Mechanism of Action of Coenzyme B_{12}. Hydrogen Transfer in the Isomerization of Methylmalonyl Coenzyme A to Succinyl Coenzyme A

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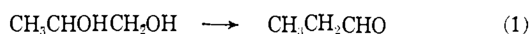
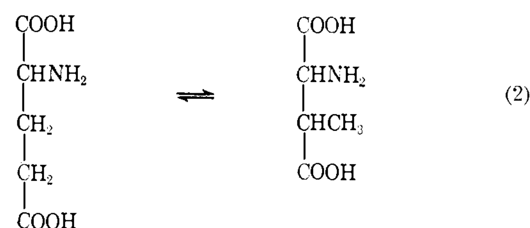
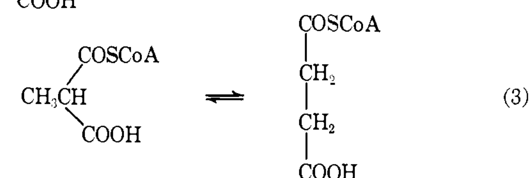
Received August 19, 1968

Abstract: The fate of the hydrogen atom that migrates during the isomerization of methylmalonyl coenzyme A to succinyl coenzyme A catalyzed by methylmalonyl coenzyme A isomerase, an enzyme which requires coenzyme B_{12} as a cofactor, has been studied by a method which follows the statistical partitioning of deuterium between substrate and product and compares the observed distribution to distributions calculated on the basis of various mechanistic models. The method is described, and the results of its application to the methylmalonyl coenzyme A mutase reaction lead to the conclusion that the hydrogen removed from methylmalonyl coenzyme A (which we find to be the rate-determining step in the over-all conversion) becomes one of three equivalent hydrogens before a hydrogen is returned to regenerate methylmalonyl coenzyme A or to yield succinyl coenzyme A. The implications of these results are discussed in terms of involvement of the bond between C-5′ of the deoxyadenosine residue and the cobalt atom of the coenzyme B_{12}.

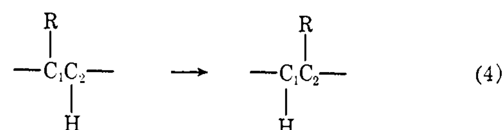
The cobalt-containing coenzyme B_{12} (*d*-dimethylbenzimidazolyl-Co-5′-deoxyadenosylcobamide) has been shown to be a cofactor in at least three rearrangement processes in which hydrogen atoms and other groups undergo 1,2 migrations: dehydration of propanediol to propionaldehyde[1,2] (eq 1), isomerization of glutamic acid to *β*-methyl aspartic acid[3] (eq 2), and isomerization of methylmalonyl coenzyme A to succinyl coenzyme A[4-7] (eq 3). These three reactions have







several features in common which can be summarized (eq 4). Work on the isomerization of methylmalonyl



coenzyme A has shown that the R group (in this case -COSCoA) is transferred intramolecularly.[8-10] Furthermore, the hydrogen which is transferred does not exchange with the hydrogens of water during the isomerization of methylmalonyl coenzyme A,[11-13] of glutamate,[14] or during the dehydration of propanediol.[1] The stereochemistry of the hydrogen migration is known in all three cases. Inversion of configuration is observed at that carbon to which hydrogen migrates for propanediol dehydration[15,16] and also for glutamate isomerization.[17] In contrast, the hydrogen which migrates from the methyl group of methymalonyl coenzyme A to C-3 of succinyl coenzyme A occupies the same steric location as was previously occupied by the -COSCoA group; in this case migration occurs with retention of configuration.[18]

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Another aspect of these reactions which has received attention is the fate of the hydrogen that migrates. Though this hydrogen does not exchange with the hydrogens of water it does exchange with similar hydrogens of other molecules of substrate. In the dehydration of propanediol this has been shown in two ways. Co-incubation of 1,2-propanediol-1-^{3}H and ethylene glycol (without any tritium) with propanediol dehydratase and coenzyme B_{12} gave propionaldehyde and acetaldehyde both of which contained tritium.[19] In another study, a mixture of 1,2-propanediol-1,1-$^2H_2$ and 1,2-propanediol without any deuterium when used as substrate yielded propionaldehyde containing no, one, and two atoms of deuterium.[20] Transfer of tritium from propanediol-1-^{3}H to coenzyme B_{12} and from coenzyme B_{12} containing tritium at C-5′ of the adenosyl moeity to propionaldehyde has also been demonstrated.[21,22] When coenzyme B_{12} containing tritium as a result of incubation with 1,2-propanediol-1-^{3}H and propanediol dehydratase is then used as cofactor with methylmalonyl coenzyme A isomerase and succinyl coenzyme A as substrate, 32% of the tritium originally in the coenzyme B_{12} can be recovered in succinic acid. When methylmalonyl coenzyme A is used as substrate in the same system, 16% of the tritium originally in the coenzyme B_{12} can be recovered in succinic acid.[20]

The purpose of the work described in this paper was to study the transfers of hydrogen from substrate to coenzyme B_{12} and then back to substrate that occur during the isomerization of methylmalonyl coenzyme A and more especially to answer the question: with how many other hydrogens on the coenzyme does the hydrogen that is removed from a molecule of substrate become equivalent before a hydrogen is transferred back to give product? Several detailed models are considered and used to calculate theoretical distributions of deuterium in succinyl coenzyme A derived from a mixture of methylmalonyl coenzyme A containing no deuterium and methylmalonyl coenzyme A containing three deuterium atoms in the methyl group. These expected distributions are then compared to those observed when a mixture of methylmalonyl coenzyme A and 4,4,4-tri-^{2}H-methylmalonyl coenzyme A is isomerized to a mixture of variously deuterated species of succinyl coenzyme A by incubation with methylmalonyl coenzyme A isomerase and coenzyme B_{12} under conditions which minimize the amount of back reaction.

Apart from the present case, the general approach outlined in this article should have general applicability to problems involving intermediates in enzyme reactions.

Results

A mixture of methylmalonyl coenzyme A and 4,4,4-tri-^{2}H-methylmalonyl coenzyme A was partially isomerized to succinyl coenzyme A by methylmalonyl coenzyme A from *Propionibacterium shermanii* and coenzyme B_{12}. The reaction was stopped by hydrolyzing the thiol esters and the methylmalonic and succinic acids were recovered by extraction with ether and purified by ion-exchange chromatography. From 55 μmol of starting methylmalonyl coenzyme A, 1.30 mg (11 μmol) of succinic acid, and 2.66 mg (23 μmol) of methylmalonic acid were recovered.

