PART I. COENZYME B_{12} AS A HYDROFORMYLATION-TYPE CATALYST

PART II. MECHANISM OF HYDROGEN TRANSFER IN THE METHYLMALONYL COENZYME A MUTASE REACTION

Thesis by

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ABSTRACT

Part I

The mechanism of the hydroformylation reaction was studied. Using cobalt deuterotetracarbonyl and 1-pentene as substrates, the first step in the reaction, addition of cobalt tetracarbonyl to an olefin, was shown to be reversible.

Part II

The role of coenzyme B_{12} in the isomerization of methylmalonyl coenzyme A to succinyl coenzyme A by methylmalonyl coenzyme A mutase was studied. The reaction was allowed to proceed to partial completion using a mixture of methylmalonyl coenzyme A and 4,4,4-tri-^{2}H-methylmalonyl coenzyme A as substrate. The deuterium distribution in the product, succinyl coenzyme A, was shown to best fit a model in which hydrogen is transferred from C-4 of methylmalonyl coenzyme A to C-5' of the adenosyl moiety of coenzyme B_{12} in the rate determining step. The three hydrogens at the 5'-adenosyl position of the coenzyme B_{12} intermediate are then able to become enzymatically equivalent before hydrogen is transferred from the coenzyme B_{12} intermediate to form succinyl coenzyme A.

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To Ramona

and

To my Parents and Parents-in-law

PART I

COENZYME B_{12} AS A HYDROFORMYLATION-TYPE CATALYST

Introduction

Vitamin B_{12} was first isolated from sheep liver in 1948 (1, 2). Ten years later, Barker and coworkers isolated a light sensitive analog of vitamin B_{12}, called coenzyme B_{12} (3, 4), which in many cases appears to be the active in vivo form of the vitamin (5).

The structure of coenzyme B_{12}, shown in Figure 1, was related to that of vitamin B_{12} by synthesis (6), and the complete spacial configuration of the coenzyme, shown in Figure 2, was elucidated by the method of X-ray crystallography (7). As in vitamin B_{12}, coenzyme B_{12} contains a pseudo-porphyrin ring system which is missing one methylene bridge, and which is more highly saturated than the normal porphyrins. These features allow the ring system to assume a non-planar configuration, as is shown in the actual three dimensional structure (8) (Figure 2a).

A second feature of coenzyme B_{12} is the existence of a stable, covalent carbon-metal bond between the central cobalt and the 5' carbon of adenosine. Evidence using coenzyme B_{12} analogues indicates that the adenosine moiety and the carbon-cobalt bond are essential for coenzyme activity. For example, when the 5'-deoxyadenosine nucleoside is replaced by a 5'-deoxyinosyl, hydroxyl, or cyano group, no coenzyme activity is observed in the methylmalonyl coenzyme A mutase reaction (see Figure 3 for structures). However, replacement of the dimethylbenzimidazolyl nucleotide by adenine, benzimidazole, or purine, or even complete removal of the nucleotide, gives a compound (see Figure 4 for structures) which is active in the methylmalonyl coenzyme A mutase reaction (9, 10, 11). Replacement of the nucleotide by 5(6)-nitrobenzimidazole, 5(6)-methylbenzimidazole, 5(6)-aminobenzimidazole, benzthiazole,

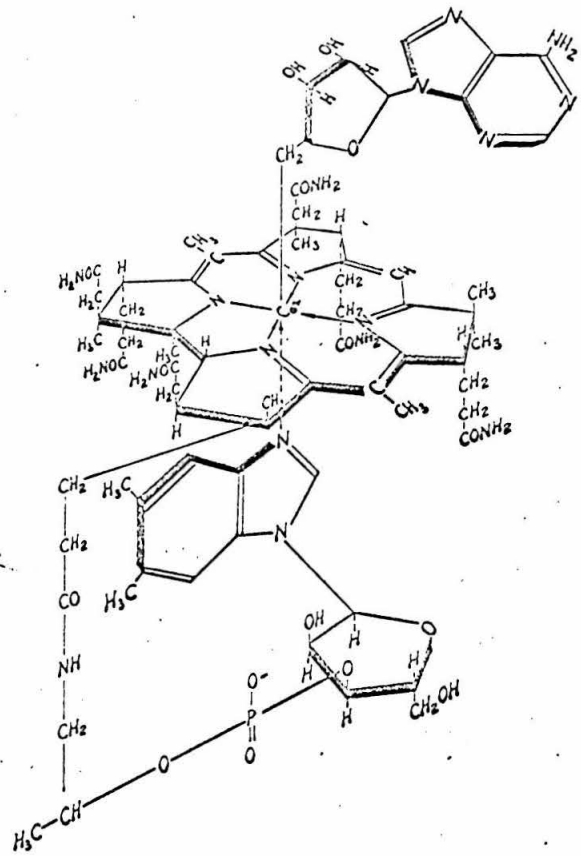
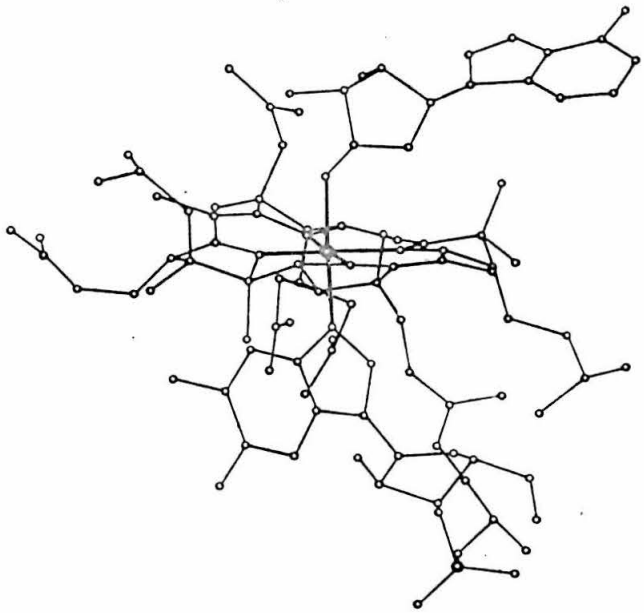
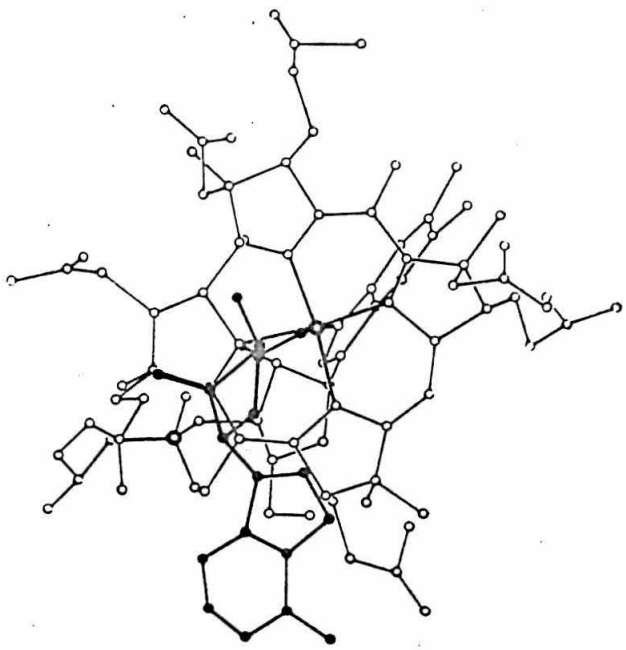


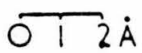
Figure 1

Structure of Coenzyme B_{12}



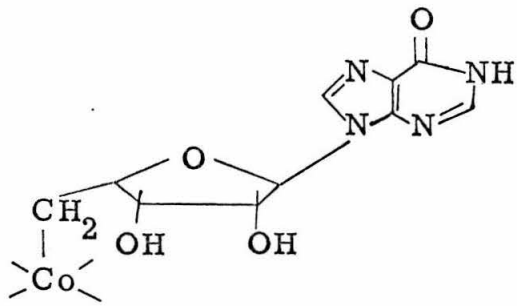
(a)



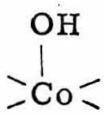


(b)

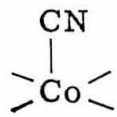
Figure 2. The atomic positions of 5, 6-dimethylbenzimidazolyl-cobamide coenzyme (coenzyme B_{12}). (a) is looking along the plane of the pseudoporphyrin ring system, and (b) is looking down the carbon-cobalt bond.



5'-inosylcobalamine
(deaminated coenzyme B_{12})



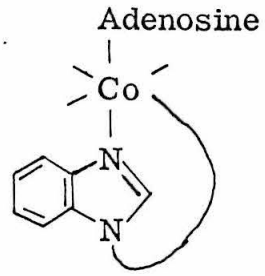
hydroxocobalamine



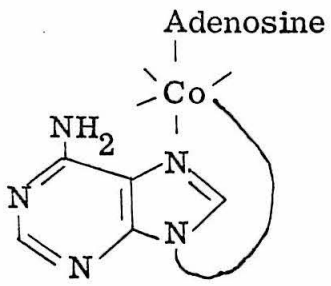
cyanocobalamine
(vitamin B_{12})

Figure 3

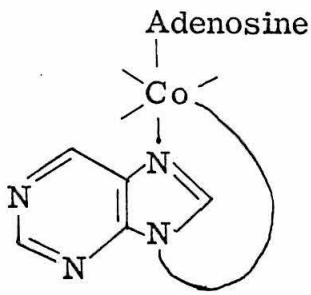
Vitamin B_{12} Analogues Inactive as Coenzymes



benzimidazolyl-
cobamide coenzyme



adenine cobamide
coenzyme



purinecobamide
coenzyme

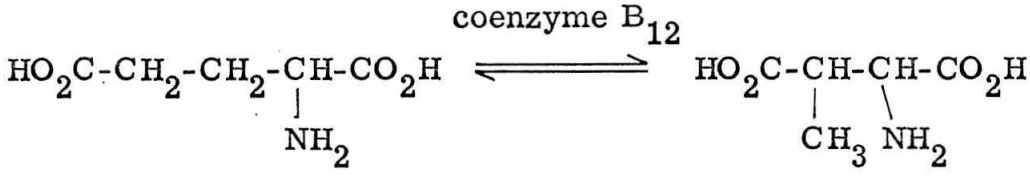
Figure 4

Vitamin B_{12} Analogues Active as Coenzymes

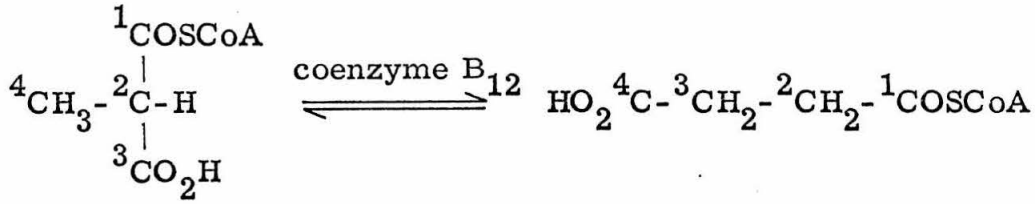
4-trifluoromethyl-1, 2-diaminobenzene, or 2, 6-diaminopurine gives a compound which is active in the glutamate mutase reaction, however, these compounds have not yet been tested for coenzyme activity with methylmalonyl coenzyme A mutase (12).

Coenzyme B_{12} and its analogues are cofactors for a number of reactions. Methylcobalamine has been implicated in the conversion of carbon dioxide to acetic acid in certain bacteria (13), in the biological synthesis of methionine from homocysteine (14), and in the formation of methane by certain anaerobic bacteria (15). 5'-Deoxyadenosylcobalamine (coenzyme B_{12}) is required for the reduction of some nucleotides to 2'-deoxynucleotides (16), and is a cofactor for at least three other enzymatic reactions (3, 17, 18):

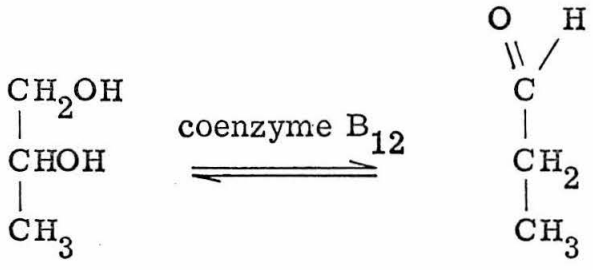
1. glutamate mutase reaction,



2. methylmalonyl coenzyme A mutase reaction (including the numbering system used throughout this thesis),



3. 1,2-propanediol dehydratase reaction.



The work in this thesis is primarily concerned with the role of coenzyme B_{12} in these last three reactions, all of which involve a hydrogen migration, and in particular with the role of coenzyme B_{12} in the isomerization of methylmalonyl coenzyme A to succinyl coenzyme A. Two of the enzymes, methylmalonyl coenzyme A mutase and 1,2-propanediol dehydratase had been obtained in pure form by 1964 (18, 19). By that time a large amount of work (20, 21, 22, 23, 24, 25, 26) and speculation (23, 27, 28, 29, 30) had been done concerning the mechanism of the methylmalonyl coenzyme A mutase reaction.

By 1964, it had been shown that the methylmalonyl coenzyme A mutase reaction proceeds by an intramolecular carbonyl transfer (20, 21), in which the thiolester group is transferred from the 2-carbon to the 4-carbon of methylmalonyl coenzyme A (22, 23, 31). Isomerization in the presence of tritiated water indicated that no substrate hydrogen exchanges with the solvent (27). More conclusively, 4, 4, 4-tri-^{2}H-methylmalonyl coenzyme A was shown to retain all three deuterium atoms during isomerization to succinyl coenzyme A (25, 26). The possibility that free acrylic acid is an intermediate in the mutase reaction was disproved when it was shown that 1-^{14}C-acrylic acid is not incorporated into the product during isomerization (24). This observation does not, however, rule out enzyme-bond acrylic acid as a possible intermediate.

Armed with this information, Whitlock suggested that the mutase mechanism might have some similarities to the mechanism of the hydroformylation reaction, and proposed the mechanism shown in Figure 5 (29). The isomerization of (d) to (f) involves the formation of a cobalt hydride and acrylic acid. The intermediate acrylic acid formed must remain bound to the enzyme, since the isomerization is known to proceed without exchange with free acrylic acid (24).

