Vagelos, P. R., Alberts, A. W., and Martin, D. B. (1963), *J. Biol. Chem. 238*, 533.

Wampler, D. E., and Westhead, E. W. (1968), *Biochemistry 7*, 1661.

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

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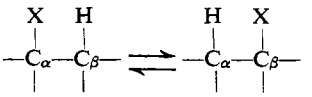
ABSTRACT: Use of a mixture of unlabeled and tetradeuterio-β-methylaspartate with coenzyme B_{12} dependent β-methylaspartate–glutamate mutase has shown that the hydrogen that migrates becomes one of three equivalent hydrogens during the isomerization. Kinetic isotope effects suggest that cleavage of 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. Mechanistic implications of these findings are discussed.

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 (Brownstein and Abeles, 1961; Lee and Abeles, 1963), deamination of ethanolamine to acetaldehyde (Bradbeer, 1965; Babior 1969), isomerization of methylmalonyl coenzyme A to succinyl coenzyme A (Smith and Monty, 1959; Wood *et al.*, 1964), isomerization of β-methylaspartate to glutamate (Barker *et al.*, 1958; Iodice and Barker, 1963; Barker *et al.*, 1964a), the isomerization of β-lysine to 3,5-diaminohexanoic acid (Stadtman and Renz, 1968), and the isomerization of α-methyleneglutarate to α-methylene-β-methylsuccinate (Kung *et al.*, 1970). These six reactions have several features in common which can be summarized (eq 1).



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In the isomerizations of both β-methylaspartate (Barker *et al.*, 1964a,b) and methylmalonyl coenzyme A (Kellermeyer and Wood, 1962; Wood *et al.*, 1964; Phares *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), the isomerization of methylmalonyl coenzyme A (Overath *et al.*, 1962; Erfle *et al.*, 1964a,b), the dehydration of propanediol (Brownstein and Abeles, 1961), or the deamination of ethanolamine (Babior and Gould, 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), methylmalonyl coenzyme A mutase (Riley and Arigoni, 1966), ethanolamine deaminase (Babior, 1969), and glutamate mutase (Switzer *et al.*, 1969). Stereochemical studies of the rearrangments 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; Zagalak *et al.*, 1966). However, configurational inversion is not 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 to the coenzyme cleaves the cobalt C-5′ bond and transforms C-5′

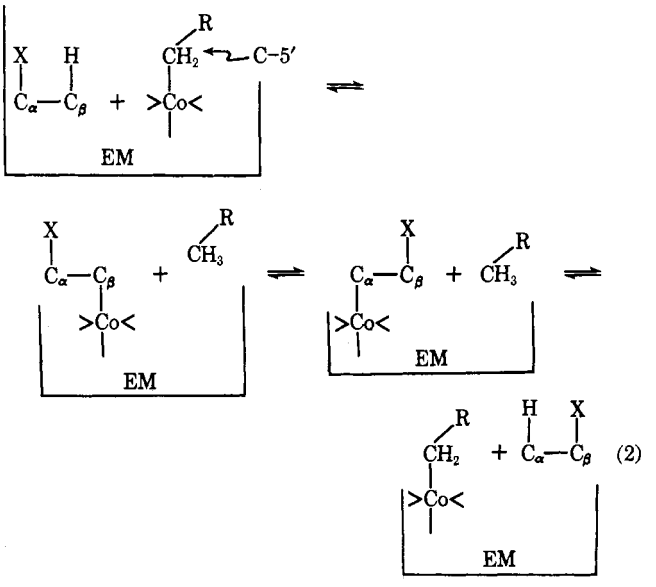

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TABLE I: Relative Intensities.

m/e	β-Methylaspartate	Tetra-[²H]-β-methyl-aspartate
	For Diethyl β-Methylaspartate	
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
	Glutamate	Penta-[²H]glutamate
	For Diethyl 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

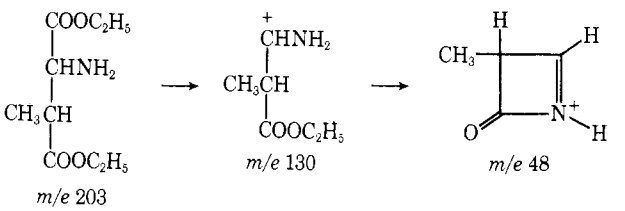
to a methyl group with three equivalent hydrogens, one of which is eventually returned to substrate (eq 2) (Miller and Richards, 1969). The purpose of the present work was to study in an analogous way the fate of the migrating hydrogen in the isomerization of β-methylaspartate to glutamate to establish the generality of an intermediate with three equivalent hydrogens, one of which is the migrating hydrogen and particularly to see if the difference is stereochemistry 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 reactions. The method was analogous to that used previously in the study of methylmalonyl coenzyme A isomerization (Miller and Richards, 1969). 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-trideuteriomethyl-α-deuterioaspartic 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 were impractical because of the strong tendency for the amino acids to dimerize to their 2,5-diketopiperazine 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°) and by determining the mass spectra as rapidly as possible after sample introduction (less than 10 min 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 of



these regions for diethyl β-methylaspartate and the tetradeuterio analog (three deuteriums in the β-methyl group, one deuterium on the α carbon) and also similar data for diethyl glutamate and its pentadeuterio analog (two deuteriums each on the β and γ carbons, one deuterium on C-α). Table II summarizes 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 tetradeuterated diethyl β-methylaspartate, one must know the mass spectra of each of these species. In fact, the spectra of only the non- and tetradeuterio analogs 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 analog comes from loss of a single deuterium and not by loss of two hydrogens from the tetradeuterio 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 the $(m-1)/e$ and $(m-2)/e$ peaks for the tetradeuteriodiethyl β-methylaspartate (*m/e* 87 and 86, respectively)