Table I. Relative Intensities for Methylmalonic Acid

	Ionizing voltage, eV		
m/e	12.5	20.0	70.0
73	37.1	57.1	60.2
74	100.0	100.0	100.0
75	4.2	4.6	4.6
76	0	0	0

Table II. Relative Intensities for 4,4,4-Tri-^{2}H-methylmalonic Acid

	Ionizing voltage, eV		
m/e	12.5	20.0	70.0
74	0	0	0
75	10.6	17.5	19.7
76	19.6	28.9	30.8
77	100.0	100.0	100.0
78	4.0	4.4	4.2
79	0	0	0

Table III. Methylmalonic Acid before Reaction

	Ionizing voltage, eV		
m/e	12.5	20.0	70.0
74	100.0	100.0	100.0
75	12.5	19.3	20.2
76	16.4	25.1	26.2
77	84.8	84.8	82.5

Table IV. Methylmalonic Acid Recovered from Reaction Mixture

	Ionizing voltage, eV		
m/e	12.5	20.0	70.0
74	74.8	74.1	76.4
75	18.1	25.9	28.5
76	25.7	34.8	37.3
77	100.0	100.0	100.0

Mass Spectra. Before introduction into the mass spectrometer, succinic acid was first converted to succinic anhydride. Table I gives the observed relative intensities in the region *m/e* 73–76 at 12.5, 20, and 70 eV for methylmalonic acid; Table II gives similar information for 4,4,4-tri-^{2}H-methylmalonic acid; Table III gives the observed intensities for the starting mixture of unlabeled and trideuteriomethylmalonic acid; Table IV gives the data for methylmalonic acid after reaction. Table V gives the observed mass spectra for unlabeled succinic anhydride and for 2,2,3,3-tetra-^{2}H-succinic acid (90% tetradeuterio, 10% trideuterio) at 12.5 eV, and Table VI gives similar data for the succinic anhydride recovered after reaction.

Calculations. To obtain the relative percentages of the variously deuterated species, spectra for mono- and dideuterated molecules must be interpolated from those for non- and tri- or tetradeuterated substances. In the case of nondeuterated methylmalonic acid, the absence of an m − 2 peak at *m/e* 72 implies that the peak at *m/e* 75 for the trideuterated species comes solely

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Table V. Mass Spectrum of Succinic Anhydride and 2,2,3,3-Tetra-^{2}H-succinic Anhydride at 12.5 eV

m/e	Anhydride: Succinic	Anhydride: 2,2,3,3-Tetra-^{2}H-succinic
27	0	0
28	100.0	100.0
29	2.3	0
30	0	0
31	0	9.7
32	0	100.0
33	0	2.1
55	0.5	0
56	100.0	0
57	3.5	0
58	0	0.8
59	0	9.9
60	0	100.0
61	0	3.3

Table VI. Succinic Anhydride Recovered from Reaction at 12.5 eV

m/e	1	2	3	4
28	100.0	100.0	100.0	100.0
29	24.4	24.1	25.6	25.4
30	17.4	16.4	18.4	18.4
31	11.3	11.1	13.1	12.9
56	100.0	100.0	100.0	100.0
57	25.6	25.9	27.0	25.4
58	18.7	18.2	20.4	19.9
59	11.9	12.2	13.1	12.3

by loss of one deuterium and not by loss of two hydrogens. The assumption is made that the m − 1 peak at 76 for the trideuterated species represents the loss of hydrogen from C-1 and C-2 of the propionic acid which is initially formed by decarboxylation of methylmalonic acid. The m − 2 peak at 75 represents loss of deuterium from C-3. If there were no primary isotope effect for loss of deuterium from C-3, the sum of m − 1 and m − 2 peaks for the trideuterated substance should equal m − 1 peak at m/e 73 for the undeuterated substance. This is not the observed result, indicating the presence of a significant isotope effect in the loss of these hydrogens.

The hypothetical spectra for mono- and dideuterated spectra were calculated taking account of the primary isotope effect for deuterium *vs.* hydrogen loss and neglecting any small secondary isotope effect. For example at 12.5 eV, the m − 1 peak for 4,4-di-^{2}H-methylmalonic acid would be 19.6 (the unobserved m − 1 peak for loss of hydrogen from C-1 and C-2) + $^1/_3$(37.1 − 19.6). In this expression (37.1 − 19.6) is the amount of hydrogen lost from C-3 when the methyl carbon of the intermediate propionic acid bears three deuteriums. If one assumes no secondary isotope effect, the loss of a hydrogen from a $-CD_2H$ group will have one-third the statistical probability of loss of a hydrogen from a $-CH_3$ group. Thus the m − 1 peak should have a relative intensity of 25.4. The m − 2 peak for 4,4-di-^{2}H-methymalonic acid at 12.5 eV will be $^2/_3 \times 10.6 = 7.1$. Similar considerations can be applied to the other deuterated possibilities and lead to Table VII which lists the observed and interpolated

Table VII. Relative Intensities of M_0, M_1, M_2, and M_3 at Ionizing Voltage 12.5 eV, Ionizing Current 60 μA

m/e	M_0 (obsd)	M_1 (calcd)	M_2 (calcd)	M_3 (obsd)
74	100.0	31.3	7.1	0
75	4.2	100.0	25.4	10.6
76	0	4.2	100.0	19.6
77	0	0	4.1	100.0

intensities in the range m/e 74 to 77 for variously deuterated methylmalonic acids (M_0 = nondeuterated methylmalonic acid, M_1 = monodeuteriomethylmalonic acid, etc.).

By use of these values and the observed intensities of the methylmalonic acid before and after reaction, the relative amounts of M_0, M_1, M_2, and M_3 can be calculated by solution of four simultaneous equations. This was done using a matrix inversion subroutine on an IBM 7094 computer. For example, for methylmalonic acid after reaction at 12.5 eV, the following set of equations is obtained.

$$74.8 = 100.0M_0^a + 31.3M_1^a + 7.2M_2^a$$
$$18.1 = 4.2M_0^a + 100.0M_1^a + 25.4M_2^a + 10.6M_3^a$$
$$25.7 = 4.2M_1^a + 100.0M_2^a + 19.6M_3^a$$
$$100.0 = 4.1M_2^a + 100.0M_3^a$$

Solution of these equations leads to: $M_0^a = 40.3\%$, $M_1^a = 1.6\%$, $M_2^a = 3.3\%$, and $M_3^a = 54.8\%$.

A similar approach was used to calculate the percentage distribution among the various deuterated succinic anhydride species, S_0 containing no deuterium, S_1 containing one deuterium, etc.

Table V gives the mass spectra of succinic anhydride and of 90% pure 2,2,3,3-tetradeuteriosuccinic anhydride. In the range m/e 28–31, the spectra of S_1, S_2, and S_3 were calculated by (i) giving the base peak a relative intensity of 100.0 (ii) assigning the m + 1 peak as the weighted average of m + 1 for S_0 at m/e 29 and for S_4 at m/e 33. For example, the spectrum of S_2 would contain a base peak at m/e 30 and an m + 1 peak at m/e 31 with relative intensity $^1/_2(2.3 + 2.1) = 2.2$.

In the range m/e 27–33, one must take into account the peaks due to O_2^+ at m/e 32 and N_2^+ at m/e 28. At an ionizing voltage of 12.5 eV, an air blank revealed a small peak due to O_2^+ at m/e 32, but no peak at m/e 28 since nitrogen is more difficult to ionize than oxygen. Since only peaks between m/e 28 to 31 were used for the actual calculation of deuterium distribution, no error was introduced by injection of a small amount of air along with the sample.