The addition of a cobalt hydride, (e), to a double bond is directly analogous to the addition of cobalt hydrocarbonyl to an olefin in the hydroformylation reaction (32). Also, the carbonyl insertion, (f) $\rightarrow$ (g), is similar to the formation of an acyl cobalt carbonyl from an alkyl cobalt carbonyl in the hydroformylation reaction (33).

The possibility that the mutase reaction and the hydroformylation reaction might have similar mechanisms gave rise to the possibility that they also might have similar deuterium and tritium isotope effects. In an effort to obtain some evidence concerning the mutase reaction an attempt was made to measure the isotope effect of the hydroformylation reaction by using 1-pentene-1-^{3}H and 1-pentene-2-^{3}H as substrates. Hopefully, these isotope effects could be measured in a straightforward manner and later compared with the isotope effects measured for the rate of the methylmalonyl coenzyme A mutase reaction using 4-^{3}H-methylmalonyl coenzyme A and 2-^{3}H-methylmalonyl coenzyme A as substrates. In actual practice, however, rapid exchange was found to occur in the hydroformylation reaction making it difficult to measure the isotope effect. A more direct approach to the methylmalonyl coenzyme A mutase mechanism problem was then taken and is discussed in Part II.

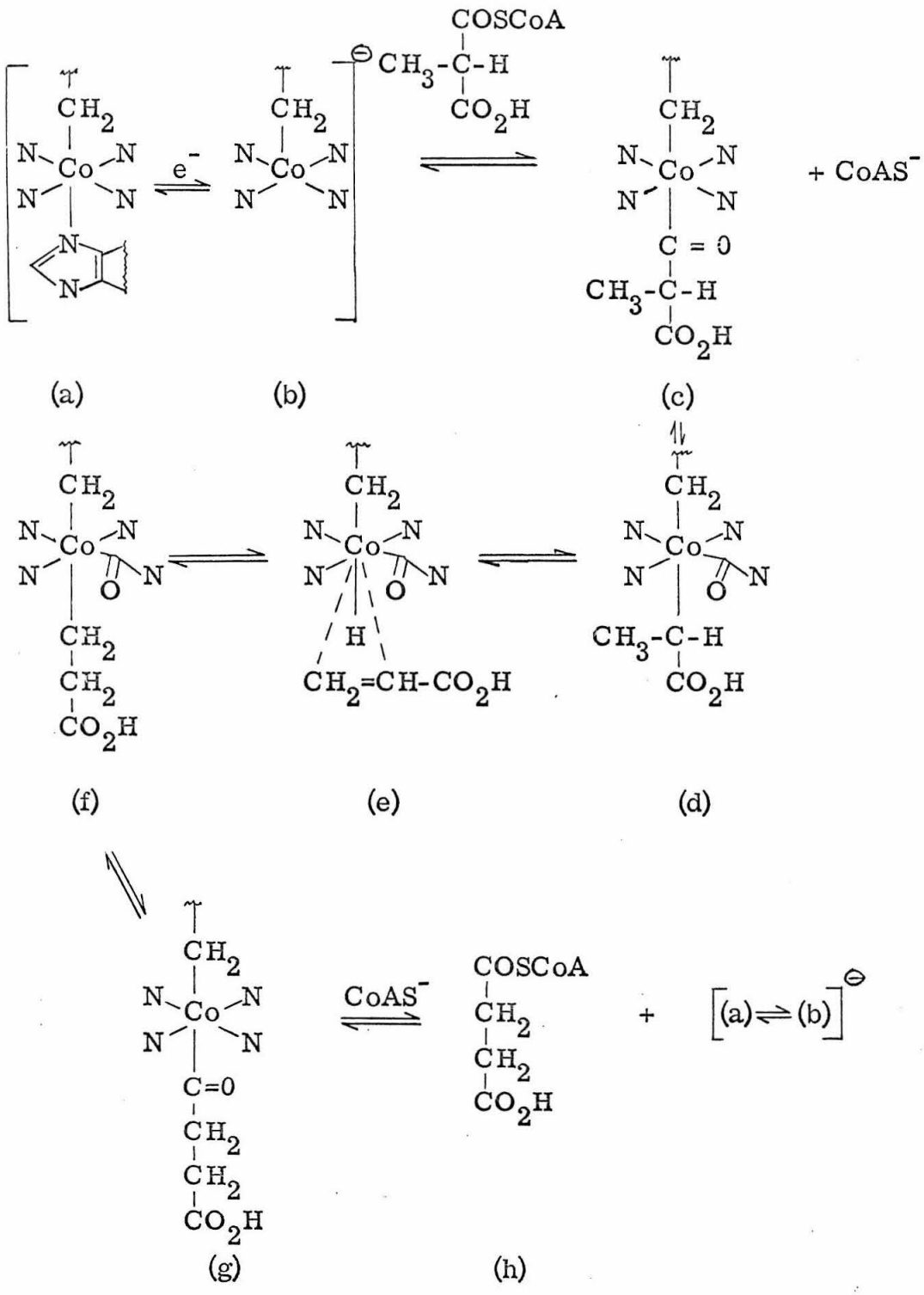


Figure 5

Whitlock's Mechanism

Results and Discussion

In the hydroformylation reaction, cobalt hydrotetracarbonyl and carbon monoxide react with an olefin to form an aldehyde according to the following overall reaction (32):

$$R\text{-}CH{=}CH_2 + 2\ HCo(CO)_4 + CO \longrightarrow RCH_2CH_2\overset{O}{\overset{\|}{C}}\text{-}H + Co_2(CO)_8 \ .$$

Although exact details of the mechanism are not known, the general reaction path has been defined (34). The first step involves the addition of cobalt hydrotetracarbonyl or cobalt hydrotricarbonyl to the olefin to form an alkyl cobalt carbonyl. The next step (or series of steps) is the insertion of carbon monoxide into the carbon-cobalt bond to form an acyl cobalt carbonyl. This acyl cobalt carbonyl is then reduced by cobalt hydrotetracarbonyl to form an aldehyde and dicobalt octacarbonyl. The entire scheme is outlined in Figure 6.

The rate of the hydroformylation reaction is inhibited by high carbon monoxide pressure (34) indicating that the mechanism is not three simple steps (Figure 6 with x = 4). Although steps (a), (b), and (c) as written in Figure 6 may not each be a single step, it is likely that one of the three, addition of cobalt hydrocarbonyl, insertion of carbon monoxide, or cleavage by cobalt hydrocarbonyl is the rate determining step.

Earlier work indicated that the addition of cobalt hydrocarbonyl to olefins may be reversible and relatively rapid. Kirch and Orchin found that the IR of the products using 1-hexene as substrate showed peaks corresponding to 2-hexene (32). This can arise by the mechanism shown in Figure 7:

(a) $R\text{-}CH{=}CH_2 + HCo(CO)_x \rightleftharpoons R\text{-}CH_2\text{-}CH_2\text{-}Co(CO)_x \quad x = 3 \text{ or } 4$

(b) $R\text{-}CH_2\text{-}CH_2\text{-}Co(CO)_x + CO \rightleftharpoons R\text{-}CH_2\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}Co(CO)_x$

(c) $R\text{-}CH_2\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}Co(CO)_x + HCo(CO)_x \rightleftharpoons R\text{-}CH_2\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}H + Co_2(CO)_8$

Figure 6

Mechanism of the Hydroformylation Reaction

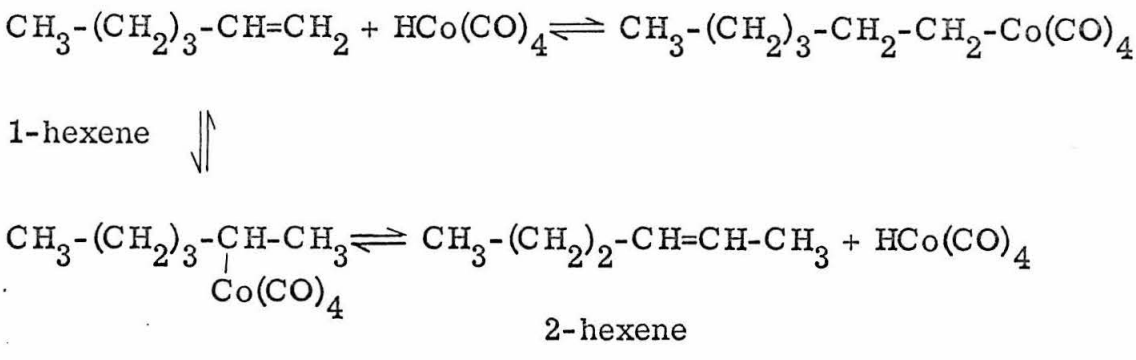


Figure 7

Conditions where these equilibria are rapid would shuffle the tritium on 1-pentene-1-^{3}H and 1-pentene-2-^{3}H, in which case an isotope effect for the rate of the hydroformylation reaction could not be measured.

In order to determine the extent of hydrogen exchange between 1-pentene and cobalt hydrotetracarbonyl, the reaction was run using cobalt deuterotetracarbonyl and excess 1-pentene as substrates. If no exchange occurred, the product aldehydes, caproaldehyde and 2-methylvaleraldehyde would be completely deuterated in the 1-position. If, however, some equilibrium of the hydrogens occurred, some cobalt hydrotetracarbonyl would be formed which could cleave the acyl cobalt carbonyl to form 1-hydro-aldehydes.

The reaction was run at 20° using cobalt deuterotetracarbonyl and excess 1-pentene. The semicarbazones of the product aldehydes were made and chromatographed. Although chromatography on silica gel did not separate the two semicarbazones, it did purify them.

Subsequently it was found that analysis of the total 1-deutero-content by NMR could be performed on the mixture. The hydrogen NMR ratios obtained for authentic semicarbazone and 1-deutero semicarbazone samples and for the products of the hydroformylation reaction are presented in Table 1.

The NMR results show that there is 40-50% hydrogen at the 1-position of the products of the hydroformylation reaction. The exchange could not have occurred after formation of the aldehydes since forming and isolating the semicarbazone derivative of 1-deuterocaproaldehyde from a mixture of 1-deuterocaproaldehyde and dicobalt octacarbonyl in pentene gave no deuterium exchange. It is most plausible that the exchange arises from the reversibility of the first step in the reaction. These results show that a tritium isotope effect cannot be measured under these conditions.

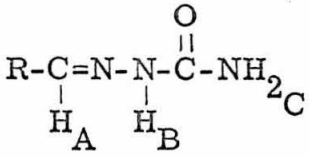
Rather than work further with the hydroformylation reaction in the hopes of finding a system in which the reverse reaction is minimized, it was decided to approach the methylmalonyl coenzyme A mutase mechanism directly by studying the enzyme system itself. The results of this study are presented in Part II.

Recent work on the hydroformylation reaction appears to contradict our results. L. Roos (35) has shown that allylbenzene, in the presence of cobalt deuterotetracarbonyl under a nitrogen atmosphere, rapidly isomerizes to propenylbenzene. Mass spectral analysis showed that a total of 5.3% deuterium was incorporated into the product. He was unable to draw any mechanistic conclusions from his results.

Although the details are not given for the above experiment, a rapid equilibrium, as we have suggested, between the olefin and cobalt deuterotetracarbonyl, should lead to substantial amounts of

Table 1

NMR Results for Semicarbazone Derivatives



	Source		Relative area (H_B = 1.00)		
			H_A	H_B	H_C
1.	Authentic sample	theor.	1.00	1.00	2.00
	R = CH_3-$(CH_2)_4$-	obs.	1.07 ± .01	1.00 ± .01	2.22 ± .01
2.	Authentic sample	theor.	1.00	1.00	2.00
	R = CH_3-$(CH_2)_2$-CH(CH_3)-	obs.	1.05 ± .01	1.00 ± .01	1.92 ± .01
3.	Authentic sample of	theor.	0	1.00	2.00
	1-deutero-capro-	obs.	0	1.0	2.4
	aldehyde				
4.	Hydroformylation	theor.	0	1.00	2.00
	product from	obs.	0.46 ± .03	1.00 ± .02	2.23 ± .06
	$DCo(CO)_4$				

deuterium in the propenylbenzene. Our experiment did not directly show that exchange occurred between the 1-pentene and cobalt deuterotetracarbonyl. However, it strongly implied exchange by virtue of the loss of deuterium from the cobalt deuterotetracarbonyl. Since the reaction was run in an aprotic solvent, it appears that the loss of deuterium can be accounted for only through a hydrogen exchange between pentene and cobalt deuterotetracarbonyl to give pentene containing carbon bound deuterium and cobalt hydrotetracarbonyl which gives rise, in turn, to caproaldehyde with hydrogen attached to the aldehydic carbon. It would be worthwhile to explore this mechanism more directly by analyzing the aldehyde product for deuterium in positions other than the 1-position. Our experiments would imply 40-50% deuterium in the 2- and 3-positions of the product aldehydes, while the experiment of L. Roos would suggest a much smaller percentage incorporation. The results of such an experiment would determine whether the discrepancy is due to a difference in the olefin used or to a difference in the experimental procedure or analysis of data.

Experimental Section

A. Reagents

1. Thionyl chloride was obtained from Matheson, Coleman and Bell, and was used without further purification.

2. Caproic acid was made by hydrolysis of methyl caproate (b. p. 144. 8-145. 2°) and distillation of the acid (b. p. 204-205°).

3. N, N-Dimethylamine was obtained from Matheson, Coleman and Bell as a 25% aqueous solution.

4. Diethyl ether was refluxed for four hours over phosphorus pentoxide and distilled (b. p. 34. 2-34. 5°). The dry ether was stored over sodium wire.

5. Ethyl acetate was refluxed for two hours over phosphorus pentoxide and distilled. The center cut was taken (b. p. 76. 8°).

6. Lithium aluminum deuteride was obtained from Metal Hydrides, Inc.

7. Dicobalt octacarbonyl was obtained from Alfa Inorganics, Inc. as a 14. 7% solution in toluene. The toluene was removed and the compound dissolved in purified n-pentane before use.

8. n-Pentane was washed with 98% sulfuric acid, dried over phosphorus pentoxide and distilled (b. p. 36. 0-36. 1°). The solvent was stored over sodium wire.

9. N, N-Dimethylformamide was distilled from barium oxide (b. p. 153-154°).

10. Deuterium chloride (38% in deuterium oxide was obtained from Volk Radiochemical Co.

B. Syntheses

1. N, N-Dimethylcaproamide (36)

A mixture of thionyl chloride (40 ml., 0.55 mol.) and caproic acid (40 ml., 0.35 mol.) was refluxed on a steam bath for fifteen minutes. The product was distilled at reduced pressure using a water aspirator. Thirty milliliters of caproyl chloride was collected with b. p. 45-55°/20-25 mm. Hg.