TABLE II: Spectral Studies.

m/e	β-Methylaspartate Before Reaction	β-Methylaspartate After 21.2% Reaction	β-Methylaspartate 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
	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	

should equal $(m - 1)/e$ for nondeuteriodiethyl β-methylaspartate (m/e 83). This is not observed, there being in the tetradeuteriodiethyl 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 $m/3$ 86 of 1.6, whereas the peak at m/e 83 for the nondeuterated analog 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 tetradeuterio diethyl β-methylaspartate will give an anticipated intensity of the $(m - 1)/e$ peak for, say the dideuterio species $[(m - 1)/e]$ of $4.6 + 2/4(9.0 - 4.6) = 6.8$. Similarly the $(m - 1)/e = 86$ peak for the trideuterio 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 nondeuterio, m of monodeuterio, $(m - 1)/e$ of dideuterio, $(m - 2)/e$ of trideuterio, and $(m - 3)/e$ of tetradeuteriodiethyl 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. (β_0 signifies β-methylaspartate with no deuterium, β_1 signifies β-methylaspartate with one deuterium, etc.): 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 $= 0.0\beta_0 + 6.7\beta_1 + 2.0\beta_2 + 8.5\beta_3 + 100.0\beta_4$.

TABLE III: Interpolated Relative Intensities.

m/e	β_0 (obsd)	β_1 (calcd)	β_2 (calcd)	β_2 (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
	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

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 130 ranges for diethyl glutamate. The relative intensities experimentally determined for non- and tetradeuteriodiethyl β-methylaspartate and for non- and pentadeuteriodiethyl glutamate are listed in Table I; the interpolated coefficients used in analyzing mixtures of deuterated species are given in Table III.

Calculations of the type just described lead from the experimentally observed relative mass spectral intensities of Table II to the relative amounts of variously deuterated species shown in Table IV for β-methylaspartate before, β_0^b, β_1^b, etc., and after β_0^a, β_1^a, etc., reaction and for glutamate after reaction G_0, G_1, etc.

Comment deserves to be made about possible errors introduced by the assumption that mass spectra for intermediately deuterated species can be predicted by simple extrapolation of spectra for diethyl non- or tetradeuterio-β-methylaspartate, for example. (The fact that hydrogen seems to be lost somewhat more readily from the tetradeuterio than from the nondeuterio derivative causes another similar worry.) Because the $(m - 1)/e$, $(m - 2)/e$, $(m + 1)/e$, etc., peaks are in general less than 10% as intense as the major peaks, they serve only as a relatively minor perturbation in determining actual isotopic distributions in experimental mixtures. In fact changing the small coefficients in Table III by as much as ±25% (*e.g.*, from 5.1 to 6.4) effects the calculated isotopic distributions by less than 5% (*e.g.*, β_0 changes from 30.7 to 30.5, β_3 changes from 3.9 to 3.8). Moreover, the very close similarities between isotopes composition of reactants and products derived by analysis of mass spectral observations in the range m/e 84–88,

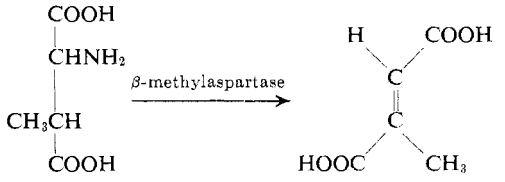
TABLE IV: Percentages of Variously Deuterated Species.

		From *m/e* 84 − *m/e* 88		From *m/e* 130 − *m/e* 134		From *m/e* 84 − *m/e* 88		From *m/e* 130 − *m/e* 134	
				Before and After 21.2% Reaction					
β_0^b	50.0	β_0^a	38.6	β_0^a	38.4	G_0	78.1	G_0	78.5
β_1^b		β_1^a	1.8	β_1^a	2.5	G_1	10.6	G_1	10.2
β_2^b		β_2^a	0.5	β_2^a	0.2	G_2	0.3	G_2	1.2
β_3^b		β_3^a	2.3	β_3^a	2.3	G_3	6.5	G_3	6.1
β_4^b	50.0	β_4^a	56.8	β_4^a	56.6	G_4	4.5	G_4	4.0
				Before and After 36.4% Reaction					
β_0^b	50.0	β_0^a	30.7	β_0^a	29.4	G_0	74.8	G_0	72.5
β_1^b		β_1^a	3.7	β_1^a	3.4	G_1	11.8	G_1	12.0
β_2^b		β_2^a	0.4	β_2^a	0.4	G_2	0.2	G_2	2.0
β_3^b		β_3^a	3.9	β_3^a	4.9	G_3	8.5	G_3	8.0
β_4^b	50.0	β_4^a	61.3	β_4^a	61.9	G_4	4.7	G_4	5.5

on the one hand, and *m/e* 130–134, on the other, further support the applicability of these extrapolated techniques in determination of isotopic composition.

Discussion

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-methylaspartate and observing the amount of mesaconate so produced in a spectrophotometric assay (Barker *et al.*, 1959). This method was used only to obtain an approximate idea of the extent of conversion of β-methylaspartate to glutamate so as to quench



the reaction at an appropriate time.

More accurate estimates were obtained by determining the concentrations of β-methylaspartate and glutamate after reaction by means of a Beckmann 120C amino acid analyzer. This method showed than in one experiment, 21.2% of the original β-methylaspartate had been converted to glutamate; in the other experiment, 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 per cent 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 can 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) only a negligible fraction of the original deuterium can remain 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} (77 nmoles of coenzyme B_{12}) that even if all the C-5′ hydrogens of the coenzyme were 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 in the first experiment the reaction went to about 20% conversion and in the second to about 30% (Table V). These numbers are in reasonable agreement with those obtained by amino acid analysis of 21 and 36%, respectively, but because we feel these later values (21 and 36%) to be the more reliable estimates of the amount of overall reaction, they have been used in subsequent computations.