In the range m/e 56–59, the calculation of spectra for S_1, S_2, and S_3 is complicated by the loss of hydrogen from the ion $(CH_2CH_2CO)^+$. Since S_4 was not isotopically pure, the absolute value for deuterium loss from S_4 cannot be calculated, and it was assumed that deuterium loss was as common as loss of hydrogen. Though this is admittedly a poor assumption, the amount of either deuterium or hydrogen loss is sufficiently small that it has only a negligible effect on the calculated percentages, which were done by (i) setting the appropriate base peak of $(CH_2CH_2CO)^+$ or its deuterated analogs equal to 100 (ii) setting m − 1 equal to 0.5 times the fraction of hydrogen in the parent com-

pound (See Table V), (iii) assigning m − 2 a value 0.5 times the fraction of deuterium in the parent ion, and (iv) calculating m + 1 as the weighted average of the isotope peak of S_0 at *m/e* 57 and of S_4 at *m/e* 61.

The observed and calculated spectra for S_0, S_1, S_2, S_3, and S_4 are summarized in Table VIII.

Table VIII. Mass Spectra of S_0, S_1, S_2, S_3, and S_4 at Ionizing Voltage 12.5 eV, Ionizing Current 60 μA

m/e	S_0(obsd)	S_1(calcd)	S_2(calcd)	S_3(calcd)	S_4(obsd)
28	100.0	0	0	0	0
29	2.3	100.0	0	0	0
30	0	2.2	100.0	0	0
31	0	0	2.2	100.0	9.7
32	0	...	...	...	100.0
56	100.0	0.4	0.2	0	0
57	3.5	100.0	0.3	0.3	0
58	0	3.4	100.0	0.2	0.8
59	0	0	3.4	100.0	9.9
60	0	...	...	...	100.0

From the values for the spectra of M_0, M_1, M_2, M_3, S_0, S_1, S_2, and S_3 in Tables VII and VIII and similarly calculated spectra for M_0, M_1, M_2, and M_3 at ionizing voltages of 20 and 70 eV (see Tables I and II) and the observed mass spectra of methylmalonic acid before reaction (M^b), of methylmalonic acid after reaction (M^a) and succinic anhydride after reaction (S) (Tables III, IV, and VI), the final results shown in Table IX were obtained.

Table IX. Percentage of Variously Deuterated Species before and after Reaction

M_0^b	54.4 ± 0.4	M_0^a	40.2 ± 0.3	S_0	65.8 ± 1.0
M_1^b		M_1^a	1.6 ± 0.1	S_1	14.8 ± 0.3
M_2^b		M_2^a	3.3 ± 0.1	S_2	11.7 ± 0.6
M_3^b	45.6 ± 0.4	M_3^a	54.9 ± 0.3	S_3	7.7 ± 0.4

Discussion

The per cent over-all reaction can be calculated in two ways. One depends on the recovery of product and assumes that methylmalonic acid and succinic acid are recovered with the same efficiency. The average molecular weights of methylmalonic acid and succinic acid can be obtained from Table IX. On this basis the reaction was found to have proceeded to 33.0% conversion of methylmalonyl coenzyme A to succinyl coenzyme A.

Another method depends on the conservation of total deuterium in the products, *i.e.*, the total deuterium in the starting methylmalonyl coenzyme A equals the sum of the deuterium in the methylmalonic acid and the succinic acid recovered from the reaction mixture. The conditions necessary for this calculation are (i) that no deuterium be lost to solvent, which has previously been demonstrated[13] and (ii) that the amount of deuterium which remains in coenzyme B_{12} be negligible, a condition which is met by virtue of the vast excess of substrate relative to coenzyme in the incubation mixture. Under these conditions, then, if x equals the mole fraction of methylmalonyl coenzyme A converted to succinyl coenzyme A

$$3M_3^b = x(S_1 + 2S_2 + 3S_3) + (1 - x)(M_1^a + 2M_2^a + 3M_3^a)$$

By use of the data in Table IX, one calculates that $x = 0.325$ or that 32.5% of the starting methylmalonyl coenzyme A has been converted to succinyl coenzyme A.

Since the reaction is, in fact, an equilibration, one must consider the possibility that succinyl coenzyme A may be converted back to methylmalonyl coenzyme A. The average concentration of methylmalonyl coenzyme A during the reaction is 100 − [0.5(33)] = 84 relative to that for succinyl coenzyme A of 16. The equilibrium constant has been shown to be 23:1 in favor of succinyl coenzyme A for that enantiomer of methylmalonyl coenzyme A that is a substrate for isomerase and in the absence of methylmalonyl coenzyme A racemase.[23] In the present experiment a *dl* mixture of methylmalonyl coenzyme A was used and the enzyme preparation contained racemase. Thus the operative equilibrium constant relative to *dl*-methylmalonyl coenzyme A would be about 12. The amount of back-reaction (conversion of free succinyl coenzyme A produced in the reaction to free methyl coenzyme A) can therefore be approximated by [(16)(33)]/[(84)(12)] = 0.5%. Thus the amount of conversion of succinyl coenzyme A back to methylmalonyl coenzyme A is negligibly small under the conditions employed.

Though a negligible amount of methylmalonyl coenzyme A present after the reaction can have ever existed as free succinyl coenzyme A, the presence of methylmalonyl coenzyme A with one and two deuteriums indicates that some intermediate in the reaction can partition yielding succinyl coenzyme A or regenerating methylmalonyl coenzyme A; M_1 and M_2 must be formed by this process. Furthermore the relative amounts of M_0 and M_3 present after reaction that at some time existed as enzyme-bound intermediate can be calculated on the assumption that an intermediate with the carbon skeleton of methylmalonyl coenzyme A abstracts a hydrogen or deuterium in the same ratio as an intermediate with the skeleton of succinyl coenzyme A. The following equations then apply.

$M_0^{BR} = M_0$ (formed by back-reaction as percentage of total M_0^a)

$$M_0^{BR} = M_1^a(S_0/S_1) = 6.6\% \text{ (see Table IX)}$$

$$M_3^{BR} = M_2^a(S_3/S_2) = 2.2\%$$

Taken together with the observed amounts of M_1 and M_2 (which can only have been formed by back-reaction from some intermediate state) this leads to a total amount of methylmalonyl coenzyme A generated by back-reaction of $M_0^{BR} + M_1 + M_2 + M_3^{BR} = 13.7\%$. As the amount of methylmalonyl coenzyme after the reaction is 67% of the total material, (0.67)(13.7) = 9% of the total material after reaction is methylmalonyl coenzyme A formed by back reaction. Thus, the total percentage of methylmalonyl coenzyme A that interacted with the enzyme and reached at least an intermediate stage is (33 + 9) = 42%. The partitioning ratio from intermediate to succinyl coenzyme A or

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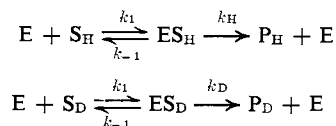
methylmalonyl coenzyme A is therefore 33/9 = 3.7; that is, 79% of the time the intermediate gives succinyl coenzyme A and 21% of the time it regenerates methylmalonyl coenzyme A.

The experimental results clearly indicate a more rapid conversion of substrate with hydrogen than of substrate with deuterium and allow a calculation of the over-all kinetic isotope for the reaction averaged over the first 42% of reaction.