The caproyl chloride was cooled to -80° in an acetone-dry ice bath and added dropwise to 60 ml. dimethylamine in 50 ml. anhydrous diethyl ether at -80°. Upon completion of the highly exothermic reaction, the mixture was filtered and washed with ether. The combined ether fractions were extracted with water and dried over anhydrous magnesium sulfate. After removal of ether, N, N-dimethylcaproamide was distilled under reduced pressure, giving 20 g. of product with b. p. 98.0-99.0°/2-3 mm.

The infrared spectrum (Perkin-Elmer IR 137) of the product showed a trace of acid impurity.

2. 1-Deuterocaproaldehyde (36)

In a 500 ml. round-bottom flask equipped with condenser, dropping funnel, mechanical stirrer and nitrogen inlet was placed lithium aluminum deuteride (0.854 g., 0.0203 mol.), partially dissolved in 50 ml. anhydrous ether. The solution was cooled to 0°, and ethyl acetate (1.787 g., 0.0203 mol.) in 25 ml. anhydrous ether was added dropwise while stirring. Upon completion of the addition, the milky white suspension was cooled to -80°, and N, N-dimethylcaproamide (4.71 g., 0.0326 mol.) in 25 ml. anhydrous

ether was added dropwise while stirring. After standing for two hours at -80°, the mixture was warmed to 0°, and excess ethyl acetate was added to destroy any remaining lithium aluminum deuteride. After addition of 4N sulfuric acid (50 ml.), the ether layer was separated and washed with water and aqueous sodium bicarbonate. After drying over magnesium sulfate and removal of the solvent, 3.1 g. of crude 1-deuterocaproaldehyde remained. Gas-liquid chromatography of the product showed that it contained a minimum of 72% aldehyde; the primary impurities were ethanol and ether. This product was used without further purification to make the semicarbazone derivative.

The IR spectrum (Perkin-Elmer IR 137) of the product showed a peak due to carbon-deuterium stretch at 2070 cm^{-1}, complete absence of the carbon-hydrogen stretch at 2720 cm^{-1}, and a shift of the carbonyl stretching frequency from 1740 cm^{-1} to 1720 cm^{-1}.

3. Hydroformylation of 1-pentene with cobalt deuterotetracarbonyl (2)

The apparatus consisted of a 100 ml. 3-necked round bottom flask equipped with a magnetic stirrer, serum stoppered sidearm, nitrogen inlet and gas outlet leading to a 2 liter gas collector. The flask was charged with dicobalt octacarbonyl (3.5 g., 0.01 mol.), n-pentane (50 ml.) and dimethylformamide (6 ml., 0.084 mol.). Disproportionation took place over a four hour period forming $Co(DMF)_6[Co(Co)_4]_2$. The complex was then acidified at 0° with 38% deuterium chloride (10 g., 0.01 mol., 99% deuterium). After stirring two hours, the aqueous layer was withdrawn and the light yellow pentane layer was washed with two 10 ml. portions of

deuterium oxide. To this cobalt deuterotetracarbonyl solution was added 15 ml. of 1-pentene and the mixture was stirred for four hours at room temperature. After cooling to -80° overnight to precipitate the dicobalt octacarbonyl, the pentane solution was withdrawn with a syringe. This solution contained pentane, 1-pentene, deuterated caproaldehyde and 1-methylvaleraldehyde, and a small amount of dicobalt octacarbonyl.

C. Analyses

1. Analysis of hydroformylation products

The pentane solution from the above reaction was evaporated in a stream of nitrogen to give a viscous red liquid which was dissolved in 10 ml. of ethanol. To this solution was added 1 g. semicarbazide hydrochloride, 1.25 g. sodium acetate and 10 ml. water. The mixture was boiled for five minutes to convert any dicobalt octacarbonyl into cobalt salts, then evaporated and extracted with two 10 ml. portions of chloroform. The chloroform portions were successively washed with water, aqueous sodium bicarbonate and water. The dried chloroform solution was evaporated to 2 ml. and chromatographed on silica gel (2 x 12 cm. column). The column was eluted with chloroform (previously passed through alumina to remove any alcohol) to give a mixture of caproaldehyde semicarbazone and 2-methylvaleraldehyde semicarbazone (200 mg. total) in fractions 10 - 30 (50 ml. fractions). The NMR of the mixture was taken in a micro-NMR tube using a Varian A60 instrument (see Figure 8). The results are tabulated in Table 1.

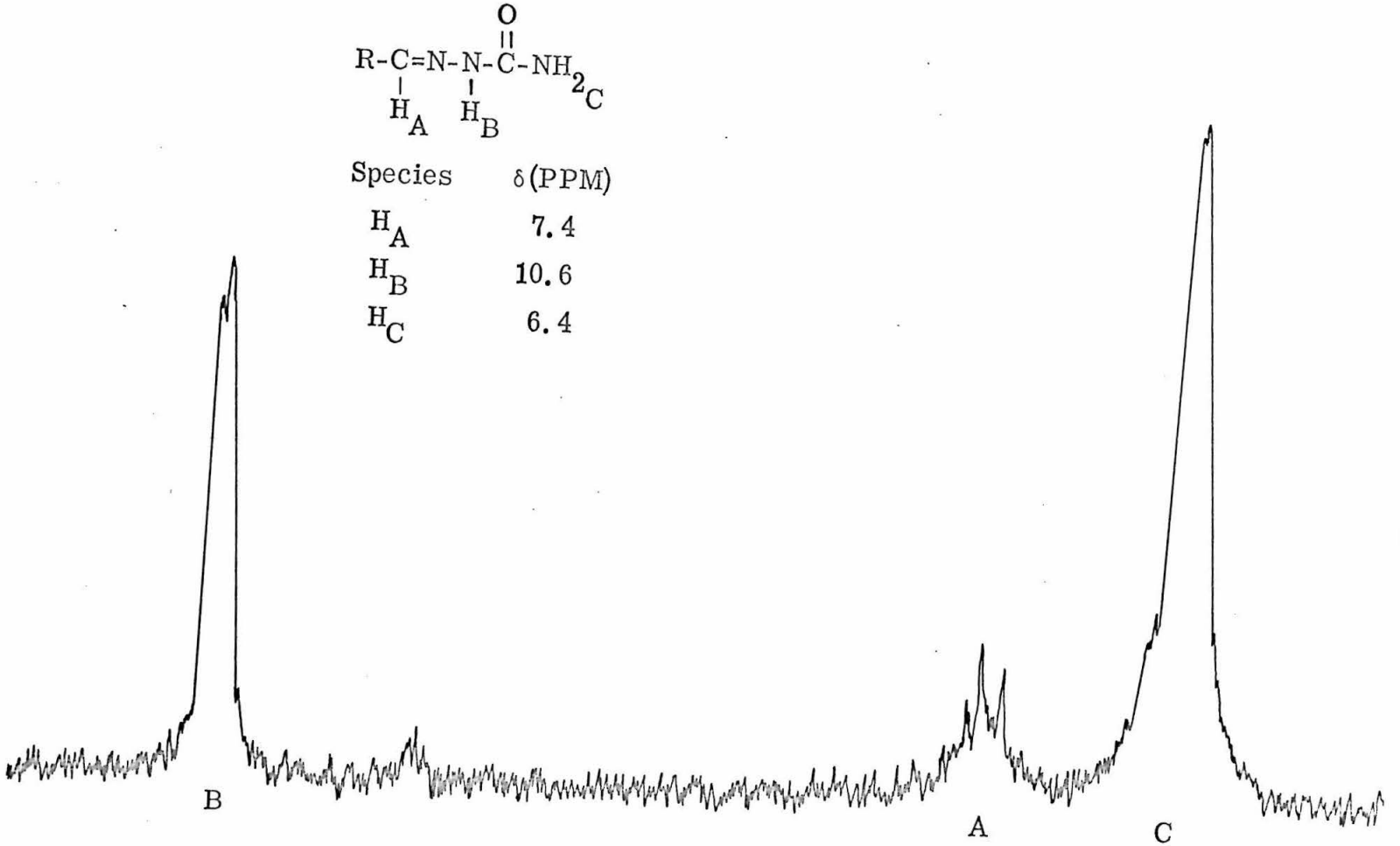
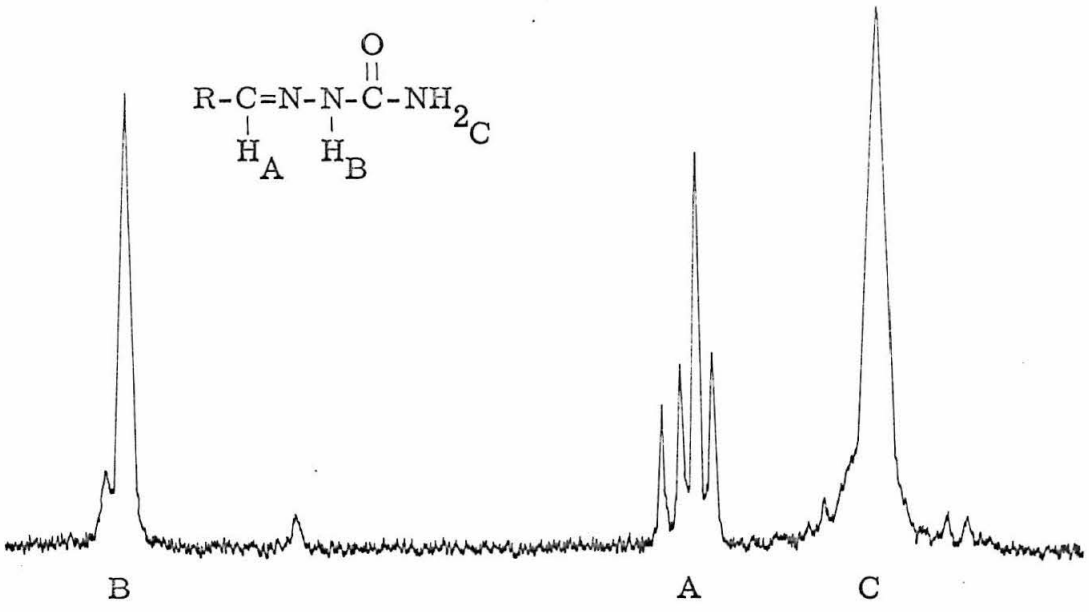


Figure 8

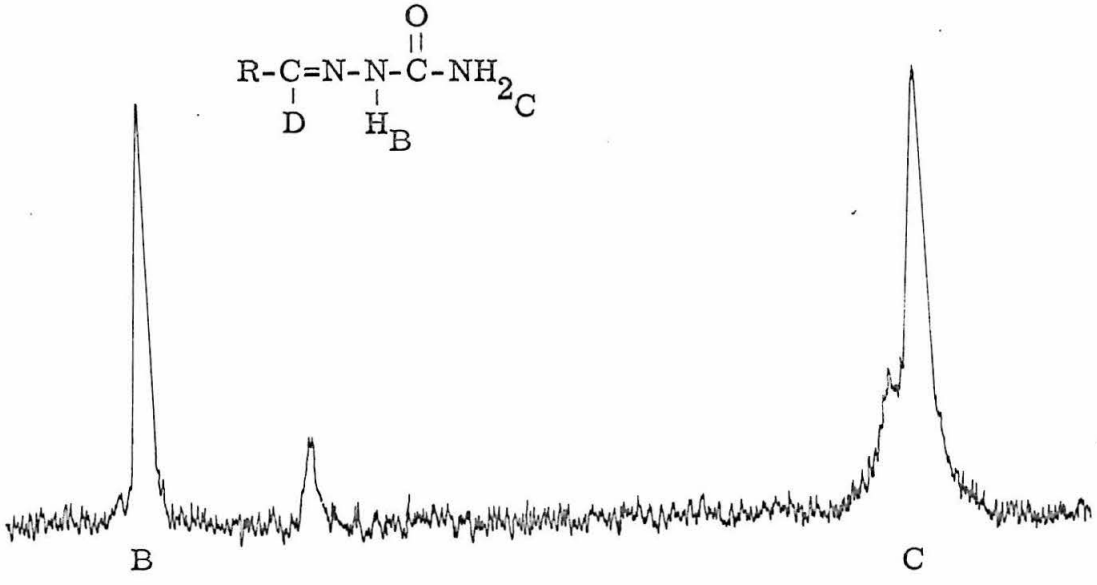
NMR Spectrum of Oxo Product Semicarbazones

2. Analysis of authentic 1-deuterocaproaldehyde

To a solution of 300 mg. of 1-deuterocaproaldehyde (crude product from synthesis 2) in 10 ml. pentane was added a small amount of dicobalt octacarbonyl. The procedure for the formation of the semicarbazones of the hydroformylation products was followed identically with this sample to give 250 mg. of 1-deuterocaproaldehyde semicarbazone. The NMR of this compound showed no hydrogen at the 1-position as is shown in Figure 9 and tabulated in Table 1.



a. NMR Spectrum of Caproaldehyde Semicarbazone



b. NMR Spectrum of 1-^{2}H-Caproaldehyde Semicarbazone

Figure 9

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PART II

MECHANISM OF HYDROGEN TRANSFER IN THE METHYLMALONYL COENZYME A MUTASE REACTION

Introduction

Since 1964 additional information has been obtained concerning the role of coenzyme B_{12} in enzymatic reactions. It was already known that coenzyme B_{12} was required for the enzymatic conversion of 1, 2-propanediol to propionaldehyde, and methylmalonyl coenzyme A to succinyl coenzyme A. The conversions were known to proceed without incorporation of hydrogen from solvent and without loss of deuterium from the substrates (1, 2).