Amount of Back-Reaction. As the reaction is in fact an equilibration with an equilibrium constant favoring glutamate of 10.6 (Barker *et al.*, 1964b) one can calculate the amount of glutamate (G) that might have been converted back to β-

$$K_{eq} = \frac{(\text{L-glutamate})}{(\text{L-}threo\text{-}\beta\text{-methylaspartate})} = \frac{k_{\beta\rightarrow G}}{k_{G\rightarrow\beta}} = 10.6$$

methylaspartate (β). For the reaction that converted 21% of 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

$$= \frac{(k_{G\rightarrow\beta})(\text{av concn of G})}{(k_{\beta\rightarrow G})(\text{av concn of }\beta)} \left(\begin{matrix} \text{amount of G formed} \\ \text{from } \beta \text{ during reaction} \end{matrix} \right)$$

Thus, for the reaction which gave 21% glutamate, the amount

TABLE V: 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

of β-methylaspartate formed by back-reaction from glutamate will be {(1/10.6)}{(10.6)/(89.4)} (21.2) = 0.24%. For the reaction which gave 36% glutamate, the amount of β-methylaspartate formed by back-reaction from glutamate will be {(1/10.6)}{(18.2)/(81.8)} (36.4) = 0.76%. 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. Therefore 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 stage[1] which can partition to regenerate β-methylaspartate, on the one hand, and to yield glutamate, on the other.

The presence of mono-, di-, and trideuterio-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, $\beta_0^{BR} = \beta_0$ 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 in turn to a value for the 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 21.2/15.4 = 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 VI), *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). 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.

TABLE VI: Partitioning Ratio for Glutamic Acid over β-Methylaspartic 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

Equilibrium between β-methylaspartate and glutamate favors glutamate by a factor of 10.6:1 (Barker *et al.*, 1964b). Between methylmalonyl coenzyme A and succinyl coenzyme A, the succinyl coenzyme A is favored by a factor of 23:1, about two 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.

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 per cent 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 trideuteriomethyl group and the α-CH 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-methylpyridine (Brown and McDonald, 1966)). Treatment of such a system by normal Michaelis–Menten kinetics leads to

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

which, on integration, yields

$$\frac{k_H\,(k_{-1} + k_D)}{k_D\,(k_{-1} + k_H)} = \frac{\ln\,(\beta_0^0/\beta_0^t)}{\ln\,(\beta_4^0/\beta_4^t)}$$

[1] By the phrase "intermediate stage" or "intermediate" we mean not necessarily a single molecular arrangement but all the entities which occur during covalent attachment of the substrate to the enzyme–coenzyme complex, an attachment which in our view (see subsequent discussion of mechanism) involves formation of a bond between the cobalt atom of the coenzyme and a carbon of the substrate.

TABLE VII: Percentage Composition of the Reaction Mixture after Reaction and the Calculated Isotope Effect.

	21.2% Reaction		36.4% Reaction	
	m/e 84 Range	*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_{0U} [a]	19.9	19.5	4.6	5.3
β_4^W [a]	43.5	43.6	37.6	37.2
β_0^{BR}	10.5	10.9	14.9	14.3
β_1^{BR}	1.4	1.4	2.4	2.4
β_2^{BR}	0.4	0.4	0.2	0.2
β_3^{BR}	1.8	1.8	2.5	2.5
β_4^{BR}	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

[a] U = unreacted.

where β_0^t = total unreacted β-methylaspartate without any deuterium at time t, β_0^0 = total initial concentration of undeuterated β-methylaspartate and β_4^0 and β_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: $\beta_0 = 50.0\%$ and $\beta_4 = 50.0\%$.

In the experiment that went to 21.2% glutamate using the mass spectral data in the *m/e* 84 range we can calculate

$$G_H = (G_0 + G_1 + 0.5G_2)0.21$$
$$= [78.1 + 10.6 + 0.5(0.3)]0.21 = 18.8\%$$
$$G_D = (G_4 + G_3 + 0.5G_2)0.21$$
$$= [4.5 + 6.5 + 0.5(0.3)]0.21 = 2.4$$

unreacted β_0	$= (\beta_0^{total} - \beta_0^{BR})0.79$	
	$= (38.6 - 13.3)0.79$	$= 19.9$
unreacted β_4	$= (56.8 - 1.6)0.79$	$= 43.5$
β_0^{BR}	$= (13.3)0.79$	$= 10.5$
β_1^{BR}	$= (1.8)0.79$	$= 1.4$
β_2^{BR}	$= (0.5)0.79$	$= 0.4$
β_3^{BR}	$= (2.3)0.79$	$= 1.8$
β_4^{BR}	$= (1.6)0.79$	$= 1.3$
		100.0%

Therefore, $k_H/k_D = \ln(50.0/19.9)/\ln(50.0/43.5) = 6.6$. Table VII collects this information for the *m/e* 130 range for the 21.2% experiment and for the *m/e* 84 and 130 ranges for the 36.4% experiment.

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: $k_H/k_D \sim 6$–10 for abstraction of hydrogen in the keto–enol conversion which is the rate-determining step in the bromination of acetone (Reitz and Kapp, 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 coenzyme A 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 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 so-called "merry-go-round" mechanisms deals with schemes in which the hydrogen is never returned to the same molecule from which it was abstracted. Four of these (i—two-equivalent hydrogen model, ii—three-equivalent hydrogen model, iii—one-hydrogen-merry-go-round model, and iv—two-hydrogen-merry-go-round model) have previously been described (Miller and Richards, 1969).