If one considers the following situation



which assumes that k_1 and k_{-1} are negligibly affected by the substitution of deuterium by hydrogen,[24] the following expression can be derived assuming normal Michaelis–Menten kinetics

$$\frac{d(P_H)/dt}{d(P_D)/dt} = \left(\frac{k_H}{k_D}\right)\left(\frac{k_{-1} + k_D}{k_{-1} + k_H}\right)$$

which yields

$$\left(\frac{k_H}{k_D}\right)\left(\frac{k_{-1} + k_D}{k_{-1} + k_H}\right) = \frac{\ln (M_0^0/M_0^t)}{\ln (M_3^0/M_0^t)}$$

where M_0^t = total unreacted methylmalonyl coenzyme A without deuterium at time t; M_0^0 = total initial amount of methylmalonyl coenzyme A without deuterium; M_3^t and M_3^0 = analogous values for trideuterated substrates, and $(k_H/k_D)/[(k_{-1} + k_D)/(k_{-1} + k_H)]$ = the isotope effect averaged from t = 0 to t. This result is equal to the kinetic isotope effect k_H/k_D for the rate-limiting, catalytic step only if k_H and k_D are significantly less than k_{-1}. In the subsequent discussion, the assumption has been made that k_{-1} is, in fact, large with respect to k_H and k_D. If this assumption should prove to be invalid, the observed isotope effects would be less than the true kinetic isotope effect, but, more importantly, the values of Δ_1 and Δ_2 appropriately used in the subsequent calculations of possible mechanistic models for coenzyme B_{12} action would not be altered. The relative concentrations are

Before reaction

$$M_H = 54.4 \text{ mol } \%$$
$$M_D = 45.6 \text{ mol } \%$$

After reaction, succinate (%) from

$$M_0 = S_H = S_0 + S_1 = (65.8 + 14.8)0.33 = 26.6$$

succinate (%) from

$$M_3 = S_0 = S_2 + S_3 = (11.7 + 7.7)0.33 = 6.4$$

unreacted M_0 = (total M_0 − M_0 formed by back-reaction) × 0.67
= (40.2 − 6.6) × 0.67 = 22.6
unreacted M_3 = (54.9 − 2.2) × 0.67 = 35.3
M_0 by back-reaction = (6.6) × 0.67 = 4.4
M_1 by back-reaction = (1.6) × 0.67 = 1.0
M_2 by back-reaction = 3.3 × 0.67 = 2.2
M_3 by back-reaction = 2.2 × 0.67 = 1.5
100.0%

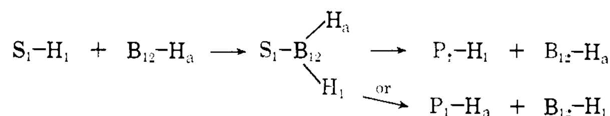
Therefore

$$\frac{k_H}{k_D} = \frac{\log (54.4/22.6)}{\log (45.6/35.3)} = 3.5$$

The meaning of this observed isotope effect is open to some speculation. Because it is averaged over the first 42% of reaction, the true kinetic isotope effect is undoubtedly somewhat larger. In any case, the observed isotope effect is surely large enough to indicate the breaking of a C–H bond in the rate-determining step which would therefore reasonably seem to be abstraction of a hydrogen from a molecule of substrate. An isotope effect of this magnitude has an interesting comparison with the recently reported k_H/k_D = 4.2 at 25° for proton transfer from 9-methylfluorene to 4,5-methylenephenanthryl anion in dimethyl sulfoxide solution.[26] An isotope effect of k_H/k_D = 12 has been observed in another reaction requiring coenzyme B_{12}, the conversion of D-propanediol-1-*d*, to propionaldehyde catalyzed by propanediol dehydratase.[27] One should emphasize that the effect observed in the present case is averaged over the first 42% of the reaction and as a result will be less than the instantaneous kinetic isotope effect.[28]

Another stage in which a kinetic effect could enter would be the readdition of a hydrogen to generate succinyl coenzyme A. The relative partitioning of an intermediate between methylmalonyl coenzyme A or succinyl coenzyme A has been seen to favor succinyl coenzyme A by a factor of 3.7(79/21). This together with the observed isotope effect (averaged over the first 42% reaction), indicates that the main rate-determining step in the isomerization of methylmalonyl coenzyme A to succinyl coenzyme A is breaking of a carbon–hydrogen bond in the methyl group of methylmalonyl coenzyme A.

Four models will be considered. (i) The hydrogen abstracted becomes equivalent with one additional hydrogen on the coenzyme (*i.e.*, in the intermediate there are two equivalent hydrogens on the coenzyme, one of which was abstracted from substrate).



(ii) The abstracted hydrogen becomes equivalent with two additional hydrogens on the coenzyme (*i.e.*, in the

(24) As the principal bonding of substrate to the enzyme–coenzyme B_{12} complex is probably primarily due to ionic, hydrogen bonding or van der Waals interactions, the only effect of replacing the methyl group by a trideuteriomethyl group should be caused by the change in "effective size." The isotope effect for the reaction of 2-(α,α,α-tri-^{2}H)-methylpyridine with methyl iodide has been measured[25] and the value k_H/k_D = 1.030 ± 0.003 reflects the extreme case where a slight difference in size of a methyl group causes a discernible change in rate.

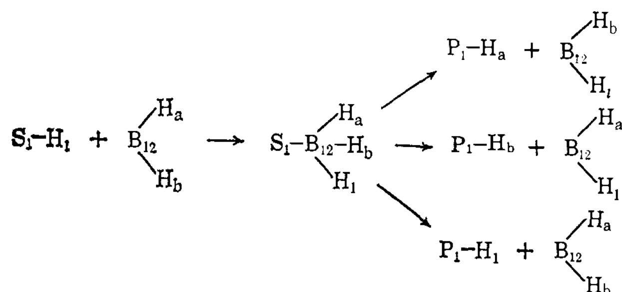
(25) H. C. Brown and G. J. McDonald, *J. Am. Chem. Soc.*, **88**, 2514 (1966).

(26) C. D. Ritchie and R. E. Uschold, *ibid.*, **89**, 1730 (1967).

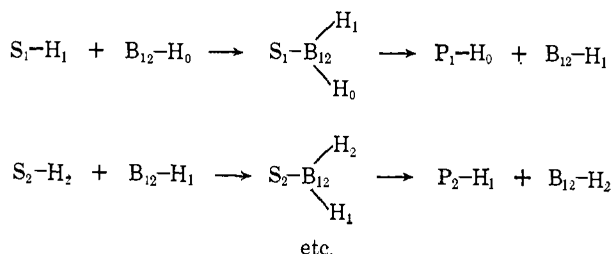
(27) P. A. Frey, G. L. Karabatsos, and R. H. Abeles, *Biochim. Biophys. Res. Commun.*, **18**, 551 (1965).

(28) C. J. Collins, *Advan. Phys. Chem.*, **2**, 1 (1964).

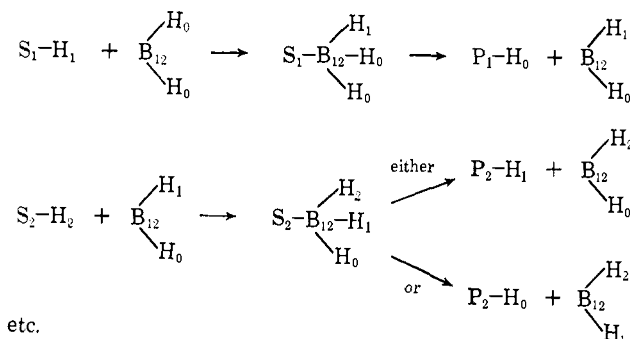
intermediate there are three equivalent hydrogens on the coenzyme).