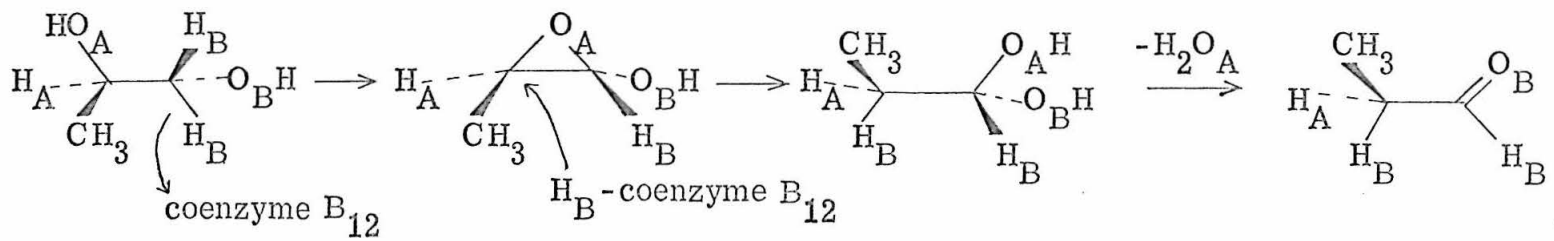
Concurrent with our work it was shown that coenzyme B_{12} acts as a hydrogen transfer agent in both reactions. In 1966, Abeles and Frey (3, 4) incubated 1, 2-propanediol-1-^{3}H with coenzyme B_{12} and diol dehydratase and isolated the coenzyme B_{12} at the end of the reaction. The quantity of tritium incorporated into the coenzyme B_{12} indicated that two moles of tritium were incorporated per mole of coenzyme B_{12}. This result can be explained on the basis of the model proposed earlier by Ingraham (5) in which the 5' carbon of the adenosyl moiety accepts a hydrogen from the substrate and later gives it up to the product. The diol dehydratase result indicates that both of the hydrogens at the 5' position of the coenzyme can be transferred to substrate during the course of the reaction.

In order to check the location of the incorporated tritium, Abeles and Frey synthesized coenzyme B_{12} containing tritium at the 5' position of the adenosyl moiety. Incubation of this synthetic coenzyme B_{12} with 1, 2-propanediol and diol dehydratase gave propionaldehyde containing all of the tritium originally in the synthetic coenzyme B_{12}. They concluded that during the course of the reaction, the carbon-cobalt bond in coenzyme B_{12} must be

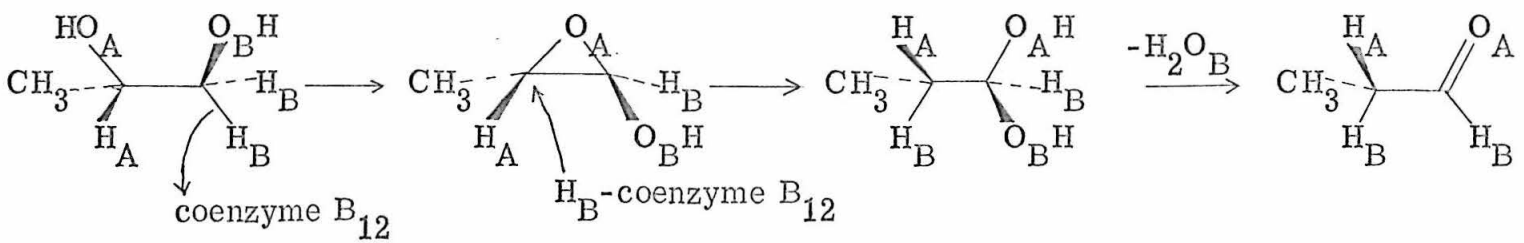
so modified that the two hydrogens attached to the carbon can become equivalent. Under the conditions of their reaction, equivalence can occur in at least two ways. There could be a transient dissociation of the carbon-cobalt bond leading to equivalence by rotation around the single bond between the 4' and 5' carbons of the adenosyl group. Or, since they used racemic 1, 2-propanediol, one of the hydrogens of the adenosyl moiety could participate when L-1, 2-propanediol is the substrate and the other hydrogen could participate when D-1, 2-propanediol is used.

The work of Arigoni and coworkers (6, 7) has shown that the two hydrogens at C-1 of 1, 2-propanediol are not enzymatically equivalent. Using optically active, labelled substrates, they showed that one of the two C-1 hydrogens is stereospecifically removed (presumably by coenzyme B_{12}). The hydroxyl group at C-2 is then transferred to C-1 while hydrogen is transferred (from coenzyme B_{12}) to the C-2 position with net inversion of configuration at C-2. The intermediate, 1, 1-propanediol, now stereospecifically looses one hydroxyl group as water to form the product, propionaldehyde (see Figure 10).

Recent work done on the role of coenzyme B_{12} in the methylmalonyl coenzyme A mutase reaction appears to be less conclusive. In late 1966, Retey and Arigoni (8) made 5'-^{3}H-deoxyadenosyl coenzyme B_{12} enzymatically from 1, 2-propanediol-1-^{3}H by the method of Abeles and Frey (3, 4). After isolation of the coenzyme B_{12} from the mixture, the labelled coenzyme B_{12} was incubated with methylmalonyl coenzyme A mutase and methylmalonyl coenzyme A or succinyl coenzyme A. The reaction mixture was allowed to proceed to equilibrium, and the succinic acid product was recovered and analyzed for tritium content. When the reaction was run



(a) Conversion of (+) - (S)-Propanediol to Propionaldehyde



(b) Conversion of (-) - (R)-Propanediol to Propionaldehyde

Figure 10

Enzymatic Conversion of 1, 2-Propanediol to Propionaldehyde

starting with methylmalonyl coenzyme A, 16% of the tritium was recovered, and starting with succinyl coenzyme A, 32% was recovered.

The results of these experiments leave the exact nature of this hydrogen transfer from coenzyme B_{12} in doubt. If the hydrogens at the 5' position of the adenosyl moiety become equivalent during the reaction, 100% incorporation of tritium would be expected, unless there was an excess of free coenzyme B_{12} which did not fully equilibrate with bound coenzyme B_{12} during the reaction. On the other hand, if hydrogen transfer is stereospecific, no incorporation would be expected.

Although our experiments were done without the knowledge of the work of Retey and Arigoni, they were designed to distinguish between various modes of hydrogen transfer between the substrate and coenzyme B_{12} in the methylmalonyl coenzyme A mutase reaction. The results of our experiments are in agreement with a model in which hydrogen is transferred from the methyl group of methylmalonyl coenzyme A to the 5' methylene carbon of the 5' adenosyl moiety in coenzyme B_{12}, to form a methyl group. The three hydrogens at the 5' position become enzymatically equivalent when one of the three hydrogens is returned to the substrate with concomitant reformation of a carbon-cobalt bond.

Results

A. Isomerization of a Mixture of Methylmalonyl Coenzyme A and 4, 4, 4-Tri-^{2}H-methylmalonyl Coenzyme A

A mixture of methylmalonyl coenzyme A and 4, 4, 4-tri-^{2}H-methylmalonyl coenzyme A was isomerized to succinyl coenzyme A. The reaction was stopped by hydrolyzing the thiolesters after about 30% conversion, and methylmalonic and succinic acids were recovered by extracting with ether and purified by ion exchange chromatography. The distribution of deuterium in the starting methylmalonic acid and in the methylmalonic acid recovered from the reaction mixture was determined by mass spectroscopy. The recovered succinic acid was converted to succinic anhydride and introduced into the mass spectrometer to determine the deuterium distribution in the isomerization product.

B. Mass Spectra of the Methylmalonic Acids

Tables 2, 3, and 4 show the observed relative intensities of methylmalonic acid and 4, 4, 4-tri-^{2}H-methylmalonic acid (> 99.5% isotopically pure) at three ionizing voltages, 12. 5, 20 and 70 ev. Table 5 shows the relative intensities of the methylmalonic acid mixture before the isomerization reaction in the range m/e 74 through 77. Table 6 shows the relative intensities at m/e 74 through 77 of the methylmalonic acid mixture recovered after the isomerization. These values were used to calculate the relative amounts of the four methylmalonic acid species (methylmalonic, 4-^{2}H-methylmalonic, 4, 4-di-^{2}H-methylmalonic and 4, 4, 4-tri-^{2}H-methylmalonic acid) present before and after the enzymatic

Table 2

Mass Spectra

Ionizing Voltage = 12.5 ev., Ionizing Current = 60 μamps

	Methylmalonic Acid	
m/e	4,4,4-tri-^{1}H-	4,4,4-tri-^{2}H-
73	37.1	0
74	100.0	0
75	4.2	10.6
76	0	19.6
77	0	100.0
78	0	4.0

Table 3

Mass Spectra

Ionizing Voltage = 20.0 ev., Ionizing Current = 60 μamps

	Methylmalonic Acid	
m/e	4,4,4-tri-^{1}H	4,4,4-tri-^{2}H-
73	57.1	0
74	100.0	0
75	4.6	17.5
76	0	28.9
77	0	100.0
78	0	4.4

Table 4

Mass Spectra

Ionizing Voltage = 70 ev., Ionizing Current = 100 μamps

	Methylmalonic Acid	
m/e	4,4,4-tri-^{1}H	4,4,4-tri-^{2}H
73	60.2	0
74	100.0	0
75	4.6	19.7
76	0	30.8
77	0	100.0
78	0	4.2

Table 5

Mass Spectra of Starting Methylmalonic Acid

	Ionizing Voltage		
m/e	12.5 ev	20 ev	70 ev
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 6

Mass Spectra of Methylmalonic Acid Recovered from the Reaction Mixture

	Ionizing Voltage		
m/e	12.5 ev	20 ev	70 ev
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

isomerization. The method used for the calculations, and the values obtained are presented in the Calculations.

C. Mass Spectra of the Succinic Anhydrides

For the mass spectral analysis of succinic anhydride two areas of the spectrum were used. The area at m/e 28 through 32 corresponds to the ethylene ion, $(CH_2\text{-}CH_2)^+$, and its deuterated analogs. Similarly, the area at m/e 56 through 59 corresponds to the ion $(CH_2CH_2CO)^+$ and its deuterated analogs.

At an ionizing voltage of 12.5 ev. almost no hydrogen was abstracted from the principle ions, so all succinic anhydride spectra were taken at this voltage. Table 7 shows the observed spectra for succinic anhydride and 2, 2, 3, 3-tetra-^{2}H-succinic anhydride (about 90% isotopically pure). The 2, 2, 3, 3-tetra-^{2}H-succinic anhydride was made by hydrolysis and decarboxylation in deuterium oxide of tetraethyl ethane-1, 1, 2, 2-tetracarboxylate (9).

Four spectra were taken of the succinic anhydride made from the enzymatically formed succinic acid. The relative intensities of the peaks at m/e 28 through 31 and m/e 56 through 59 are shown in Table 8. In each of the two areas of interest the largest peak has been given the relative intensity of 100.0. From the relative intensities in Table 8, eight values of the relative amounts of each of the four succinic anhydride species (succinic anhydride, 2-^{2}H-succinic anhydride, 2, 2-di-^{2}H-succinic anhydride, and 2, 2, 3-tri-^{2}H-succinic anhydride) were calculated. The method used to calculate these values, and the ratios obtained are presented in the Calculations.

Table 7

Mass Spectra of Succinic Anhydride and 2, 2, 3, 3-Tetra-^{2}H-succinic Anhydride[1]

m/e	Succinic anhydride	2, 2, 3, 3-Tetra-^{2}H succinic anhydride
27	0	0
28	100.0	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

[1]Ionizing Voltage = 12.5 ev., Ionizing Current = 60 μamps.

Table 8

Mass Spectra of Succinic Anhydride Obtained from Enzymatically Formed Succinic Acid[1]

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

[1]Ionizing Voltage = 12.5 ev., Ionizing Current = 60 μamps

D. Attempted Enzymatic Isomerization of N-Acetyl-S-Methylmalonylcysteamine

An attempt was made to enzymatically isomerize N-acetyl-S-methylmalonylcysteamine to N-acetyl-S-succinylcysteamine. The isomerization was attempted at two enzyme concentrations. After incubating for 20 minutes each sample was heated to 100^{o} for two minutes to destroy any N-acetyl-S-succinylcysteamine formed. The amount of residual N-acetyl-S-methylmalonylcysteamine was measured by converting it to methylmalonylmonohydroxamic acid (10).

The concentration of methylmalonyl monohydroxamate was measured with a calibrated Klett colorimeter. The errors due to pipetting and reading the absorbance at 500 mμ. amount to about ±2%.

The results in Table 9 show that no isomerization of N-acetyl-S-methylmalonylcysteamine occurred up to an enzyme concentration of 0.100 units. The last column in Table 9 shows that two μmoles of methylmalonyl coenzyme A would be converted to succinyl coenzyme A under the same conditions. This means that N-acetyl-S-methylmalonylcysteamine is isomerized at a rate which is slower than the rate of isomerization of methylmalonyl coenzyme A by at least a factor of 2.00/0.02 = 100.

E. Inhibition of Methylmalonyl Coenzyme A Mutase by N-Acetyl-S-Methylmalonylcysteamine

Although N-acetyl-S-methylmalonylcysteamine does not appear to be a substrate for methylmalonyl coenzyme A mutase, the thiolester might be an inhibitor of the mutase enzyme. To determine the amount of mutase inhibition caused by N-acetyl-S-methylmalonylcysteamine, a constant amount of enzyme and 4-^{3}H-

Table 9

Attempted Isomerization of N-Acetyl-S-Methylmalonylcysteamine

Enzyme[1] (units)	Thiolester[2] (μmoles)			Methylmalonyl coenzyme A[3] (μmoles)
	before reaction	after reaction	μmoles isomerized	
0	1.06 ± .02	1.06 ± .02	0	0
0.034	1.06 ± .02	1.04 ± .02	.02 ± .02	0.70 ± .01
0.100	1.06 ± .02	1.05 ± .02	.01 ± .02	2.00 ± .04

[1] 1 unit = 1 μmole methylmalonyl coenzyme A isomerized/min./mg. protein.

[2] N-acetyl-S-methylmalonylcysteamine.

[3] Amount theoretically isomerized under identical conditions of methylmalonyl coenzyme A were present in excess.

methylmalonyl coenzyme A was incubated with varying amounts of N-acetyl-S-methylmalonylcysteamine. After quenching and adding 10.0 mg. of succinic acid, the product was chromatographed to obtain pure succinic acid. Weighed amounts of the succinic acid were analyzed for tritium content which is directly proportional to the percent isomerization.

The results of the inhibition study are presented in Table 10. The error due to pipetting and tritium counting amounts to about 5%. No inhibition is observed at 2 molar excess of inhibitor within experimental error. However, at 10 molar excess, 43% inhibition is observed which is well outside of the experimental error.

Table 10

Inhibition of Methylmalonyl Coenzyme A Mutase by N-Acetyl-S-Methylmalonylcysteamine

Mole Inhibitor[1] / Mole Substrate[2]	m. p. of Recovered Succinic Acid	Total cpm.	% Inhibition
0	186.5 - 187.5°	1310 ± 65	0
2	187 - 188°	1430 ± 72	0
10	189 - 190°	745 ± 35	43 ± 5

[1] N-Acetyl-S-methylmalonylcysteamine.