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 in earlier work on methylmalonyl coenzyme A mutase (Miller and Richards, 1969) and can be applied in the present cases as well. In the present case unlabeled and tetradeuterio-β-methylaspartate was used; in the work on methylmalonyl coenzyme A mutase, unlabeled and trideuterated substrate was used. Accordingly, in the present case, equations will be in terms of β_0, β_1, β_3, and β_4 and G_0, G_1, G_2, and G_4 (β_2 and G_2 commonly arise as the result of at least two reactions).

Appropriate equations were derived for each of the models 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 and taking explicit account of the formation of G_2. These equations were programmed 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

TABLE VIII: Best Fits for Various Models (for *m/e* 84 Range of 21.2% Reaction).

Model	Calculated							
	Δ_1	Δ_2	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 equations used to derive these distributions are

$$G_0 = \beta_0\Delta_1\Delta_2(B_0 + {}^2/_3B_1 + {}^1/_3B_2) + {}^1/_3\beta_1\Delta_2({}^2/_3B_0 + {}^1/_3B_1)$$

$$G_1 = \beta_0\Delta_1({}^1/_3B_1 + {}^2/_3B_2) + {}^2/_3\beta_1\Delta_1\Delta_2(B_0 + {}^2/_3B_1 + {}^1/_3B_2) + {}^1/_3\beta_1({}^1/_3\beta_0 + {}^2/_3B_1 + B_2) + {}^1/_2\beta_2\Delta_2({}^2/_3B_0 + {}^1/_3B_1)$$

$$G_2 = {}^2/_3\beta_1\Delta_1({}^1/_3B_1 + {}^2/_3B_2) + {}^1/_2\beta_2\Delta_1\Delta_2(B_0 + {}^2/_3\beta_1 + {}^1/_3B_2) + {}^1/_2\beta_2({}^1/_3B_0 + {}^2/_3B_1 + B_2) + {}^2/_3\beta_3\Delta_2({}^2/_3B_0 + {}^1/_3B_1)$$

$$G_3 = {}^1/_2\beta_2\Delta_1({}^1/_3B_1 + {}^2/_3B_2) + {}^1/_3\beta_3\Delta_1\Delta_2(B_0 + {}^2/_3B_1 + {}^1/_3B_2) + {}^2/_3\beta_3({}^1/_3B_0 + {}^2/_3B_1 + B_2) + \beta_4\Delta_2({}^2/_3B_0 + {}^1/_3B_1)$$

$$G_4 = {}^1/_3\beta_3\Delta_1({}^1/_3B_1 + {}^2/_3B_2) + \beta_4({}^1/_3B_0 + {}^2/_3B_1 + B_2)$$

$$B_0 = ({}^2/_3\beta_0 + {}^4/_9\beta_1 + {}^1/_3\beta_2 + {}^2/_9\beta_3)^2\Delta_1{}^2$$

$$B_1 = 2({}^2/_3\beta_0 + {}^4/_9\beta_1 + {}^1/_3\beta_2 + {}^2/_9\beta_3)({}^2/_9\beta_1 + {}^1/_3\beta_2 + {}^4/_9\beta_3 + {}^2/_3\beta_4)\Delta_1\Delta_2$$

$$B_2 = ({}^2/_9\beta_1 + {}^1/_3\beta_2 + {}^4/_9\beta_3 + {}^2/_3\beta_4)^2\Delta_2{}^2$$

TABLE IX: Best Fits for Various Models (for *m/e* 130 Range of 21.2% Reaction).

Model	Calculated							
	Δ_1	Δ_2	G_0	G_1	G_2	G_3	G_4	Error
i Two equivalent hydrogens	4.8	4.0	81.4	6.8		6.8	5.1	5.24
ib Two equivalent hydrogens with back-reactions	5.0	4.1	80.1	7.6	0.4	7.2	4.7	2.36
ii Three equivalent hydrogens	6.9	1.9	79.2	8.2		8.2	4.3	2.13
iib Three equivalent[b] hydrogens with back-reaction	7.8	1.6	78.7	8.6	0.4	8.2	4.1	1.55
iii One-hydrogen merry-go-round	5.9	*a*	69.1	14.0		14.0	2.9	41.92
iv Two-hydrogen merry-go-round	5.9	*a*	69.1	14.0		14.0	2.9	41.92
v Three hydrogen	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	

[a] See Table VIII, footnote *a*. [b] See Table VIII footnote b.

distribution were made using the following equation for absolute error

$$\text{error} = 1\Big/n\sum_{i=1}^{n}(G_{i-1}{}^{\text{obsd}} - G_{i-1}{}^{\text{calcd}})^2$$

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 VIII–XI.

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-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.

TABLE X: Best Fits for Various Models (for *m/e* 84 Range of 36.4% Reaction).

Model	Calculated							
	Δ_1	Δ_2	G_0	G_1	G_2	G_3	G_4	Error
i Two equivalent hydrogens	4.9	3.5	79.0	7.4		7.4	6.2	9.89
ib Two equivalent hydrogens with back-reactions	8.0	2.2	79.5	8.1	0.5	7.2	4.7	7.41
ii Three equivalent hydrogens	5.3	4.0	75.7	9.7		9.7	4.9	1.59
iib Three equivalent[b] hydrogens with back-reaction	7.3	2.1	74.8	10.5	0.6	9.7	4.4	0.62
iii One-hydrogen merry-go-round	6.0	*a*	66.4	15.1		15.1	3.4	31.88
iv Two-hydrogen merry-go-round	6.0	*a*	66.4	15.1		15.1	3.4	31.88
v Three hydrogen	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	

[a] See Table VIII, footnote *a*. [b] See Table VIII, footnote *b*.