(iii) a "one hydrogen merry-go-round" in which the hydrogen removed from the methylmalonyl coenzyme A is never readded to the same molecule of substrate; the hydrogen added to liberate product comes from the coenzyme and the hydrogen the coenzyme just acquired from substrate now becomes the hydrogen given to the next molecule of substrate to interact with the coenzyme, *i.e.*



(iv) two hydrogen merry-go-round which is analogous to iii except that in this case there are two hydrogens on the coenzyme neither of which has come from the substrate then bound to the coenzyme, but either of which can be released.



The following symbols will be used in deriving the various equations: M_0 = average concentration of methylmalonyl coenzyme A during the first 42% of reaction; M_1 = average concentration of 4-^{2}H-methylmalonyl coenzyme A; M_2 = average concentration of 4,4-di-^{2}H-methylmalonyl coenzyme A; M_3 = average concentration of 4,4,4-tri-^{2}H-methylmalonyl coenzyme A; S_0 = relative concentration of succinyl coenzyme A; S_1 = relative concentration of 3-^{2}H-succinyl coenzyme A; S_2 = relative concentration of 2,2-di-^{2}H-succinyl coenzyme A; S_3 = relative concentration of 2,2,3-tri-^{2}H-succinyl coenzyme A; B_0 = average concentration of coenzyme B_{12} without deuterium; B_1 = average concentration of monodeuterio coenzyme B_{12}; B_2 = average concentration of dideuterio coenzyme B_{12}; Δ_1 = average isotope effect (k_H/k_D) for the first 42% of reaction for transfer of hydrogen or deuterium from M_0, M_1, M_2, or M_3 to give B_0, B_1, or B_2; Δ_2 = average isotope effect (k_H/k_D) for transfer of hydrogen or deuterium from B_0, B_1, or B_2 to give S_0, S_1, S_2, or S_3.

In order to simplify the equations, we assumed in most cases the absence of any back-reaction. To a first approximation, M_1 can only be obtained from M_0 and M_2 only from M_3; therefore

$$M_0 = {}^1/_2(54.4 + 40.2) + {}^1/_2(1.6) = 48\%$$

$$M_3 = {}^1/_2(45.6 + 54.9) + {}^1/_2(3.3) = 52\%$$

Calculation of model i: Each term in equations for S_0, S_1, S_2, and S_3 was calculated as follows. S_i = (mole fraction of M_j) × (probability of hydrogen, or deuterium transfer from M_j to B_k) × (mole fraction of B_k) × (probability of hydrogen, or deuterium, transfer from B_k to give S_i). The probability of a hydrogen transfer equals the relative rate of hydrogen transfer, Δ_1 or Δ_2, times the statistical probability that a hydrogen can be transferred. The probability of a deuterium transfer equals the relative rate of deuterium transfer, one, times the statistical probability that deuterium can be transferred.

Therefore, for M_0 reacting with B_1

$$S_0 = M_0(\Delta_1)(1)B_1(\Delta_2)({}^1/_2) = {}^1/_2 M_0 B_1 \Delta_1 \Delta_2$$

S_0 can also be generated by reaction of M_0 with B_0

$$S_0 = M_0(\Delta_1)(1)B_0(\Delta_2)(1) = M_0 B_0 \Delta_1 \Delta_2$$

Consideration of the various possible permutations, leads to the following set of equations for the relative values of S_0, S_1, S_2, and S_3

$$S_0 = M_0 B_0 \Delta_1 \Delta_2 + {}^1/_2 M_0 B_1 \Delta_1 \Delta_2$$

$$S_1 = {}^1/_2 M_0 B_1 \Delta_1$$

$$S_2 = {}^1/_2 M_3 B_0 \Delta_2$$

$$S_3 = M_3 B_1 + {}^1/_2 M_3 B_0$$

In order to obtain B_0 and B_1 as functions of M_0, M_3, Δ_1, and Δ_2, the steady-state approximation can be applied

$$\frac{d(B_0)}{dt} = \frac{d(B_1)}{dt} = 0$$

$$\frac{d(B_0)}{dt} = {}^1/_2 M_0 B_1 \Delta_1 - {}^1/_2 M_3 B_0 \Delta_2 = 0$$

$$\frac{B_0}{B_1} = \frac{M_0 \Delta_1}{M_3 \Delta_2}$$

By combination of these equations and by use of the values for M_0 and M_3 discussed above, the predicted results in Table X are obtained.

Table X. Two Equivalent Hydrogen Model

	S_0	S_1	S_2	S_3
$\Delta_1 = 3.0$, $\Delta_2 = 3.0$	74.2	8.5	8.5	8.8
$\Delta_1 = 3.5$, $\Delta_2 = 3.5$	78.0	7.6	7.6	6.8
$\Delta_1 = 4.0$, $\Delta_2 = 4.0$	80.8	6.9	6.9	5.4

Table XI. Best Fits for Various Models

	Model	Δ_1	Δ_2	Calculated S_0	Calculated S_1	Calculated S_2	Calculated S_3	Error
i	Two equivalent hydrogen	2.8	2.7	71.5	9.1	9.1	10.3	0.39
ii	Three equivalent hydrogen	3.1	2.8	67.9	11.9	11.9	8.3	0.055
iib	Three equivalent[b] hydrogen with back-reaction	3.1	2.9	66.9	12.6	12.5	8.0	0.034
iii	One hydrogen merry go round	3.4	*a*	57.6	18.3	18.3	5.8	0.33
iv	Two hydrogen merry go round	3.8	*a*	69.3	8.6	8.6	13.5	0.67
ivb	Two hydrogen merry go round with back-reaction	3.4	*a*	56.7	18.8	18.7	5.8	0.37
Obsd distribution		...	...	65.8 ± 1.0	14.8 ± 0.3	11.7 ± 0.6	7.7 ± 0.4	...

[a] The calculated values for these models are independent of the value assigned to Δ_2. [b] The equations used to derive these distributions are

$$S_0 = M_0\Delta_1\Delta_2(B_0 + {}^2/_3B_1 + {}^1/_3B_2) + M_1\Delta_2({}^2/_9B_0 + {}^1/_9B_1)$$
$$S_1 = M_0\Delta_1({}^1/_3B_1 + {}^2/_3B_2) + M_1({}^2/_3B_0\Delta_1\Delta_2 + {}^1/_9B_0 + {}^4/_9B_1\Delta_1\Delta_2 + {}^2/_9B_1 + {}^2/_9B_2\Delta_1\Delta_2 + {}^1/_3B_2) + M_2\Delta_2({}^4/_9B_0 + {}^2/_9B_1)$$
$$S_2 = M_3\Delta_2({}^2/_3B_0 + {}^1/_3B_1) + M_2({}^1/_3B_0\Delta_1\Delta_2 + {}^2/_9B_0 + {}^2/_9B_1\Delta_1\Delta_2 + {}^4/_9B_1 + {}^1/_9B_2\Delta_1\Delta_2 + {}^2/_3B_2) + M_1\Delta_1({}^2/_9B_1 + {}^4/_9B_2)$$
$$S_3 = M_3({}^1/_3B_0 + {}^2/_3B_1 + B_2) + M_2\Delta_1({}^1/_9B_1 + {}^2/_9B_2)$$
$$B_0 = \Delta_1{}^2(3M_0 + 2M_1 + M_2)^2$$
$$B_1 = 2\Delta_1\Delta_2(3M_0 + 2M_1 + M_2)(M_1 + 2M_2 + 3M_3)$$
$$B_2 = \Delta_2{}^2(M_1 + 2M_2 + 3M_3)^2$$