[2] 4-^{3}H-Methylmalonyl coenzyme A.

Calculations

A. Calculation of Methylmalonic Acid Distribution

The following notations will be used throughout this section:

M_0 = methylmalonic acid

M_1 = 4-^{2}H-methylmalonic acid

M_2 = 4, 4-di-^{2}H-methylmalonic acid

M_3 = 4, 4, 4-tri-^{2}H-methylmalonic acid

Superscript "b" will denote a species before the enzymatic isomerization, and superscript "a" will denote a species after the isomerization. For example, M_1^b equals the amount of 4-^{2}H-methylmalonic acid present before the isomerization.

In order to calculate the relative amounts of M_0, M_1, M_2, and M_3 from a mass spectrum of a mixture of the four species, the mass spectrum of each of the compounds M_0, M_1, M_2, and M_3 must be known. Tables 2, 3, and 4 in the results show the spectra of pure M_0 and M_3. From these spectra, calculated spectra for M_1 and M_2 were obtained.

The spectrum of M_0 gives a peak at m/e 74 corresponding to the molecular ion of propionic acid (see Experimental Section). The peak at m/e 73 corresponds to loss of one hydrogen from propionic acid, and the peak at m/e 75 is the isotope peak for the molecular ion. The spectrum of M_3 is similar to that of M_0, except that it is shifted three mass units higher, and there are peaks due to the loss of hydrogen and the loss of deuterium. The peak at m/e 77 corresponds to the molecular ion peak of 3, 3, 3-tri-^{2}H-propionic acid.

This ion can lose either hydrogen or deuterium giving peaks at m/e 76 and 75 respectively. The peak at m/e 75 is not likely due to loss of two hydrogens, since this type of fragmentation is not observed in the spectrum of M_0.

To calculate the spectra of M_1 and M_2, the following method was used:

1. The appropriate molecular ion was given the value 100.0;
2. The M-1 peak was calculated as (relative amount of methyl hydrogen) x (relative intensity of M-1 for M_0) + (relative amount of methyl deuterium) x (relative intensity of M-1 for M_3);
3. The M-2 peak was calculated as (relative amount of methyl deuterium) x (relative intensity of M-2 for M_3);
4. The M+1 peak was calculated as the weighted average of the M+1 peak for M_0 and the M+1 peak for M_3.

For example, the calculated mass spectrum of M_2 at an ionizing voltage of 12.5 ev. and a current of 60 μamps. would be obtained as follows:

1. The molecular ion of M_2, containing two methyl deuterium atoms, is at m/e 76. Therefore the relative intensity at m/e 76 is 100.0.
2. M-1 = 1/3 x 37.1 (from Table 2)+2/3 x 19.6 (from Table 2). The relative intensity at m/e 75 is thus 25.4.
3. M-2 = 2/3 x 10.6 (from Table 2). The relative intensity at m/e 74 is thus 7.1.

4. M+1 = 1/2 (4.2 + 4.0) = 4.1. The relative intensity at m/e 77 is thus 4.1.

The calculated mass spectra for M_1 and M_2 along with the observed spectra of M_0 and M_3 at ionizing voltages of 12.5, 20, and 70 ev. are tabulated in Tables 11, 12, and 13. The tables include only the relative intensities at m/e 74 through 77, since only these values are needed to calculate the relative amounts of M_0, M_1, M_2, and M_3 from the spectrum of a mixture of the four species.

The mass spectrum of the methylmalonic acid mixture before the isomerization, and the spectrum of the methylmalonic acid isolated after the isomerization were taken at an ionizing voltage of 12.5, 20, and 70 ev. Using the relative intensities at m/e 74 through 77, four linear equations in the four unknowns M_0, M_1, M_2, and M_3 were derived. The relative intensities used for these calculations are shown in Tables 5 and 6. The four linear equations were solved by the determinant method using a matrix inversion sub-routine of an IBM 7094 computer. An example of a set of linear equations derived for the solution of M_0^a, M_1^a, M_2^a and M_3^a at an ionizing voltage of 12.5 ev. is given below (see Tables 6 and 11):

$$74.8 = 100.0\ M_0^a + 31.3\ M_1^a + 7.1\ M_2^a$$

$$18.1 = 4.2\ M_0^a + 100.0\ M_1^a + 25.4\ M_2^a + 10.6\ M_3^a$$

$$25.7 = 4.2\ M_1^a + 100.0\ M_2^a + 19.6\ M_3^a$$

$$100.0 = 4.1\ M_2^a + 100.0\ M_3^a\ .$$

Table 11

Relative Intensities of M_0, M_1, M_2, and M_3

Ionizing Voltage = 12.5 ev., Ionizing Current = 60 μamps

m/e	M_0 (obs.)	M_1 (calc.)	M_2 (calc.)	M_3 (obs.)
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

Table 12

Relative Intensities of M_0, M_1, M_2, and M_3

Ionizing Voltage = 20 ev., Ionizing Current = 60 μamps

m/e	M_0 (obs.)	M_1 (calc.)	M_2 (calc.)	M_3 (obs.)
74	100.0	47.7	11.7	0
75	4.6	100.0	38.3	17.5
76	0	4.5	100.0	28.9
77	0	0	4.5	100.0

Table 13

Relative Intensities of M_0, M_1, M_2, and M_3
Ionizing Voltage = 70 ev., Ionizing Current = 100 μamps

m/e	M_0 (obs.)	M_1 (calc.)	M_2 (calc.)	M_3 (obs.)
74	100.0	50.4	13.1	0
75	4.6	100.0	40.6	19.7
76	0	4.5	100.0	30.8
77	0	0	4.4	100.0

The values obtained from this set of linear equations are given by,

$$M_0^a = 73.5 = 40.4\%$$

$$M_1^a = 3.0 = 1.6\%$$

$$M_2^a = 5.8 = 3.2\%$$

$$M_3^a = 99.8 = 54.8\% \quad .$$

In a similar manner, values at 12.5, 20 and 70 ev. were obtained for M_0^b, M_1^b, M_2^b, M_3^b, and M_0^a, M_1^a, M_2^a, and M_3^a. These values along with the averages and standard deviations are given in Tables 14 and 15.

From the results in Tables 14 and 15, two important facts can be obtained. During about 30% of the reaction (see p. 65 for computation of the percent reaction) the relative amount of M_0 dropped from 54.4% to 40.3% while M_3 increased from 45.6% to 54.9%. Evidently, there is an isotope effect present which favors reaction of methylmalonyl coenzyme A (M_0^b) over 4,4,4-tri-^{2}H-methylmalonyl coenzyme A (M_3^b) during the isomerization. As will be shown later, the isotope effect has a magnitude characteristic of a primary rather than a secondary effect. Consequently, the rate determining step in the isomerization must be the formation or rupture of an H-X bond.

The results presented in Table 15 show that there is less than 5% scrambling in the starting material after 30% reaction. If there is a mechanism by which scrambling can occur, then the lack of scrambling in the starting material indicates that little back reaction has occurred and that once hydrogen is abstracted from

Table 14

Relative Amounts of Methylmalonic Acid Before the Isomerization Reaction[1]

Ionizing Voltage (ev.)	M_0^b (%)	M_3^b (%)
12.5	54.2	45.8
20.0	54.1	45.9
70.0	54.9	45.1
Average =	54.4 ± 0.4	45.6 ± 0.4

[1] M_1^b and M_2^b = 0.

Table 15

Relative Amounts of Methylmalonic Acid Found in Starting Material Recovered from Isomerization Reaction

Ionizing Voltage (ev.)	M_0[a] (%)	M_1[a] (%)	M_2[a] (%)	M_3[a] (%)
12.5	40.4	1.6	3.2	54.8
20.0	39.9	1.6	3.2	55.3
70.0	40.5	1.5	3.5	54.5
Average=	40.3 ± 0.3	1.6 ± 0.1	3.3 ± 0.1	54.9 ± 0.3

the substrate (methylmalonyl coenzyme A), the intermediate goes on to form product at a faster rate than it can regain a hydrogen to give back starting material (see p. 60 for quantitative calculation of the relative amount of back reaction).

B. Calculation of Succinic Acid Distribution

The following notations will be used to designate the different succinic anhydride species throughout this section:

S_0 = succinic anhydride

S_1 = 2-^{2}H-succinic anhydride

S_2 = 2, 2, di-^{2}H-succinic anhydride

S_3 = 2, 2, 3-tri-^{2}H-succinic anhydride

S_4 = 2, 2, 3, 3-tetra-^{2}H-succinic anhydride .

For the calculation of the relative amounts of S_0, S_1, S_2, and S_3 obtained from the isomerization product, the spectra of pure S_0, S_1, S_2, and S_3 must be known. Table 7 gives the spectra of pure S_0 and of 90% pure S_4 in the ranges m/e 27 through 33 and 55 through 61. Since the spectrum of S_0 is exceedingly simple at an ionizing voltage of 12.5 ev., all extrapolations were made from this spectrum with the exception of the isotope peaks.

The spectra of S_1, S_2, and S_3 in the range m/e 28 through 31 were calculated in the following manner:

1. The base peak was given the relative value of 100.0;
2. The isotope peak was calculated as the weighted average of the isotope peaks of S_0 at m/e 29 and S_4 at m/e 33.

For example, the calculated spectrum of S_2 in the range m/e 28 through 31 would contain a base peak at m/e 30 (S_2 contains two deuterium atoms) and an isotope peak of 1/2 (2.3 + 2.1) = 2.2 at m/e 31 (see Table 7).

In the range m/e 27 through 33 one must take into account the peaks due to O_2^+ at m/e 32 and N_2^+ at m/e 28. Using an air blank, it was found that O_2^+ gives a small peak while N_2^+, since it is more difficult to ionize, gave no detectable peak at an ionizing voltage of 12.5 ev. Since only the peaks at m/e 28 through 31 were used for the actual calculation of the deuterium distribution, no error was introduced by the injection of a small amount of air along with the sample.

The calculation of the spectra of S_1, S_2, and S_3 in the range m/e 56 through 59 is slightly more complicated. From Table 7, one can see that, in addition to a parent peak and an isotope peak, S_0 also contains a small peak at m/e 55 due to loss of hydrogen from the ion $(CH_2CH_2CO)^+$. Since S_4 was not isotopically pure, no value for the loss of deuterium from S_4 can be obtained. However, it was assumed that the loss of deuterium from S_4 will approximate the loss of hydrogen from S_0.

The approximation that loss of hydrogen or deuterium from the $(CH_2CH_2CO)^+$ ion occurs with equal probability leads to the spectra of S_1, S_2,and S_3 calculated in the following manner:

1. The appropriate base peak (B) of $(CH_2CH_2CO)^+$ or its deuterated analogues, was given the relative value of 100.0.

2. The B-1 peak was calculated as 0.5 times the fraction of hydrogen in the parent ion (see Table 7).

3. The B-2 peak was calculated as 0.5 times the fraction of deuterium in the parent ion (see Table 7).

4. The B+1 isotope peak was calculated as the weighted average of the isotope peaks of S_0 at m/e 57 and S_4 at m/e 61.

A sample calculation for the spectrum of S_2 is given below:

1. The base peak at m/e 58 is given a relative intensity of 100.0.

2. B-1 = 0.5 x 1/2 = 0.25. The relative intensity at m/e 57 is thus 0.3 [1].

3. B-2 = 0.5 x 1/2 = 0.25. The relative intensity at m/e 56 is thus 0.2 [1].

4. B+1 = 1/2 (3.5 + 3.3) = 3.4. The relative intensity at m/e 59 is thus 3.4.

The observed spectrum of S_0 and the calculated spectra for S_1, S_2, and S_3 in the ranges m/e 28 through 31 and m/e 56 through 59 are given in Table 16.

The mass spectrum of the succinic anhydride obtained from the isomerization product was taken at an ionizing voltage of 12.5 ev. Four spectra were obtained giving four sets of relative intensities at m/e 28 through 31 and four sets of relative intensities at m/e 56 through 59 which are reported in Table 8. The relative amounts of

---

[1] Values for loss of hydrogen were rounded off upward while the values for loss of deuterium were rounded off downward since hydrogen is more easily abstracted than deuterium (13).

Table 16

Mass Spectra of S_0, S_1, S_2, and S_3
Ionizing Voltage = 12.5 ev., Ionizing Current = 60 μamps

m/e	S_0 (obs.)	S_1 (calc.)	S_2 (calc.)	S_3 (calc.)
28	100.0	0	0	0
29	2.3	100.0	0	0
30	0	2.2	100.0	0
31	0	0	2.2	100.0
56	100.0	0.4	0.2	0
57	3.5	100.0	0.3	0.3
58	0	3.4	100.0	0.2
59	0	0	3.4	100.0

Table 17

Relative Amounts of S_0, S_1, S_2, and S_3 Obtained from the Product of the Isomerization Reaction

Range (m/e)	S_0 (%)	S_1 (%)	S_2 (%)	S_3 (%)
28-31	66.7	14.7	11.3	7.3
	67.5	14.7	10.7	7.1
	65.0	15.1	11.6	8.3
	65.2	15.1	11.7	8.1
56-59	66.2	14.6	11.8	7.5
	66.1	14.7	11.5	7.7
	64.4	15.1	12.6	8.0
	65.6	14.3	12.5	7.6
Average =	65.8 ± 1.0	14.8 ± 0.3	11.7 ± 0.6	7.7 ± 0.4

S_0, S_1, S_2, and S_3 were calculated from these intensities by the same determinant method used for the methylmalonic acid calculations. The results of the calculations along with the average and standard deviation for each species are given in Table 17.

The results in Table 17 lead to two important conclusions. The fact that substantial amounts of 4-^{2}H-succinyl coenzyme A and 4,4-di-^{2}H-succinyl coenzyme A are formed shows that there is a mechanism by which the hydrogens can become scrambled. Thus, the lack of substantial scrambling in the methylmalonic acid recovered after 30% isomerization is valid evidence for the lack of rapid hydrogen equilibration between the 4-position of methylmalonyl coenzyme A and coenzyme B_{12}. The small amount of S_3 and the large amount of S_0 again verifies the earlier observation that an isotope effect is present which favors reaction of methylmalonyl coenzyme A over 4,4,4-tri-^{2}H-methylmalonyl coenzyme A. The fractionation of deuterium between the starting material and product during the course of the reaction gives a convenient method for calculating the percent reaction. These calculations and results are presented in the next section.