TABLE IX: Best Fits for Various Models (for *m/e* 130 Range of 36.4% Reaction).

Model	Calculated							
	Δ_1	Δ_2	G_0	G_1	G_2	G_3	G_4	Error
i Two equivalent hydrogens	4.4	3.2	76.7	8.0		8.0	7.4	9.40
ib Two equivalent hydrogens with back-reactions	4.6	3.3	74.1	9.6	0.6	8.9	6.8	2.61
ii Three equivalent hydrogens	4.4	4.5	73.3	10.4		10.4	6.0	2.07
iib Three equivalent[b] hydrogens with back-reaction	7.0	1.5	72.9	10.8	0.7	10.1	5.6	1.51
iii One-hydrogen merry-go-round	5.4	*a*	63.7	16.1		16.1	4.1	39.66
iv Two-hydrogen merry-go-round	5.3	*a*	63.2	16.3		16.3	4.2	42.84
v Three hydrogen	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	

[a] See Table VIII, footnote *a*. [b] See Table VIII, footnote *b*.

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.

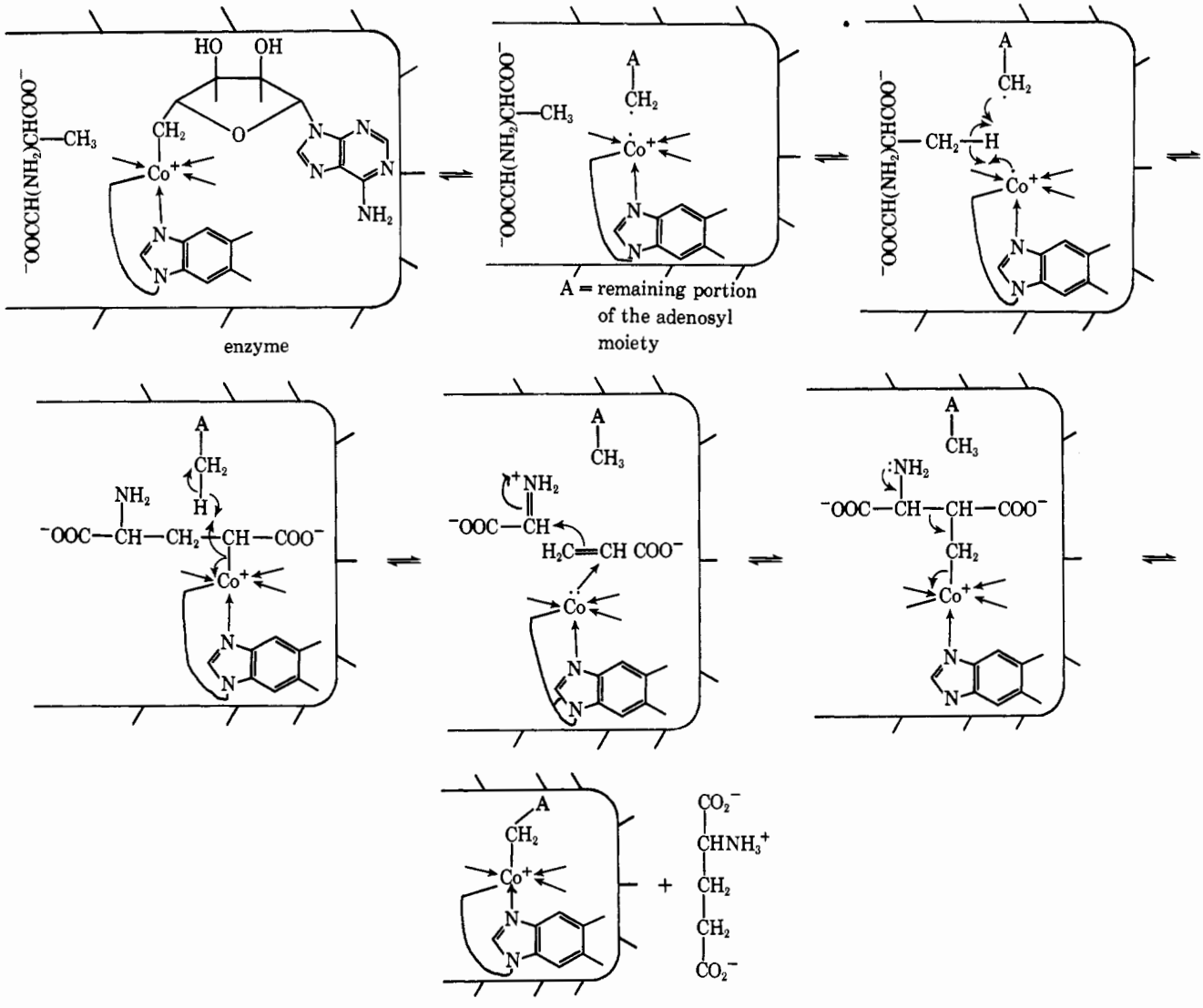
In addition to the poor agreement between the calculated and observed isotope distribution for merry-go-round models, another objection against these models can be raised. They fail to provide a clear pathway for formation of β-methylaspartate containing one, two, or three deuteriums (β_1, β_2, β_3). For example, if deuterium is removed from β_4, and this particular deuterium maintains its identity as required by the assumptions of the merry-go-round model, then the principle of microscopic reversibility will require that this same deuterium atom should be transferred back if the intermediate reverts to β-methylaspartate. Only if the deuterium first acquired from β-methylaspartate can become equivalent with hydrogens on the coenzyme (which hydrogens can be transferred back to β-methylaspartate) does a pathway exist for conversion of β_4 to β_3, etc.