Appropriate equations were derived in a similar way for each of the models discussed above. In addition, equations were derived for model iv (two hydrogen merry go round) and model ii (three equivalent hydrogens) that took account of the fact that back-reaction generating M_1 and M_2 does occur and that these species can also react further to give succinyl coenzyme A. The various theoretical predictions were programmed for the computer which varied, when appropriate, both Δ_1 and Δ_2 (from 1.0 to 5.0) and sought the theoretical values which gave the smallest least-square percentage error between the calculated and experimental values for S_0, S_1, S_2, and S_3 (error $= [2(S_0(\text{calcd}) - S_0(\text{exptl}))/(S_0(\text{calcd}) + S_0(\text{exptl}))]^2 +$ etc.). The minima so obtained, with the resultant errors are shown in Table XI.

The data in Table XI lead clearly to the conclusion that by far the most satisfactory model is that one in which the hydrogen abstracted from the substrate becomes one of three equivalent hydrogens before one of these three equivalent hydrogens is transferred back to give product. When, in addition, the small amount of back-reaction is taken into consideration, the agreement between the calculated and observed distribution is very good. Moreover, the isotope effects that give the best agreement between the calculated and observed distribution are within quite good agreement with $k_H/k_D = 3.5$ for the rate-determining step of hydrogen abstraction from methylmalonyl coenzyme A. In this regard, the variation is relatively small in calculated distribution near the minima given in Table XI as a function of the assigned values of Δ_1 and Δ_2. This variation is shown for model iib (three equivalent hydrogens with back-reaction) in Table XII and gives a quantitative demonstration of the fact that though the calculations clearly implicate only one model, they do not themselves within narrow limits define a single combination of isotope effects which will give acceptable agreement between the calculated and observed distributions but only set Δ_1 and Δ_2 in the vicinity of 3.

Table XII. Variation in Calculated Distribution as Function of Δ_1 and Δ_2 for Model iib (Three Equivalent Hydrogens with Back-Reaction)[a]

Δ_1	Δ_2	S_0	S_1	S_2	S_3	Error
2.8	3.5	66.2	12.7	12.7	8.4	0.038
	3.8	66.9	12.5	12.5	8.2	0.036
	4.1	67.5	12.3	12.3	7.9	0.038
3.1	2.6	66.2	12.7	12.7	8.4	0.037
	2.9	66.9	12.6	12.5	8.0	0.034
	3.2	67.6	12.4	12.3	7.7	0.035
3.5	1.8	66.6	12.5	12.4	8.5	0.043
	2.1	67.4	12.3	12.3	7.9	0.037
	2.4	68.2	12.2	12.1	7.5	0.041

[a] Variation of Δ_1 and Δ_2 each from 1 to 100 does not generate any new combinations which give acceptable agreement between the calculated and observed distribution of deuterated succinyl coenzyme A. Indeed, as the values of either Δ_1 or Δ_2 are increased, the agreement becomes increasingly worse.

Another argument, aside from the poor agreement between the calculated and observed distributions, can be raised against model iii (one hydrogen merry go round) and iv (two hydrogen merry go round). Neither of these models would give rise to methylmalonyl coenzyme A containing one or two deuterium atoms (M_1 or M_2) because the hydrogen abstracted does not become equivalent with any other hydrogens of the coenzyme until that molecule of substrate from which this hydrogen was removed has been released. Thus if deuterium is removed from M_3, this same deuterium atom would necessarily be readded if the intermediate reverted to methylmalonyl coenzyme A rather than going on to succinyl coenzyme A.

The meaning of the isotope effect for that step which readds hydrogen or deuterium to the coenzyme–substrate complex to generate succinyl coenzyme A (D_2) is open to discussion. If the succinyl coenzyme A, once formed, could not interact with the coenzyme B_{12} to reform a coenzyme–substrate complex, Δ_2 would be the isotope effect averaged over the observed extent of reaction. However, to the extent that such back-reaction occurs, Δ_2 as determined experimentally, will

approach a value which will represent the difference in energies of the coenzyme–substrate complex and free succinyl coenzyme A with hydrogen or deuterium bonded to the appropriate atoms. In this case, Δ_2 will probably approach one. As actually done, the experiment described in this paper probably falls somewhere between these two extreme cases. Some of the succinyl coenzyme A once formed probably interacts with coenzyme B_{12}, but the degree of this back-reaction is not sufficient to amount to equilibration. Thus, that value of Δ_2 which gives the best fit with the experimentally observed distribution of deuterium in succinyl coenzyme A will be lower than a true kinetic value. In the absence of this equilibration, one might expect that Δ_1 and Δ_2 would have very similar values, since, in the conversion of methylmalonyl coenzyme A or succinyl coenzyme A to coenzyme–substrate intermediate, similar carbon–hydrogen (or deuterium) bonds are being broken and made and the difference in energies between reactants and intermediates with hydrogen or deuterium is probably small.

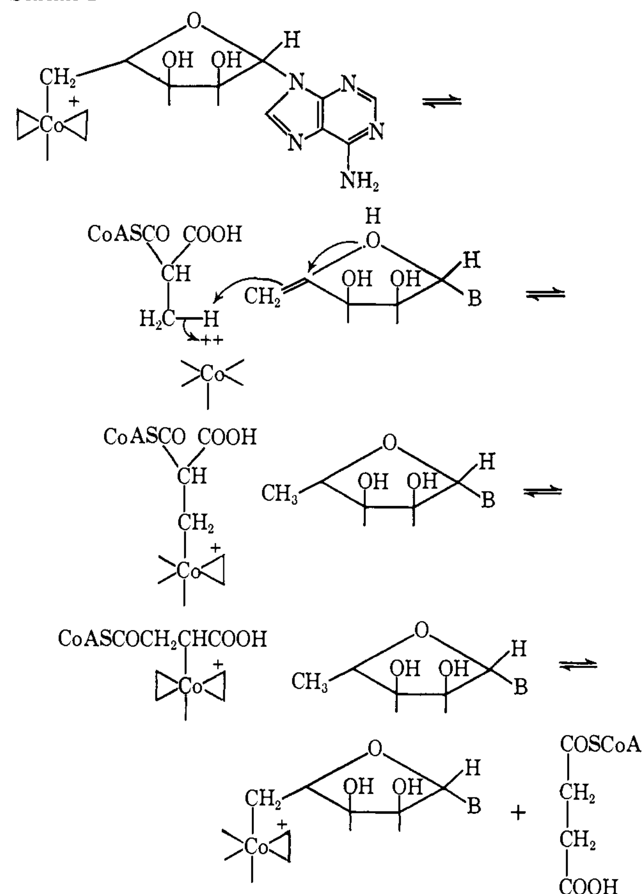
Thus this experiment confirms that the transfer of hydrogen from substrate to product during the isomerization of methylmalonyl coenzyme A to succinyl coenzyme A is intermolecular and that an intermediate stage probably involves a species with three equivalent hydrogen atoms, one from the substrate and two from the coenzyme B_{12}.