C. Calculation of the Magnitude of the Isotope Effect

1. Kinetics

The calculation of the isotope effect rests on a number of assumptions. The work of Abeles (3, 4) and of Arigoni (8) indicates that hydrogen transfer occurs from the 4-position of methylmalonyl coenzyme A to the 5'-adenosyl position of coenzyme B_{12}. After rearrangement of the substrate intermediate, a hydrogen is transferred from the 5'-adenosyl position of coenzyme B_{12} to the

3-position of an intermediate having the carbon skeleton of succinyl coenzyme A.

When the reaction is allowed to proceed to 32.7% completion (see below for calculation of the percent reaction), 4.9% ($M_1^a + M_2^a$ from Table 15) of the starting methylmalonyl coenzyme A has reversibly exchanged hydrogen with coenzyme B_{12} to give scrambled starting material. If the methylmalonyl coenzyme A intermediate (Figure 11a) abstracts a hydrogen or deuterium from the coenzyme B_{12} intermediate (Figure 11d) in the same ratio that the succinyl coenzyme A intermediate (Figure 11c) abstracts a hydrogen or deuterium from intermediate d, the total amount of back reaction can be calculated knowing M_1^a and M_2^a. The following equations are then valid for the percent of M_0, M_1, M_2, and M_3 formed by back reaction:

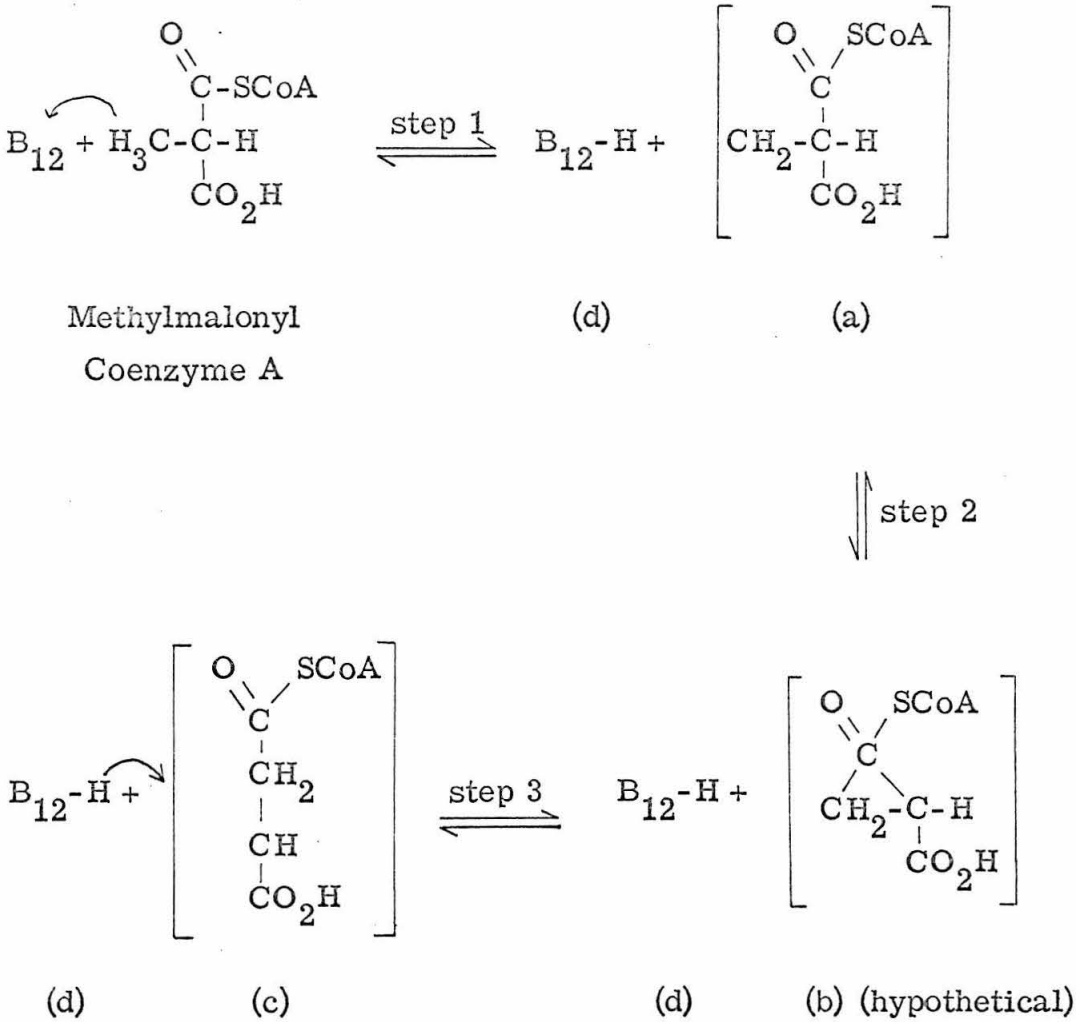
$$M_0 = M_1^a \frac{S_0}{S_1} = 6.6\% \text{ (see Table 17 for } S_0 \text{ and } S_1)$$

$$M_1 = 1.6\%$$

$$M_2 = 3.3\%$$

$$M_3 = M_2^a \frac{S_3}{S_2} = 2.2\% \text{ (see Table 17 for } S_2 \text{ and } S_3) \ .$$

Therefore, the total amount of back reaction is 13.7%. After the formation of 46.4% (32.7% + 13.7%) of intermediate a (Figure 11), 70% of the time the intermediate goes on to form succinyl coenzyme



Methylmalonyl Coenzyme A

(d) (a)

(d) (c) (d) (b) (hypothetical)

Succinyl Coenzyme A

Figure 11

Intermediates in the Isomerization of Methylmalonyl Coenzyme A

A, while 30% of the time it abstracts a hydrogen of deuterium from intermediate d (Figure 11) to give back methylmalonyl coenzyme A.

Since the isotope effect appears to be too large to be caused by a secondary effect, step 1 or step 4 (Figure 11) must be the rate determining step. If step 4 is the rate determining step in the isomerization, then all prior steps, including step 1, would be in reversible equilibrium. But if step 1 were in equilibrium then a statistical distribution of deuterium would be expected in M_0^a, M_1^a, M_2^a, and M_3^a of $(M_0^b)^3$: $3(M_0^b)^2(M_3^b)$: $3(M_0^b)(M_3^b)^2$: $(M_3^b)^3$ or about 1:3:3:1 ($M_0^b \approx M_3^b$). Since the actual distribution of M_0^a, M_1^a, M_2^a, M_3^a (see Table 15) is quite different from the statistical distribution, step 1 cannot be a rapid equilibrium. Thus, step 4 is not the rate determining step.

Since only step 1 and step 4 (Figure 11) involve the formation or rupture of a carbon-hydrogen bond, and the deuterium distribution in M_0^a, M_1^a, M_2^a, and M_3^a argue that step 4 cannot be the rate determining step, step 1 must therefore be rate determining[1]. If this is true, the following derivation of the magnitude of the isotope effect can be made.

$$M_H + EB \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} M_HEB \xrightarrow[\text{slow}]{k_H} M_HEBH \qquad \ldots \qquad (a)$$

---

[1] The amount of back reaction observed (13.7%) reflects the relative values of $\Delta F^{\ddagger}$ for the reverse of step 1 and the $\Delta F^{\ddagger}$ of steps 2, 3, or 4, whichever is largest. If $\Delta F^{\ddagger}$ for step 4, the hydrogen transfer step, is the largest of the three, then the ratio of 70% forward to 30% back reaction indicates that step 1 is rate determining, but step 4 is not much faster than step 1.

$$M_D + EB \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} M_HEB \xrightarrow[\text{slow}]{k_D} M_DEBD \qquad \ldots \qquad (b)$$

where,

M_H = methylmalonyl coenzyme A;

M_D = 4, 4, 4-tri-^{2}H-methylmalonyl coenzyme A;

EB = enzyme-coenzyme B_{12} complex;

M_HEB and M_DEB = respective substrate-enzyme-coenzyme B_{12} complexes before hydrogen or deuterium transfer;

M_HEBH and M_DEBD = respective substrate-enzyme-coenzyme B_{12} complex after hydrogen or deuterium transfer.

The steady state approximation when applied to M_HEB and M_DEB gives the following equations:

$$\frac{d(M_H)}{dt} = \frac{k_H}{K_M}(EB)(M_H) \qquad (c)$$

$$\frac{d(M_D)}{dt} = \frac{k_D}{K'_M}(EB)(M_D) \qquad (d)$$

where

$$K_M = \frac{k_{-1}}{k_1} \quad \text{and} \quad K'_M = \frac{k'_{-1}}{k'_1} \quad .$$

K_M is the equilibrium constant for the dissociation of methylmalonyl coenzyme A and the enzyme-coenzyme B_{12} complex, while K'_M is the equilibrium constant for the dissociation of 4, 4, 4-tri-^{2}H-methylmalonyl coenzyme A and the enzyme-coenzyme B_{12} complex. The principle bonding of the substrate to the enzyme-coenzyme B_{12} complex is probably primarily ionic (see Conclusion). The only effect of replacing the methyl group by a trideuteromethyl group would, therefore, be caused by the change in "effective size" of the methyl group. The isotope effect for the reaction of 2-(α, α, α-tri-^{2}H)-methyl pyridine with methyl iodide has been measured (11). The value of $k_D/k_H = 1.030 \pm .003$ reflects the extreme case where the slight difference in size of the methyl group causes a measurable change in the rate.

Ordinarily, K_M cannot be measured accurately to three significant figures, thus, the substitution of deuterium for hydrogen in a substrate should not cause a measurable change in the binding constant of the substrate and enzyme. Therefore, it is valid to assume that $K_M = K'_M$.

Dividing (c) by (d),

$$\frac{d(M_H)}{(M_H)} = \frac{k_H}{k_D} \frac{d(M_D)}{(M_D)} \quad . \qquad \text{(e)}$$

Integration from t = 0 to t = t gives,

$$\log \frac{M_H^t}{M_H^0} = \frac{k_H}{k_D} \log \frac{M_D^t}{M_D^0} \quad . \qquad (f)$$

2. Calculation of the percent reaction

In order to calculate M_H^t and M_D^t, the percent reaction must be known. The percent reaction may be calculated by two independent methods: 1. from the conservation of deuterium in the products, and 2. from the relative amount of succinic and methylmalonic acid isolated from the reaction mixture.

By the conservation of deuterium, the total deuterium in the starting methylmalonic acid equals the sum of the deuterium in the methylmalonic acid and succinic acid after partial isomerization.

If x equals the mole fraction of methylmalonyl coenzyme A converted to succinyl coenzyme A, then

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

Using Tables 15 and 17,

$$3(45.6) = (x)[14.8 + 2(11.7) + 3(7.7)] + (1-x)[1.6 + 2(3.3) + 3(54.9)]$$

$$x = 0.325 = 32.5\% \text{ reaction} \, .$$

The percent reaction can also be estimated independently from the amount of each product recovered. Assuming the methylmalonic and succinic acids were recovered with the same efficiency, the percent reaction will be given by the following equation:

$$x = \frac{\dfrac{\text{wt. of succinic acid}}{\text{ave. molecular wt.}}}{\dfrac{\text{wt. of succinic acid}}{\text{ave. molecular wt.}} + \dfrac{\text{wt. of methylmalonic acid}}{\text{ave. molecular wt.}}} .$$

Using the values from Tables 15 and 17, the average molecular weight of succinic acid and methylmalonic acid was calculated. The average molecular weight was computed as the sum of the fraction of each species times the molecular weight of that species. For succinic acid,

$$S_0 = 0.658(118) = 77.6$$
$$S_1 = 0.148(119) = 17.6$$
$$S_2 = 0.117(120) = 14.0$$
$$S_3 = 0.077(121) = \underline{\ 9.3}$$
$$118.5 = \text{average molecular weight of succinic acid.}$$

Similarly, for methylmalonic acid,

$$M_0^a = 0.403(118) = 47.5$$
$$M_1^a = 0.016(119) = 1.9$$
$$M_2^a = 0.033(120) = 4.0$$

$$M_3^a = 0.549(121) = 66.4$$

119.8 = average molecular weight of methylmalonic acid.

Since 1.30 mg. of succinic acid and 2.66 mg. of methylmalonic acid were recovered (see Experimental Section),

$$x = \frac{\frac{1.30}{118.5}}{\frac{1.30}{118.5} + \frac{2.66}{119.8}} = 0.330 = 33.0\% \text{ reaction} .$$

3. Calculation of the deuterium isotope effect

From the two independent calculations of the fraction of product formed, an average of 32.7 ± 0.2% was obtained for the percent reaction. It is now possible to calculate k_H/k_D. From Table 14,

$$M_H^0 = 54.4$$

$$M_D^0 = 45.6$$

and using Table 15,

$$M_H^t = (x)(40.3 + 1.6) = 28.2$$

$$M_D^t = (x)(54.9 + 3.3) = 39.2 .$$

For the purpose of the above calculation, M_0^a and M_1^a were combined to give M_H^t since M_1^a can only be formed from M_0^a. Similarly, M_2^a and M_3^a were combined to give M_D^t. Using equation (f),

$$\frac{k_H}{k_D} = \frac{\log\left(\frac{28.2}{54.4}\right)}{\log\left(\frac{39.2}{45.6}\right)} = \frac{\log 0.518}{\log 0.860} = 4.4 .$$

The calculated isotope effect of 4.4 verifies the assumption made earlier that the isotope effect is primary and not secondary. This calculated isotope effect is really the minimum deuterium isotope effect for the reaction, since it is the isotope effect averaged over the first 32.7% of the reaction. The true deuterium isotope effect is probably greater by several percent. All calculations of models made for this system are based on the average of the first 32.7% reaction, making this calculated isotope effect the value needed for incorporation into the equations derived in the next section.

D. Calculation of Deuterium Distribution in Succinyl Coenzyme A for Various Models

1. Abbreviations and assumptions to be made in the calculations

Three possible mechanisms for the isomerization of methylmalonyl coenzyme A to succinyl coenzyme A will be considered. When 5'-^{3}H-adenosyl coenzyme B_{12} is incubated with succinyl coenzyme A or methylmalonyl coenzyme A, Retey and Arigoni (8) showed that some tritium is transferred to succinyl

coenzyme A. These results imply that hydrogen scrambling can occur during the isomerization by equilibration of some or all of the hydrogens at the 5'-adenosyl position of coenzyme B_{12}. The three mechanisms proposed involve equilibration of the 5'-adenosyl hydrogens with the hydrogen abstracted from the methyl group of methylmalonyl coenzyme A.