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 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 causes only a negligible perturbation to the contention that Δ_2 represents the kinetic isotope effect in the second transfer to hydrogen from intermediate to glutamate.

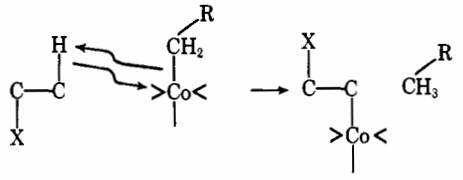
One might anticipate that Δ_1 and Δ_2 would have similar values (as they did in the earlier work on methylmalonyl coenzyme A mutase (Miller and Richards, 1969)) but Δ_2 is computed to be significantly smaller than Δ_1. This may indicate that the differences in the ground-state vibrational energies of CH *vs.* CD 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, transfer of hydrogen by β-methylaspartate mutase is intermolecular and involves an intermediate state where the migrating hydrogen becomes 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. Moreover, the difference in stereochemistry at the carbon to which hydrogen migrates (retention for methylmalonyl coenzyme A mutase, inversion for glutamate mutase) does not cause an apparent change in mechanism for hydrogen transfer as observed by the techniques of this work.

SCHEME I: MECHANISTIC SCHEME



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 $k_H/k_D \sim 2$–8 are observed when the migrating hydrogen is replaced by deuterium, and (iii) in both the methylmalonyl coenzyme A mutase and the glutamate mutase reactions, the migrating hydrogen becomes 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 of substrate 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 unprecedented 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 electron paramagnetic resonance (epr) signals characteristic of B_{12r} in ethanolamine deaminase (Babior, 1970b; Babior and Gould, 1969) and ribonucleotide reductase (Hamilton *et al.*, 1971) suggest that homolytic scission of the carbon–cobalt bond may be important, although the advent of the epr signal in ethanolamine deaminase lags behind the onset of enzymatic conversion, which might suggest that in this case the epr signal is not a direct result of the enzymatic process. 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 which would make the cobalt more accessible to substrate.

This mechanism 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 re-forms the cobalt–C-5′ bond. Some evidence for the intermediate presence of the 5′-deoxynucleoside comes from the observation that the use of 5′-deoxyinosylcobalamin 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.) Babior (1970a) has also observed the release of 5′-deoxyadenosine when ethanolamine deaminase–coenzyme B_{12} complex is treated with ethylene glycol; moreover one of the three hydrogens of the 5′-methyl group of 5′-deoxyadenosine comes from the ethylene glycol.

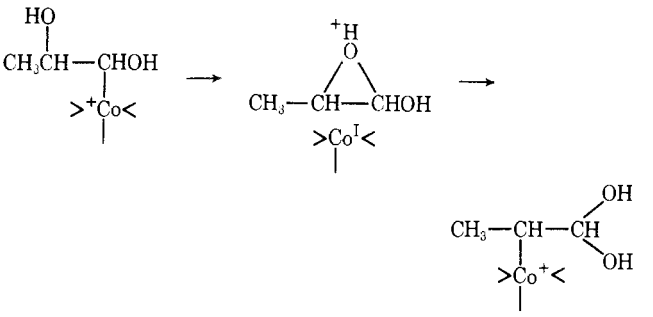
The observed kinetic isotope effect is also in the region of some reported for radical-induced hydrogen abstractions. For example, $k_H/k_D = 4.5$ has been observed for the reaction of phenyl radicals with toluene (Bridger and Russell, 1963) and $k_H/k_D = 7$ for reaction of methyl radicals with toluene (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, which now seems eliminated by the unanticipated finding that an analog of coenzyme B_{12} with a methylene group in place of this ether oxygen (Kerwar *et al.*, 1970) has coenzyme activity.

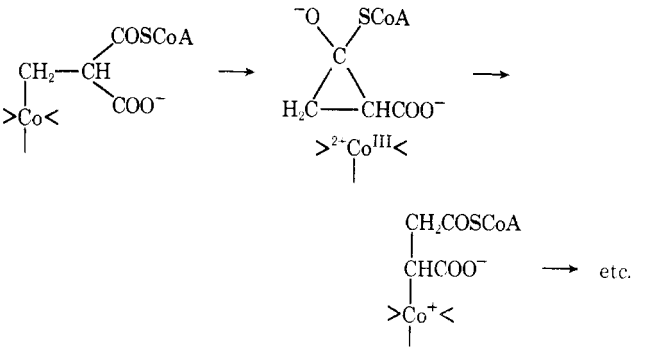
The importance of cobalt in rearrangement processes has been demonstrated in interconversion of model substances, for example, α- and β-substituted ethylcobaloximes (Schrauzer and Windgassen, 1967). The importance of a Co^I nucelophile in rearrangement processes has claimed to be significant in propanediol dehydrase which is reported to be appreciably inhibited by the presence of nitrous oxide (Schrauzer *et al.*, 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. Subsequent intermediates in the rearrangement sequence of Scheme I might explain such a result as the relative instability of the carbonyl coenzyme A (or glycyl) fragments as migrating groups might limit the completeness of acquisition of the electron pair by cobalt and therefore its character as a Co^I nucleophile. This in turn, could render the cobalt less susceptible to attack by nitrous oxide. However, in the case of propanediol dehydrase, the intermediate might have sufficient Co^I character to react with nitrous oxide which could account, thereby, for the partial inhibition observed. Though glycyl and acrylate fragments may exist as separate molecular entities during rearrangement, they must at all times be firmly bound to the enzyme–coenzyme complex, as neither free glycine or free acrylate can be detected (Barker *et al.*, 1964a).