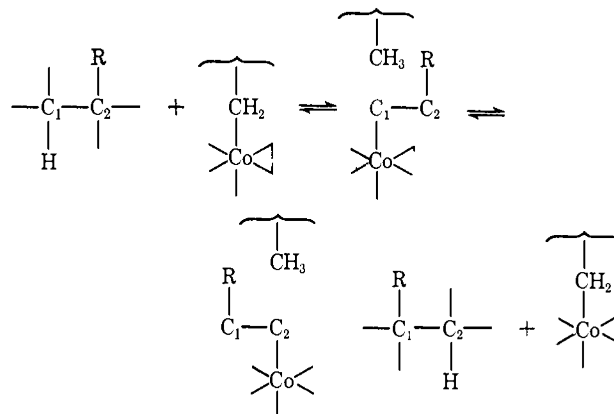
The demonstration that tritium at C-5′ of the 5′-deoxyadenosyl moiety of coenzyme B_{12} can be incorporated into propionaldehyde[21,22] and also into succinyl coenzyme A,[20] can lead, as one possibility, to the mechanism shown in Scheme I, in which the three hydrogens of the methyl group of 5′-deoxyadenosine are equivalent so that hydrogen transfer occurs with an activation energy higher than that of rotation of the methyl group (3–4 kcal).[29]

The transfers of hydrogen from methylmalonyl coenzyme A to coenzyme B_{12} and from the methyl group of 5′-deoxyadenosine to substrate occur from carbons that are inactivated in any classical sense, though the anion obtained by removal of a proton from C-4 of methylmalonyl coenzyme A may be stabilized in a manner analagous to the homoenolate anion intermediate invoked to explain the racemization of (+)-camphenilone in basic solution.[30] Also, biological attack at C–H bonds, inactivated in any classical chemical sense, has ample precedence in the many introductions of hydroxyl groups during the biosynthetic conversion of cholesterol to bile acids and steroid hormones; these generally occur with the new hydroxyl group occupying the same stereochemical site as the hydrogen that is replaced.[31] Another aspect of interest in the reaction between methylmalonyl coenzyme A and the C-5′ carbon of the deoxyadenosyl group of coenzyme B_{12} to give the intermediate shown in Scheme I, is that the net change should be nearly isoenergetic. On the other hand, there is no evidence for the intermediate formation of 5′-deoxyadenosine which this mechanism suggests.

The plausibility of the rearrangement of the methylmalonyl coenzyme A–cobalamin to succinyl coenzyme A–cobalamin requires comment. Similar processes can be invoked in other rearrangements involving coenzyme B_{12}. Thus

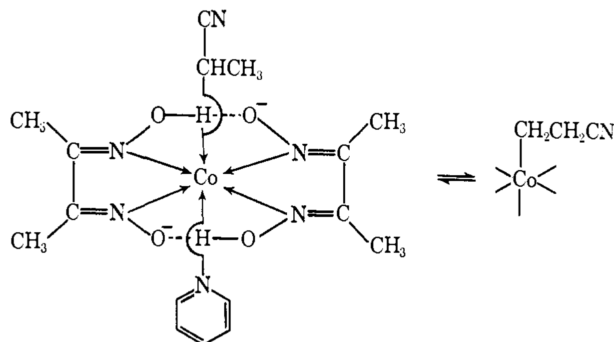
Scheme I



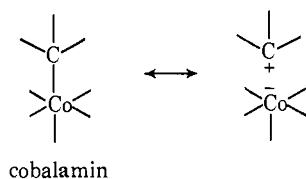


Schrauzer and Windgassen[32] have reported the interconversion of α- and β-substituted ethylcobaloximes (*e.g.*, α-cyanoethylpyridinatocobaloxime ⇌ β-cyanoethylpyridinatocobaloxime). On the other hand, in this system they were unable to observe a rearrangement which involved concomitant migration of a carboethoxyl residue analogous to the migration of the carbonyl coenzyme A residue postulated in the above sequence. Thus, degradation under a variety of

(29) L. L. Ingraham, *Ann. N. Y. Acad. Sci.*, **112**, 713 (1964).
(30) A. Nickon and J. L. Lambert, *J. Am. Chem. Soc.*, **84**, 4694 (1962).
(31) For a review of these processes, *cf.* J. H. Richards and J. B. Hendrickson, "The Biosynthesis of Steroids, Terpenes and Acetogenins," W. A. Benjamin, Inc., New York, N. Y., 1964.
(32) G. N. Schrauzer and R. J. Windgassen, *J. Am. Chem. Soc.*, **89**, 1999 (1967).



conditions of 1-carbethoxy-1-carboxyethylcobaloxime gave no decarboxylation or carboxyl group migration. Furthermore, degradation of carbomethoxyisopropylcobaloxime gave only *n*-butyric acid. One could argue that the cobalamin derivatives, because of the lower electronegativity of nitrogen relative to oxygen, will have a higher electron density around the cobalt which will facilitate conversion of d^6 (hexacoordinate cobalt + 3) to d^8 (pentacoordinate cobalt + 1). Thus the carbon–cobalt bond of cobalamin derivatives will have significantly greater contributions of ionic forms with positive carbon and negative cobalt than will cobaloxime derivatives; thus resonance of the type shown will be



cobalamin

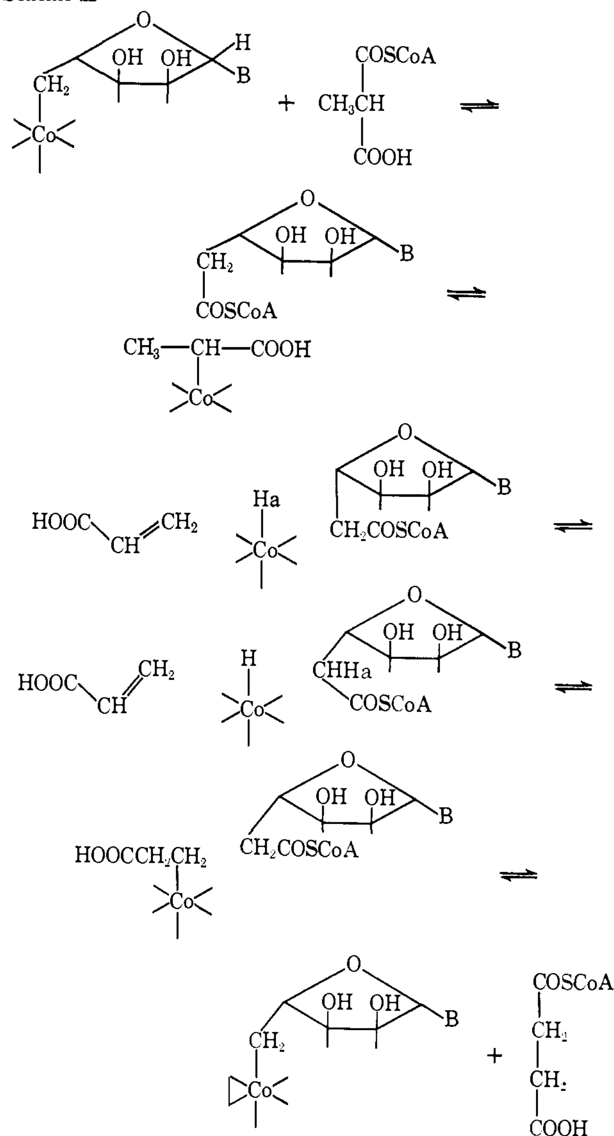
more favorable than analogous resonance in cobaloxime derivatives. Accordingly, rearrangements should be facilitated in cobalamin derivatives relative to cobaloxime derivatives; and to this extent, the chemistry of cobaloximes may be unrepresentative of some of the reactions possible in cobalamin derivatives.