The following symbols will be used in the derivations:

M_0 = average concentration of methylmalonyl coenzyme A present during the first 32.7% of the 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 amount of 4,4,4-tri-^{2}H-methylmalonyl coenzyme A;

S_0 = average amount of succinyl coenzyme A in the product during the first 32.7% of the reaction;

S_1 = average concentration of 2-^{2}H-succinyl coenzyme A;

S_2 = average concentration of 2,2-di-^{2}H-succinyl coenzyme A;

S_3 = average concentration of 2,2,3-tri-^{2}H-succinyl coenzyme A;

B_0 = average concentration of coenzyme B_{12} during the first 32.7% of the reaction;

B_1 = average concentration of 5'-^{2}H-adenosyl coenzyme B_{12};

B_2 = average concentration of 5', 5'-di-^{2}H-adenosyl coenzyme B_{12};

Δ_1 = average isotope effect (k_H/k_D) for the first 32.7% of the reaction for transfer of hydrogen or deuterium from M_0, M_1, M_2, or M_3 to B_0, B_1, or B_2;

Δ_2 = average isotope effect (k'_H/k'_D) for the first 32.7% of the reaction for transfer of hydrogen or deuterium from B_0, B_1, or B_2 to S_0, S_1, S_2, or S_3.

To obtain M_0, M_1, M_2, and M_3, the values for each species before and after 32.7% of the substrate was isomerized were averaged. Using the values found in Tables 14 and 15 the following average values are obtained:

$$\begin{aligned} M_0 &= 1/2\ (40.3 + 54.4) = 47.3\% \\ M_1 &= 1/2\ (1.6) = 0.8\% \\ M_2 &= 1/2\ (3.3) = 1.6\% \\ M_3 &= 1/2\ (54.9 + 45.6) = 50.3\% . \end{aligned} \qquad \text{(g)}$$

In order to simplify the equations, it was assumed that no back reaction had occurred. To a first approximation, M_1 can only be obtained from M_0, and M_2 can only be obtained from M_3 [1], so the approximation was made that,

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[1] Strictly speaking, M_1 can be formed from M_3, and M_2 can be formed from M_0 by a minimum of two turnovers; however, the probability of such an occurrence is vanishingly small (0.01 to 0.1% under the conditions of the reaction).

$$M_0 = 47.3 + 0.8 = 48.1\%$$
$$M_1 = M_2 = 0 \qquad \text{(h)}$$
$$M_3 = 50.3 + 1.6 = 51.9\% \ .$$

This assumption will result in a maximum error of 2%, as will be shown later when more exact calculations are made.

2. Calculation of succinyl coenzyme A distribution assuming that only one 5'-adenosyl hydrogen on coenzyme B_{12} can exchange (1 hydrogen mechanism)

A look at the model of coenzyme B_{12} (see Figure 1) will show that the two 5'-adenosyl hydrogens are in different steric environments. Since the two hydrogens are not identical, it is possible that only one can enter into any exchange during the reaction while the other is always buried in the enzyme and cannot react.

Using the above model it is possible to calculate the values for S_0, S_1, S_2, and S_3 knowing M_0, M_1, M_2, M_3, and Δ_1 or Δ_2. Each term in the equations for S_0, S_1, S_2, and S_3 was calculated as follows: S_i = (fraction of M_j) x (probability of a hydrogen or deuterium transfer from M_j to B_k) x (fraction of B_k) x (probability of a hydrogen or deuterium transfer from B_k to S_i). In general, the probability of a hydrogen transfer equals the relative rate of hydrogen transfer, Δ_1 or Δ_2, times the probability that a hydrogen can be transferred. The probability of a deuterium transfer equals the relative rate of deuterium transfer, one, times the probability that a deuterium can be transferred.

The calculation and explanation of the terms derived from the reaction of M_0 with B_1 (in this model, B_1 is considered to have one 5'-adenosyl deuterium available for transfer and one 5'-adenosyl hydrogen always inaccessible and unavailable for transfer) follows:

a. Fraction of M_0 = M_0 (from equations in h);

b. Probability of a hydrogen transfer from M_0 to B_1 = relative rate, Δ_1, times one (all three methyl positions of M_0 contain hydrogen);

c. Probability of a deuterium transfer from M_0 to B_1 = 0 (no methyl position of M_0 contains deuterium);

d. Fraction of B_1 = B_1 (calculated from the steady state approximation, see below);

e. Probability of transfer of hydrogen from B_1 to give S_0 = relative rate of transfer of hydrogen, Δ_2, times probability that hydrogen can be transferred, one-half (there are two positions containing transferable atoms, one contains the original deuterium of B_1, and the other contains the hydrogen transferred to B_1 from M_0) = $1/2\ \Delta_2$;

f. Probability of transfer of deuterium from B_1 to give S_1 = relative rate of transfer of deuterium, one, times the probability that deuterium can be transferred, one-half = 1/2 .

Therefore, for M_0 reacting with B_1,

$$S_0 = (M_0)(\Delta_1)(1)(B_1)(\Delta_2)(1/2) = 1/2\ M_0B_1\Delta_1\Delta_2$$

$$S_1 = (M_0)(\Delta_1)(1)(B_1)(1)(1/2) = 1/2\ M_0 B_1 \Delta_1 .$$

The permutation of M_0 and M_3 with B_0 and B_1, shown graphically in Figure 12, gives the following set of equations for the relative values of S_0, S_1, S_2, and S_3:

$$\begin{aligned} S_0 &= M_0 B_0 \Delta_1 \Delta_2 + 1/2\ M_0 B_1 \Delta_1 \Delta_2 \\ S_1 &= 1/2\ M_0 B_1 \Delta_1 \\ S_2 &= 1/2\ M_3 B_0 \Delta_2 \\ S_3 &= M_3 B_1 + 1/2\ M_3 B_0 \end{aligned} \qquad \text{(i)}$$

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 is applied[1]:

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

$$\frac{d(B_0)}{dt} = 1/2\ M_0 B_1 \Delta_1 - 1/2\ M_3 B_0 \Delta_2 = 0$$

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

Therefore, the relative values of B_0 and B_1 are given by:

$$B_0 = M_0 \Delta_1$$

$$B_1 = M_3 \Delta_2 .$$

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[1] See next section for justification of steady state approximation.

Methylmalonyl Coenzyme A | Coenzyme B_{12} | Succinyl Coenzyme A

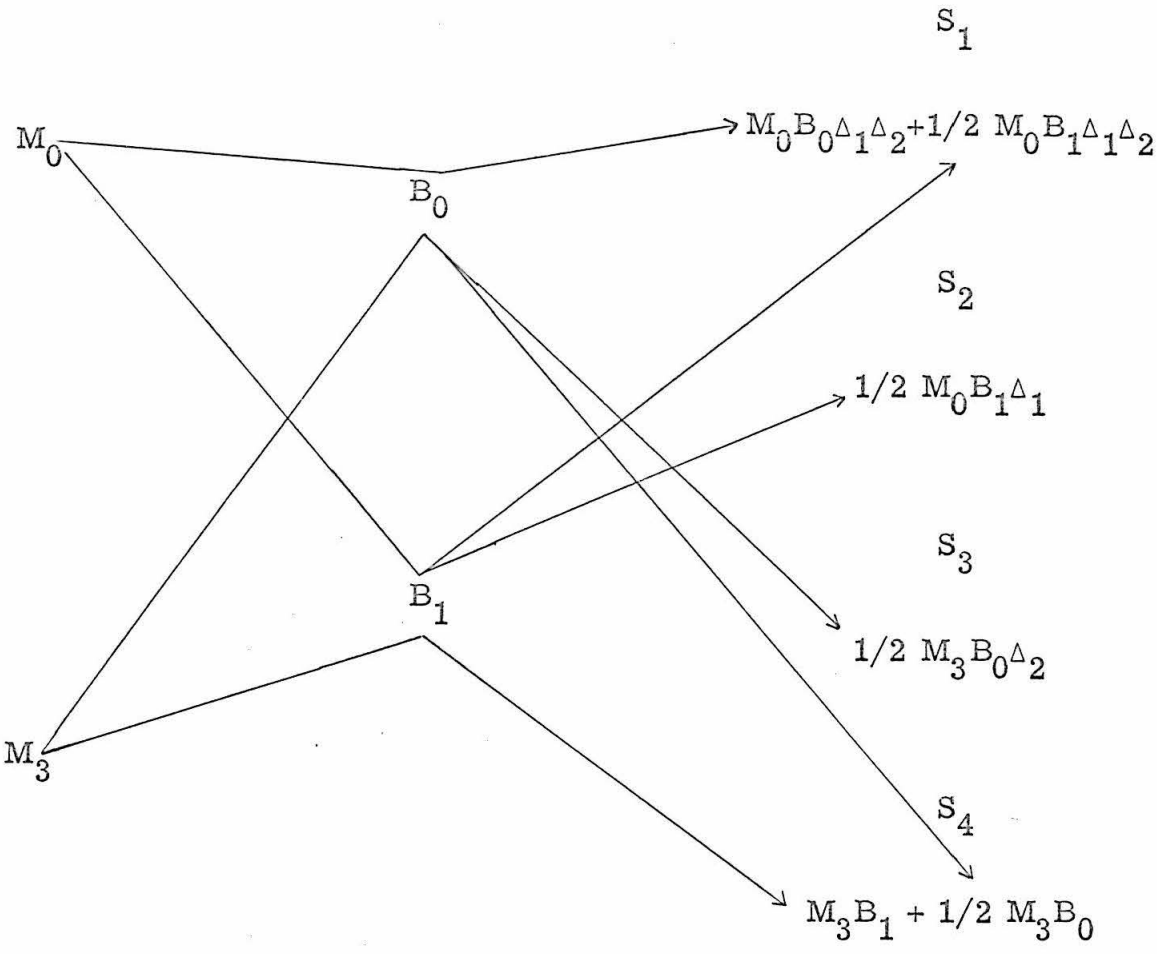


Figure 12

Calculation of S_0, S_1, S_2, and S_3 for One Hydrogen, Equilibrium Model

Using the equations in (i) to solve for S_0, S_1, S_2, and S_3, we obtain:

$$\begin{aligned} S_0 &= M_0^2 \Delta_1^2 \Delta_2 + 1/2\ M_0 M_3 \Delta_1 \Delta_2^2 \\ S_1 &= 1/2\ M_0 M_3 \Delta_1 \Delta_2 \\ S_2 &= 1/2\ M_0 M_3 \Delta_1 \Delta_2 \\ S_3 &= M_3^2 \Delta_2^2 + 1/2\ M_0 M_3 \Delta_1 \end{aligned} \qquad \text{(j)}$$

Using the values for M_0 and M_3 obtained in (h) and the calculated isotope effect of 4.4, we can now calculate the theoretical amounts of S_0, S_1, S_2, and S_3 for this proposed mechanism. First, let us assume that the isotope effect is caused by the abstraction of hydrogen or deuterium from methylmalonyl coenzyme A. Then,

$$\Delta_1 = 4.4,\ \Delta_2 = 1,\ M_0 = 0.481, \text{ and } M_3 = 0.519.$$

Using the equations in (j), the following results were obtained:

$$\begin{aligned} S_0 &= 5.026 = 72.4\% \\ S_1 &= 0.549 = 7.9\% \\ S_2 &= 0.549 = 7.9\% \\ S_3 &= 0.818 = 11.8\% \ . \end{aligned} \qquad \text{(k)}$$

If we assume that the isotope effect is caused by abstraction of hydrogen from the 5'-adenosyl position of coenzyme B_{12} (this seems less likely from earlier arguments concerning the amount of back reaction found), then,

$\Delta_1 = 1$, $\Delta_2 = 4.4$, $M_0 = 0.481$, and $M_3 = 0.519$.

Using the equations in (j), the following results were obtained:

$$\begin{aligned} S_0 &= 3.432 = 34.8\% \\ S_1 &= 0.549 = 5.6\% \\ S_2 &= 0.549 = 5.6\% \\ S_3 &= 5.342 = 54.1\% \end{aligned} \qquad (1)$$

3. Calculation of succinyl coenzyme A distribution assuming both 5'-adenosyl hydrogens on coenzyme B_{12} can exchange (2 hydrogen equilibrium)

This model assumes that both 5'-adenosyl hydrogens can equilibrate at a rapid rate with the hydrogen transferred to the 5'-position from methylmalonyl coenzyme A. The actual rate of equilibration is assumed to be faster than the rate of rearrangement of the intermediate so that the three hydrogens attached to the 5' position of the coenzyme B_{12} intermediate reach equilibrium during each turnover of the coenzyme B_{12}.

