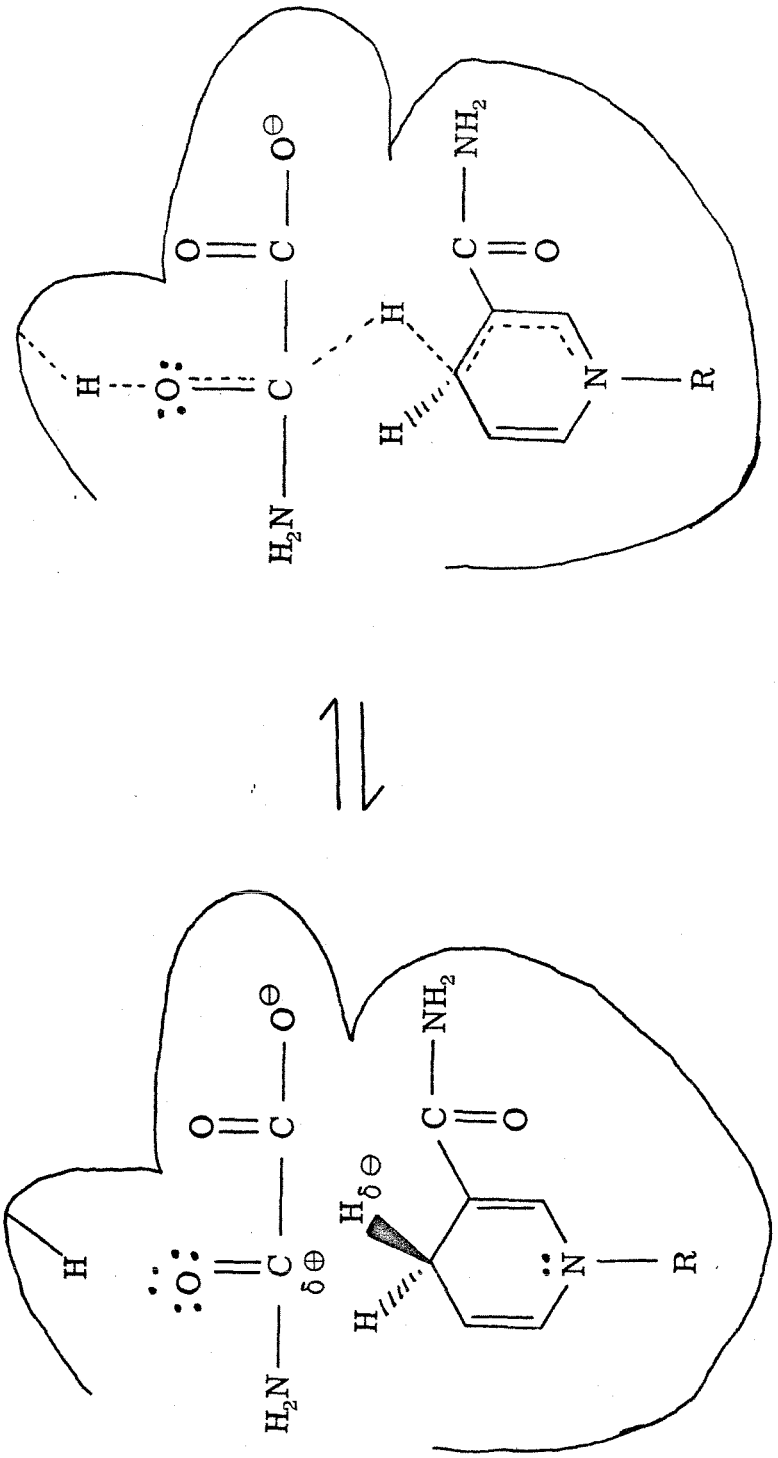


Figure 4. Interaction of oxamate with NADH in the LDH complex.

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PROPOSITION V

Bacteria which metabolize both D- and L-lactate have two distinctly different mechanisms for accomplishing this task. One method is to synthesize two different lactate dehydrogenases (Mulvena et al., 1972). One enzyme is specific for L-lactate, and the other is specific for the D-enantiomer. Other bacteria use only one type of lactate dehydrogenase which is coupled to lactate racemase (EC 5.1.2.1.). This enzyme interconverts L- and D-lactic acid.

Lactate racemase has been isolated and purified from Clostridium butylicium (Dennis and Kaplan, 1963) and from Lactobacillus sake (Tetsuo et al., 1968). Neither protein shows any cofactor requirement. Both are inhibited by oximate, hydroxylamine, semicarbazide, and phenylhydrazine. Pyruvate does not react with either enzyme. Neither enzyme exchanges the α-hydrogen of lactate with solvent.

The racemase from Clostridium butylicium has a molecular weight of 82, 400 and a pH range of 4.5-8.5 with an optimum at 5.5 (Cantwell and Dennis, 1974). Both enantiomers of lactic acid react at identical rates and bind equally well ($K_M = 8. \times 10^{-3}$ M) (Dennis and Kaplan, 1963).

In contrast, the racemase from Lacobacillus sake has a molecular weight of 25, 000 and a pH optimum between 5.8 and 6.2. K_M values of 1.7×10^{-2} M for D-lactate and 8×10^{-2} for L-lactate

were reported. The catalytic rates showed $k_{cat}^{D\text{-lactate}}/k_{cat}^{L\text{-lactate}} = 2.5$.*

Regardless of these differences, the two enzymes appear to racemize lactic acid by a similar mechanism. A considerable number of experiments suggest that the reaction of the Clostridium racemase involves a direct internal hydride shift (Dennis and Kaplan, 1963; Shapiro and Dennis, 1965; Shapiro and Dennis, 1966). Preliminary reports suggest that this mechanism also holds for the Lactobacillus enzyme (Tetsuo et al., 1968).