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, and though the pH optimum is 8.5, the reaction occurs at a significant rate down to pH 5.5–6.0 (Barker *et al.*, 1964b).

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 carbanion skeleton needs to be considered.



In the case of the rearrangement of β-methylaspartate, such migration could be facilitated by interaction of the amino acid fragment with pyridoxal phosphate, a possibility which was suggested by Eggerer *et al.* (1960), but no evidence for B_6 involvement could be obtained (Suzuki and Barker, 1966). Conceivably some other carbonyl group from the enzyme itself may play such a role but no evidence on this point is presumably available. 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 can rationalize all the known facts of coenzyme B_{12} dependent reactions. Moreover the ability of the cobalt to exist readily in three formal oxidation states (Co^I, Co^{II}, and Co^{III}) may account for the versatility of coenzyme B_{12} in catalyzing these seemingly unrelated 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, 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 Section

3-Tri-[^2H]Methyl-[2-^2H]Aspartic Acid. The deuterated amino acid was made by an analogous scheme as the reported preparation of the tritiated 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 nuclear magnetic resonance (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 mmoles) was converted to L-*threo*-3-tri-[$^2H_2$]-methyl-[2-^2H]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. The reaction was followed by the decrease in 240-mμ absorbance. After incubation at 35° for 45 min, the reaction was complete. The solution was deproteinized by chilling, adding glacial acetic acid to pH 5.0, and heating at 90–95° for 3 min. After filtering, concentrated hydrochloric acid was added to pH 3.1. Absolute ethanol (9 ml) was then 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%) of 3-tri-[^2H]methyl-[2-^2H]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% or less mesaconate.

Unlabeled β-methylaspartic acid was obtained in the same way by enzymatic transformation of diammonium mesaconate.

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

Isomerization of the Above Mixture. The reaction mixture (2.4 ml) contained 40 mM Tris-Cl buffer (pH 8.2), 20 mM mercaptoethanol, 2 mM calcium chloride, 0.5 ml of the above β-methylaspartate mixture (40 mM), 1×10^{-8} M cyanocobinamide, 3.2×10^{-5} M deoxyadenosylcobalamin, 4.08 units of component S, and 0.84 unit of component E. The mixture was prepared by the method of Barker *et al.* (1964b) and incubated in a 5-ml syringe in the dark at 37° (Switzer *et al.*, 1969). The reaction was followed by removing 2-μl portions and determining the amount of residual β-methylaspartate by an enzymic assay (Barker et al., 1959). After approximately one-third 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° for 2 min, centrifuged, and the solution decanted. The deproteinized reaction mixture was acidified to pH 1–2 with 2 drops of 4 N hydrochloric acid. A small portion of the reaction mixture was then adsorbed to a 0.9 × 53 cm column of AA-15 custom spherical cation-exchange resin of a Beckman 120C amino acid analyzer and eluted at a rate of 68 ml/hr with a sodium citrate buffer (0.20 M Na^+; 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 and 2.27-ml fractions were collected. The amino acid peaks 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.* (1954) on a 0.9 × 12 cm column 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 then charged with 100 ml of 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 *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 min). The molar intensities of both β-methylaspartate and deuterio-β-methylaspartate and both glutamate and deuterioglutamate were shown to be the same within experimental error.

References

Abeles, R. H., and Frey, P. A. (1966), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 25*, 1639.

Abeles, R. H., and Zagalak, B. (1966), *J. Biol. Chem. 241*, 1245.

Babior, B. M. (1969), *J. Biol. Chem. 244*, 449.

Babior, B. M. (1970a), *J. Biol. Chem. 245*, 1755.

Babior, B. M. (1970b), *J. Biol. Chem. 245*, 6125.

Babior, B. M., and Gould, D. C. (1969), *Biochem. Biophys. Res. Commun. 34*, 441.

Barker, H. A., Rooze, V., Suzuki, F., and Iodice, A. A. (1964b), *J. Biol. Chem. 239*, 3260.

Barker, H. A., Smyth, R. D., Wilson, R. M., and Weissbach, H. (1959), *J. Biol. Chem. 234*, 320.

Barker, H. A., Suzuki, F., Iodice, A. A., and Rooze, V. (1964a), *Ann. N. Y. Acad. Sci. 112*, 644.

Barker, H. A., Weissbach, H., and Smyth, R. D. (1958), *Proc. Nat. Acad. Sci. U. S. 44*, 1093.

Biemann, K., Seibl, J., and Gapp, F. (1961), *J. Amer. Chem. Soc. 83*, 3795.

Bradbeer, C. (1965), *J. Biol. Chem. 240*, 4765.

Bridger, R. F., and Russell, G. A. (1963), *J. Amer. Chem. Soc. 85*, 3754.

Brodie, J. D. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 461.

Brown, H. C., and McDonald, G. J. (1966), *J. Amer. Chem. Soc. 88*, 2514.

Brownstein, A. M., and Abeles, R. H. (1961), *J. Biol. Chem. 236*, 1199.

Dréze, A., Moore, S., and Bigwood, E. J. (1954), *Anal. Chim. Acta 11*, 554.

Eggerer, H., Stadtmann, E. R., Overath, P., and Lynen, F. (1960), *Biochem. Z. 333*, 1.

Erfle, J. D., Clark, J. M., Jr., and Johnson, B. C. (1964a), *Ann. N. Y. Acad. Sci. 112*, 684.