Another mechanism can, however, be proposed which involves a 1,2 migration of cobalt and a 2,1 migration of hydrogen (Scheme II). In this mechanism, the COSCoA group is transferred from the substrate to C-5′ of the adenosyl residue and a carbon cobalt bond is formed with the substrate. (In the general case, the R group would be transferred to C-5′.) During the isomerization of the substrate, now bonded to the cobalt, the hydrogen which migrates has an opportunity to exchange (and thus become equilibrated) with the other two hydrogens at C-5′ of the adenosyl residue. In the methylmalonyl coenzyme A mutase reaction this exchange would be rapid relative to structural rearrangement and thus account for the results reported in this article. In other cases this may not be true and one might observe situations in which the migrating hydrogen of the substrate does not become equivalent with two other hydrogens of the enzyme–coenzyme complex.

The mechanism of Scheme II has, however, two possible drawbacks. During isomerization of methylmalonyl coenzyme A, ^{14}C-acrylic acid added to the reaction medium is not incorporated into succinate thus eliminating *free* acrylic acid as an intermediate[33]

(33) R. W. Swick, *Proc. Natl. Acad. Sci. U. S.*, **48**, 288 (1962).

Scheme II



though the intermediacy of a form of acrylic acid that is always bound to the enzyme is possible. The second defect of Scheme II is that the exchange equilibrium though possible seems a somewhat artificial explanation of the observed results. Thus, we prefer the general outlines of Scheme I as the mechanism of coenzyme B_{12} action.

The discussion has, hitherto, concentrated on the role of coenzyme B_{12} in the methylmalonyl coenzyme A isomerization and other processes for which coenzyme B_{12} is an obligatory cofactor. Surely the function of the enzyme itself is of importance and, at minimum, must account for the specificity of binding and stereochemical arrangement of the coenzyme and a particular substrate. Whether, in addition, the protein is involved in specific catalytic interactions between the substrate and coenzyme is, based on the present work, unknown. However, the evidence of this paper eliminates an exchange of the hydrogen that migrates during rearrangement of substrate with any hydrogen atoms bound to the protein itself.

Experimental Section

General. All melting points and boiling points are uncorrected. Coenzyme A was obtained from CalBiochem Co. as a lyophilized powder containing 75 to 82% reduced coenzyme A. Methylmalonyl coenzyme A mutase (specific activity 0.31 μmol/min/mg of protein) was kindly supplied by Dr. R. W. Kellermeyer, Department of Medicine, Western Research University, Cleveland, Ohio. Coenzyme B_{12} was supplied by Dr. H. A. Barker, Department of Biochemistry, University of California, Berkeley, Calif., and by Dr. Perlman, Squibb Institute for Medical Research, New Brunswick, N. J.

4,4,4,-Tri-^{2}H-methylmalonic Acid. The deuterated carboxylic acid was made from perdeuteromethyl iodide and sodium diethyl malonate by the method in Organic Syntheses.[34] The crude product had mp 125–126°. The nmr showed this acid contained less than 0.5% hydrogen in the methyl position.

The crude 4,4,4-tri-^{2}H-methylmalonic acid was mixed with an equal amount of methylmalonic acid and chromatographed by the method of Erfle and coworkers.[27] The purified methylmalonic acid mixture had mp 133.5–134.5°. The mass spectrum of the mixture showed that no deuterium scrambling had occurred during the chromatography.

Labeled Methylmalonyl Coenzyme A Mixture. A mixture of methylmalonyl coenzyme A and 4,4,4-tri-^{2}H-methylmalonyl coenzyme A was made from the above methylmalonic acid mixture by the method of Trams and Brady.[35] A total of 55 μmol of methylmalonyl coenzyme A was obtained starting with 200 μmol of coenzyme A (28% yield).

Isomerization of the above Mixture. The following mixture was incubated for 15 min at 37°: 55 μmol of the above methylmalonyl coenzyme A mixture, 0.016 μmol of coenzyme B_{12}, 4.4 mg of mutase preparation (specific activity 0.31 μmol/min/mg of protein), and 1250 μmol of trishydrochloride buffer, pH 7.3, in a total volume of 20 ml. The reaction was quenched by the addition of 3 ml of 1 *N* sodium hydroxide. After 30 min, the solution was acidified with 3.5 ml of 1 *N* HCl, and the volume was reduced to 5 ml. By continuous extraction with ether for 24 hr, the organic acids were removed. After removal of the ether the acid was dissolved in 0.5 ml of water and chromatographed on a Biorad AG-1-X2 ion-exchange column (20 × 1.0 cm) eluting with a linear formic acid gradient of 0.2 to 2.0 *N* formic acid in a total volume of 300 ml. Fractions (10 ml) were collected; succinic (1.30 mg, mp 182–184°) was eluted in fractions 6–9 and methylmalonic acid (2.66 mg, mp 132–133.5°) was eluted in fractions 20–23. The reported melting points are 185 and 131.5–132.5°, respectively.[27]

The succinic acid (1.30 mg) was converted to succinic anhydride (0.5 mg) by the micro method developed by G. Popjak and coworkers[36] before introduction into the mass spectrometer.

Mass Spectra. All mass spectra were taken with a Consolidated Electrodynamic Corp. mass spectrometer, Model #21-103C. The molar intensities of methylmalonic acid and 4,4,4-tri-^{2}H-methylmalonic acid were shown to be the same within experimental error using known mixtures of the two acids. Extrapolated spectra were calculated for 4-^{2}H-methylmalonic acid and 4,4-di-^{2}H-methylmalonic acid from the observed spectra of methylmalonic acid and 4,4,4-tri-^{2}H-methylmalonic acid.

The spectra of succinic anhydride was taken at 12.5 eV and extrapolated for the mono-, di-, and trideuteriosuccinic acids. Again, use of known samples showed that the molar intensities of deuterated and undeuterated succinic acid are the same within experimental error.

Acknowledgment. We are grateful to Dr. Kellermeyer for a generous gift of methylmalonyl coenzyme A isomerase, to Dr. M. A. Barker and Dr. Perlman for generous gifts of coenzyme B_{12}, to the USPHS (Grant GM-10218) for financial support, to the National Science Foundation and National Institutes of Health for fellowship support for William W. Miller, and to Professor H. B. Gray for interesting comments.

(34) A. H. Blatt, "Organic Syntheses," Coll. Vol. II, John Wiley & Sons, Inc., New York, N. Y., 1943, p 279.

(35) E. G. Trams and R. O. Brady, *J. Am. Chem. Soc.*, **82**, 2972 (1960).

(36) G. Popjak, D. W. S. Goodman, J. W. Cornforth, R. H. Cornforth, and R. Ryhage, *J. Biol. Chem.*, **236**, 1934 (1961).