The isotope effect for the racemization of both D- and L-α-deuteriolactate is 2.15 (Shapiro and Dennis, 1965). There is no loss of the α-hydroxyl to solution (Shapiro and Dennis, 1966). Although early reports claimed that carboxyl reagents did not inhibit the racemase reaction by trapping the reactive intermediate, recently reported evidence has shown that hydroxylamine inhibits the reaction by forming an enzyme-bound oxime (Cantwell and Dennis, 1974). These results all support the internal hydride shift mechanism shown in Scheme I.

The substrate specificity of lactate racemase has not been studied. If the enzyme will racemize other α-hydroxy acids, the effect of changing the electronic nature of the substrate may be examined. For example, substituted mandelic acids would be

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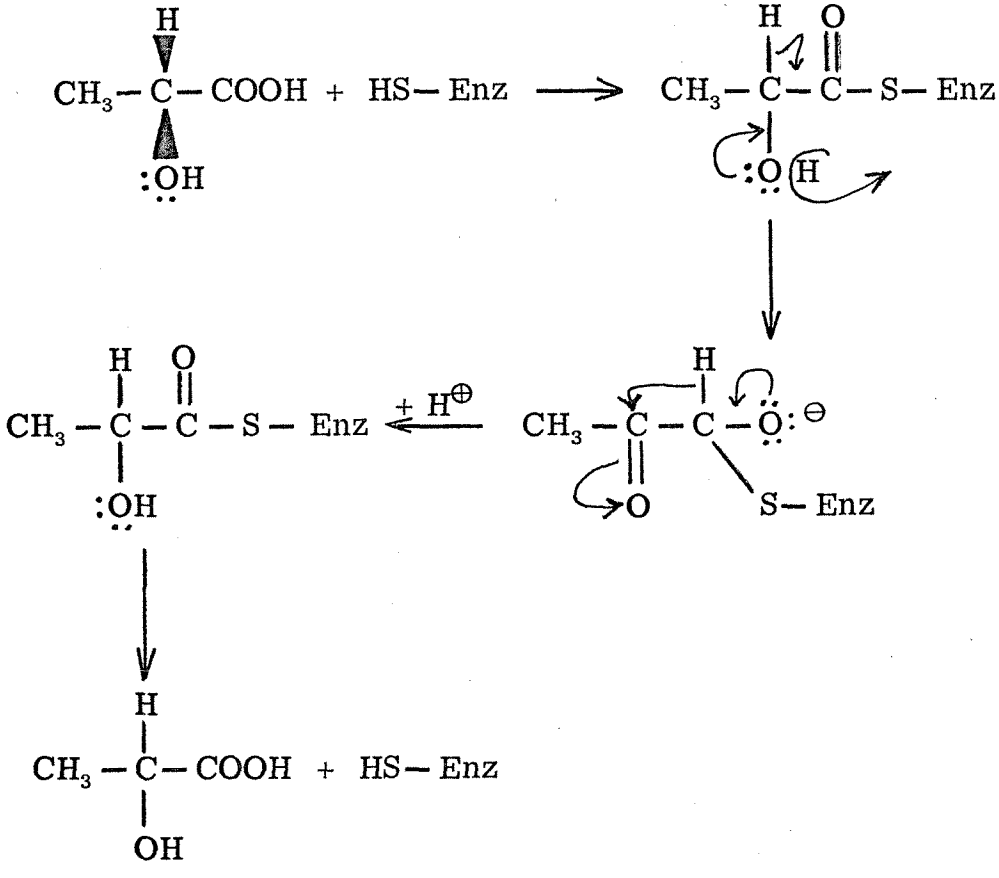
* The purity of the isomers of lactic acid used is somewhat uncertain. No purity data was given. Consequently, the differences in enzymic behavior of the two enantiomers may be a result of purity differences in the two isomers.

Scheme I


CH3 – C – COOH + HS – Enz
H
:OH
CH3 – C – C – S – Enz
H O
:O H
CH3 – C – C – O: ⊖
H
O
S – Enz
± H⊕
CH3 – C – C – S – Enz
H O
:OH
CH3 – C – COOH + HS – Enz
H
OH

ideally suited to comparisons by Hammet plots. Inhibition experiments with racemic mixtures of these substrate analogues could be used to determine the substrate specificity of the enzyme.

Even if these substrate analogues do not bind to the enzyme, β-fluorolactate should bind to lactate racemase as a result of its similar spatial requirements as the natural substrate. If the racemization involves a hydride transfer, this substrate analogue should definitely react more slowly than the natural substrate (Ayling and Kun, 1965). β, β, β-trifluorolactate might be expected to totally inhibit the reaction as it does for lactate dehydrogenase (Pogolotti and Rupley, 1973). Thus, a study of the reactivity of these 3-fluorinated lactic acids could confirm or reject the hydride transfer hypothesis.

Both L- and D-β-fluorolactate would be necessary for rate comparisons. These could be prepared enzymatically from β-fluoropyruvate (Eisman et al., 1965; Shapiro and Dennis, 1965; Grassetti et al., 1966). The two enantiomers of β, β, β-trifluorolactic acid may be prepared from β, β, β-trifluoropyruvic acid by a similar procedure (Dipple and Heidelberger, 1966).

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