Erfle, J. D., Clark, J. M., Jr., Mystrom, R. F., and Johnson, B. C. (1964b), *J. Biol. Chem. 239*, 1920.

Frey, P. A., and Abeles, R. H. (1966), *J. Biol. Chem. 241*, 2732.

Frey, P. A., Karabatsos, G. L., and Abeles, R. H. (1965), *Biochem. Biophys. Res. Commun. 18*, 551.
Hamilton, J. A., Yamada, R., Blakley, R. L., Hogenkamp, H. P. C., Looney, F. D., and Winfield, M. E. (1971), *Biochemistry 10*, 347.
Iodice, A. A., and Barker, H. A. (1963), *J. Biol. Chem. 238*, 2094.
Jayme, M., and Richards, J. N. (1971), *Biochem. Biophys. Res. Commun., 43*, 1399.
Kellermeyer, R. W., Allen, S. H., Stjernholm, R., and Wood, H. G. (1964), *J. Biol. Chem. 239*, 2562.
Kellermeyer, R. W., and Wood, H. G. (1962), *Biochemistry 1*, 1124.
Kerwar, S. S., Smith, T. A., and Abeles, R. H. (1970), *J. Biol. Chem. 245*, 1169.
Kung, H. F., Cederbaum, S., Tsai, L., and Stadtman, T. C. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 978.
Lee, J. A., Jr., and Abeles, R. N. (1963), *J. Biol. Chem. 238*, 2367.
Miller, W. W., and Richards, J. H. (1969), *J. Amer. Chem. Soc. 91*, 1498.
Overath, P., Kellerman, G. M., Lynen, F., Fritz, H. P., and Keller, H. J. (1962), *Biochem. Z. 335*, 500.
Phares, E. F., Long, M. V., and Carson, S. F. (1964), *Ann. N. Y. Acad. Sci. 112*, 680.
Reitz, O., and Kapp, J. (1939), *Z. Physik. Chem. 184A*, 429.
Retey, J., Umani-Ronchi, A., and Arigoni, D. (1966), *Experientia 22*, 72.
Riley, J., and Arigoni, D. (1966), *Experientia 22*, 783.
Schrauzer, G. N., Holland, R. J., and Seck, J. A. (1971), *J. Amer. Chem. Soc. 93*, 1503.
Schrauzer, G. N., and Windgassen, R. J. (1967), *J. Amer. Chem. Soc. 89*, 1999.
Smith, R. M., and Monty, K. J. (1959), *Biochem. Biophys. Res. Commun. 1*, 105.
Sprecher, M., Clark, M. J., and Sprinson, D. B. (1964), *Biochem. Biophys. Res. Commun. 15*, 881.
Sprecher, M., and Sprinson, D. B. (1964), *Ann. N. Y. Acad. Sci. 112*, 655.
Stadtman, T. C., and Renz, P. (1968), *Arch. Biochem. Biophys. 125*, 226.
Suzuki, F., and Barker, H. A. (1966), *J. Biol. Chem. 241*, 878.
Switzer, R. L., Baltimore, B. G., and Barker, H. A. (1969), *J. Biol. Chem. 244*, 5263.
Wilen, S. H., and Eliel, E. L. (1958), *J. Amer. Chem. Soc. 80*, 3309.
Wood, H. G., Kellermeyer, R. W., Stjernholm, P., and Allen, S. H. G. (1964), *Ann. N. Y. Acad. Sci. 112*, 661.
Zagalak, B., Frey, P. A., Karabatsos, G. L., and Abeles, R. H. (1966), *J. Biol. Chem. 241*, 3028.

Conformational Studies of the Human Vitamin A–Transporting Protein Complex*

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ABSTRACT: The human vitamin A–transporting protein complex and its two component proteins, prealbumin and the retinol-binding protein (RBP), have been studied by optical rotatory dispersion (ORD) and circular dichroism (CD). Some information about the absolute conformation of the two proteins was obtained from these measurements. Thus neither protein seems to contain any appreciable amount of α helix. Prealbumin may contain some regions of β structure, but more probable is that some nonperiodic, unordered, yet rigid, structure dominates both in this protein and in RBP. The major features of the ORD and CD spectra of RBP are caused by the protein moiety, vitamin A being responsible only for certain minor characteristics. Dissociation of the prealbumin–RBP complex is effected at low ionic strength, as has been described earlier; a major result of this study is that a conformational change associated with this phenomenon occurs in RBP and not in prealbumin. Another conformational change, distinct from the previous one, takes place when COOH-terminal arginine is cleaved from the polypeptide chain of RBP, a reaction that occurs physiologically. Finally, measurements of ORD and CD of the RBP–prealbumin complex reveal that at most minimal alterations of the conformations of the two proteins occur when they interact.

The human vitamin A–transporting protein complex consists of two proteins, the thyroxine-binding prealbumin and the retinol-binding protein (RBP),[1] which is the actual vitamin carrier (Kanai *et al.*, 1968; Peterson, 1969, 1971a). The two proteins are attached to each other by noncovalent bonds, but their interaction is very strong under physiological conditions, the apparent association constant being 2×10^7 M^{-1}. The complex is an example of a protein–protein interaction with a high degree of specificity (Peterson and Rask, 1971). The binding between prealbumin and RBP is abolished at low ionic strength, and concomitantly the fluorescent properties of the retinol bound to RBP are also altered (Peterson, 1971b, Peterson and Rask, 1971). This finding, together with the fact that there exists a modified form of RBP in normal serum which cannot bind to prealbumin (Peterson, 1971c)

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[1] Abbreviations used are: RBP, retinol-binding protein; ORD, optical rotatory dispersion; CD, circular dichroism.