Again, M_1 and M_2, formed by back reaction, are neglected for the purposes of this preliminary calculation. The method used for calculating the relative amounts of S_0, S_1, S_2, and S_3 is identical to the one used above. Now, however, there are three coenzyme B_{12} intermediates instead of two. Permuting M_0 and M_3 with B_0, B_1, and B_2 gives the following values for S_0, S_1, S_2, and S_3:

$$S_0 = M_0B_0\Delta_1\Delta_2 + 2/3\ M_0B_1\Delta_1\Delta_2 + 1/3\ M_0B_2\Delta_1\Delta_2$$
$$S_1 = 1/3\ M_0B_1\Delta_1 + 2/3\ M_0B_2\Delta_1$$
$$S_2 = 1/3\ M_3B_1\Delta_2 + 2/3\ M_3B_0\Delta_2 \qquad \text{(m)}$$
$$S_3 = M_3B_2 + 2/3\ M_3B_1 + 1/3\ M_3B_0 .$$

Applying the steady state approximation,

$$\frac{d(B_0)}{dt} = \frac{d(B_1)}{dt} = \frac{d(B_2)}{dt} = 0$$

the relative values for B_0, B_1, and B_2 were calculated as,

$$B_0 = M_0^2\Delta_1^2$$
$$B_1 = 2M_0M_3\Delta_1\Delta_2 \qquad \text{(n)}$$
$$B_2 = M_3^2\Delta_2^2 .$$

Eliminating B_0, B_1, and B_2 from the equations in (m), the following values are obtained:

$$S_0 = M_0\Delta_1\Delta_2\ (M_0^2\Delta_1^2 + 4/3\ M_0M_3\Delta_1\Delta_2 + 1/3\ M_3^2\Delta_2^2)$$
$$S_1 = 2/3\ M_0M_3\Delta_1\Delta_2\ (M_0\Delta_1 + M_3\Delta_2)$$
$$S_2 = 2/3\ M_0M_3\Delta_1\Delta_2\ (M_0\Delta_1 + M_3\Delta_2) \qquad \text{(o)}$$
$$S_3 = M_3\ (M_3^2\Delta_2^2 + 4/3\ M_0M_3\Delta_1\Delta_2 + 1/3\ M_0^2\Delta_1^2) .$$

Using the values of M_0 and M_3 obtained in equation (h) and the calculated isotope effect of 4.4, we can now calculate the theoretical amounts of S_0, S_1, S_2, and S_3 for this proposed mechanism. If the isotope effect is caused by the abstraction of hydrogen or deuterium from methylmalonyl coenzyme A, then,

$$\Delta_1 = 4.4, \quad \Delta_2 = 1, \quad M_0 = 0.481, \text{ and } M_3 = 0.519 .$$

Using the equations in (o), the following results are obtained:

$$\begin{aligned} S_0 &= 14.530 = 70.2\% \\ S_1 &= 2.197 = 10.6\% \\ S_2 &= 2.197 = 10.6\% \\ S_3 &= 1.763 = 8.5\% . \end{aligned} \qquad \text{(p)}$$

If it is assumed that the isotope effect is due to abstraction of hydrogen or deuterium from the coenzyme B_{12} intermediate, then $\Delta_1 = 1$, and $\Delta_2 = 4.4$. Under these conditions the following results are obtained for this model:

$$\begin{aligned} S_0 &= 7.888 = 49.0\% \\ S_1 &= 2.197 = 13.7\% \\ S_2 &= 2.197 = 13.7\% \\ S_3 &= 3.805 = 23.7\% . \end{aligned} \qquad \text{(q)}$$

4. Calculation of deuterium distribution in succinyl coenzyme A using the "merry-go-round" model

The "merry-go-round" model is similar to the previous model, which allows complete equilibration of the 5'-adenosyl hydrogens of coenzyme B_{12}, except the hydrogen transferred to coenzyme B_{12} from the substrate cannot then be reacquired by the same rearranged substrate molecule. In other words, it takes a minimum of two turnovers of substrate for a specific hydrogen to be transferred from substrate to coenzyme B_{12} and back to the product again.

For the calculation of the deuterium distribution for this model, all three coenzyme B_{12} species are present at equilibrium; however, the relative number of hydrogens or deuteriums available for transfer from B_k to S_i is now two instead of three. The calculations for this model give the following equations for the deuterium distribution:

$$
\begin{aligned}
S_0 &= M_0B_0\Delta_1\Delta_2 + 1/2\ M_0B_1\Delta_1\Delta_2 \\
S_1 &= 1/2\ M_0B_1\Delta_1 + M_0B_2\Delta_1 \\
S_2 &= 1/2\ M_3B_1\Delta_2 + M_3B_0\Delta_2 \\
S_3 &= M_3B_2 + 1/2\ M_3B_1
\end{aligned}
\qquad (r)
$$

where,

$$B_0 = M_0^2\Delta_1^2$$

$$B_1 = 2M_0M_3\Delta_1\Delta_2$$

$$B_2 = M_3^2 \Delta_2^2 .$$

Eliminating B_0, B_1, and B_2 from the equations in (r), the following results were obtained:

$$\begin{aligned}
S_0 &= M_0\Delta_1\Delta_2 (M_0^2\Delta_1^2 + M_0M_3\Delta_1\Delta_2) = M_0^2\Delta_1^2 \\
S_1 &= M_0\Delta_1 (M_0M_3\Delta_1\Delta_2 + M_3^2\Delta_2^2) = M_0M_3\Delta_1 \\
S_2 &= M_3\Delta_2 (M_0^2\Delta_1^2 + M_0M_3\Delta_1\Delta_2) = M_0M_3\Delta_1 \\
S_3 &= M_3 (M_0M_3\Delta_1\Delta_2 + M_3^2\Delta_2^2) = M_3^2 .
\end{aligned} \qquad \text{(s)}$$

The equations in (s) are independent of Δ_2, so to calculate the deuterium distribution for this model we shall assume $\Delta_1 = 4.4$, $M_0 = 0.481$, and $M_3 = 0.519$. Using the equations in (s), the following results are obtained:

$$\begin{aligned}
S_0 &= 4.477 = 63.6\% \\
S_1 &= 1.148 = 16.3\% \\
S_2 &= 1.148 = 16.3\% \\
S_3 &= 0.269 = 3.8\% .
\end{aligned} \qquad \text{(t)}$$

5. Comparison of calculated and observed results

The results of the calculations for the three proposed mechanisms are presented in Table 18 along with the observed succinic anhydride distribution taken from Table 17. Only two sets of calculated distributions appear to fit the observed

Table 18

Summary of Calculated Distributions

Mechanism	S_0 (%)	S_1 (%)	S_2 (%)	S_3 (%)
One Hydrogen				
$\Delta_1 = 4.4$, $\Delta_2 = 1$	72.4	7.9	7.9	11.8
$\Delta_1 = 1$, $\Delta_2 = 4.4$	34.8	5.6	5.6	54.1
Two Hydrogen Equilibrium				
$\Delta_1 = 4.4$, $\Delta_2 = 1$	70.2	10.6	10.6	8.5
$\Delta_1 = 1$, $\Delta_2 = 4.4$	49.0	13.7	13.7	23.7
Two Hydrogen "Merry-go-round"				
$\Delta_1 = 4.4$	63.6	16.3	16.3	3.8
Observed Values	65.8	14.8	11.7	7.7

distribution within reasonable limits. These two are the two hydrogen "merry-go-round" mechanism and the two hydrogen equilibrium mechanism with $\Delta_1 = 4.4$ and $\Delta_2 = 1$.

For simplicity of calculation, the above results were obtained using the assumption that no back reaction had occurred, and that $M_1 = M_2 = 0$. In order to determine how well the two most likely mechanisms fit the observed data, the calculations were repeated using the average values of M_0, M_1, M_2, and M_3 obtained from the equations in (g).

6. Detailed calculation for the two hydrogen equilibrium model

The calculation of the deuterium distribution in the succinyl coenzyme A was repeated taking into account the small amount of back reaction leading to M_1 and M_2. The calculation was done in the same manner as those described earlier. The equations derived for the two hydrogen equilibrium are given below:

$$S_0 = M_0\Delta_1\Delta_2\ (B_0 + 2/3\ B_1 + 1/3\ B_2) + M_1\Delta_2\ (2/9\ B_0 + 1/9\ B_1)$$

$$S_1 = M_0\Delta_1\ (1/3\ B_1 + 2/3\ B_2) + M_1\ (2/3\ B_0\Delta_1\Delta_2 + 1/9\ B_0 + 4/9\ B_1\Delta_1\Delta_2 + 2/9\ B_1 + 2/9\ B_2\Delta_1\Delta_2 + 1/3\ B_2) + M_2\Delta_2\ (4/9\ B_0 + 2/9\ B_1) \qquad (u)$$

$$S_2 = M_3\Delta_2\ (2/3\ B_0 + 1/3\ B_1) + M_2\ (1/3\ B_0\Delta_1\Delta_2 + 2/9\ B_0 + 2/9\ B_1\Delta_1\Delta_2 + 4/9\ B_1 + 1/9\ B_2\Delta_1\Delta_2 + 2/3\ B_2) + M_1\Delta_1\ (2/9\ B_1 + 4/9\ B_2)$$

$$S_3 = M_3\ (1/3\ B_0 + 2/3\ B_1 + B_2) + M_2\Delta_1\ (1/9\ B_1 + 2/9\ B_2)\ .$$

Applying the steady-state approximation, the values of B_0, B_1, and B_2 were obtained:

$$B_0 = \Delta_1^2 (3M_0 + 2M_1 + M_2)^2$$

$$B_1 = 2\Delta_1\Delta_2 (3M_0 + 2M_1 + M_2)(M_1 + 2M_2 + 3M_3) \qquad (v)$$

$$B_2 = \Delta_2^2 (M_1 + 2M_2 + 3M_3)^2 .$$

From the equations in (u) and (v), the relative amounts of S_0, S_1, S_2, and S_3 can be calculated. Using the values $M_0 = 0.473$, $M_1 = 0.008$, $M_2 = 0.016$, $M_3 = 0.503$, $\Delta_1 = 4.4$, and $\Delta_2 = 1$, the following results were obtained:

$$S_0 = 1.803 = 67.5\%$$

$$S_1 = 0.333 = 12.5\% \qquad (w)$$

$$S_2 = 0.296 = 11.1\%$$

$$S_3 = 0.238 = 8.9\% .$$

7. Detailed calculation for two hydrogen "merry-go-round" model

The distribution of deuterium in succinyl coenzyme A was calculated for the two hydrogen "merry-go-round" model using a more rigorous derivation which takes into account the small amount of back reaction leading to M_1 and M_2. Using the method applied above, and assuming a steady state approximation, the following equations were obtained:

$$S_0 = \Delta_1(M_0\Delta_1 + 1/3\ M_1)(3M_0 + 2M_1 + M_2)$$

$$S_1 = \Delta_1(2/3\ M_1\Delta_1 + 2/3\ M_2)(3M_0 + 2M_1 + M_2) + (M_0\Delta_1 + 1/3\ M_1)$$
$$(M_1 + 2M_2 + 3M_3)$$

$$S_2 = \Delta_1(1/3\ M_2\Delta_1 + M_3)(3M_0 + 2M_1 + M_2) + (2/3\ M_1\Delta_1 + 2/3\ M_2) \qquad (x)$$
$$(M_1 + 2M_2 + 3M_3)$$

$$S_3 = (1/3\ M_2\Delta_1 + M_3)(M_1 + 2M_2 + 3M_3)\ .$$

The relative amounts of S_0, S_1, S_2, and S_3 were calculated using the values $M_0 = 0.473$, $M_1 = 0.008$, $M_2 = 0.016$, $M_3 = 0.503$, and $\Delta_1 = 4.4$:

$$\begin{aligned} S_0 &= 13.304 = 63.4\% \\ S_1 &= 3.446 = 16.4\% \\ S_2 &= 3.414 = 16.3\% \\ S_3 &= 0.816 = 3.9\% \ . \end{aligned} \qquad (y)$$

8. Summary of detailed calculations

The results of the detailed calculations for the two models, along with the observed deuterium distribution in the succinic anhydride, are presented in Table 19. The implications of these calculations will be discussed in the Conclusion.

Table 19

Calculated Deuterium Distribution in Succinyl Coenzyme A, Taking into Account the Back Reaction

Species	Two Hydrogen, Equilibrium (%) $\Delta_1 = 4.4$, $\Delta_2 = 1$	Two Hydrogen, "Merry-go-round" (%) $\Delta_1 = 4.4$	Observed Distribution (%)
S_0	67.5	63.4	65.8 ± 1.0
S_1	12.5	16.4	14.8 ± 0.3
S_2	11.1	16.3	11.7 ± 0.6
S_3	8.9	3.9	7.7 ± 0.4

E. Justification of the Steady State Approximation for the Coenzyme B_{12} Species, B_0, B_1, and B_2

In order to justify the use of the steady state approximation for B_0, B_1, and B_2, the number of turnovers required to reach a steady state equilibrium must be small compared to the total number of turnovers which occurred during the reaction. To obtain the number of turnovers required to reach equilibrium, the distribution of B_0, B_1, and B_2 was calculated as a function of the number of turnovers using the two hydrogen equilibrium model.

The calculations were made assuming that no back reaction had occurred, and that all coenzyme B_{12} was in the B_0 form before the first turnover ($B_1 = B_2 = 0$ at this point). For calculating the relative amounts of B_0, B_1, and B_2 after n turnovers, it can be shown that,

$$B_{0_n} = r_n - s_n x + t_n x^2$$

$$B_{1_n} = s_n x - 2t_n x^2 \qquad (z)$$

$$B_{2_n} = t_n x \ ,$$

where,

n = number of turnovers

B_{0_n} = fraction of B_0 present after the nth turnover

B_{1_n} = fraction of B_1 present after the nth turnover

B_{2_n} = fraction of B_2 present after the nth turnover

$r_n = r_{n-1}$ $(r_o = 1)$

$s_n = 2/3\ (r_{n-1} + s_{n-1})$

$t_n = 1/3\ (s_{n-1} + t_{n-1})$

$x = M_3/(M_0\Delta_1 + M_3)$= effective fraction of M_3 "seen" by B_0, B_1, or B_2.

Using the equations in (z), the fraction of B_0, B_1, and B_2 were calculated for the first eight turnovers. The results are recorded in Table 20 and presented graphically in Figure 13, for $M_0 = 48.1\%$, $M_3 = 51.9\%$, and $\Delta_1 = 4.4$.

After the eighth turnover, the deviation from equilibrium is 1.2% for B_0, 0.9% for B_1, and 0.3% for B_2; therefore, the total deviation from equilibrium at this point is only 2.4%. From the Experimental Section, one finds that a total of 0.016 μmole of coenzyme B_{12} was used. Eight turnovers of substrate would, therefore, represent $8 \times .016 = 0.128$ μmole of succinyl coenzyme A formed. Since a total of 55 μmole of methylmalonyl coenzyme A was allowed to react to 32.7% completion, $55 \times .327 = 18.0$ μmole of succinyl coenzyme A was formed during the reaction. Eight turnovers represents $0.128/18.0 = 0.7\%$ reaction. So, in the first 0.7% of reaction, B_0, B_1, and B_2 have come to within 97.6% of their relative equilibrium concentrations.

The above calculations assume that the coenzyme B_{12} is in rapid equilibrium with the enzyme so that all coenzyme B_{12} is able to react with substrate. The work of Retey and Arigoni (8) indicates

Table 20

Fraction of B_0, B_1, and B_2 Versus Number of Turnovers of Substrate

	n = number of turnovers									
Species	0	1	2	3	4	5	6	7	8	∞
B_0 (%)	1	86.9	79.0	74.0	70.8	68.7	67.3	66.3	65.7	64.5
B_1 (%)	0	13.1	20.1	24.3	26.8	28.4	29.5	30.3	30.7	31.6
B_2 (%)	0	0	0.9	1.7	2.4	2.9	3.2	3.4	3.6	3.9

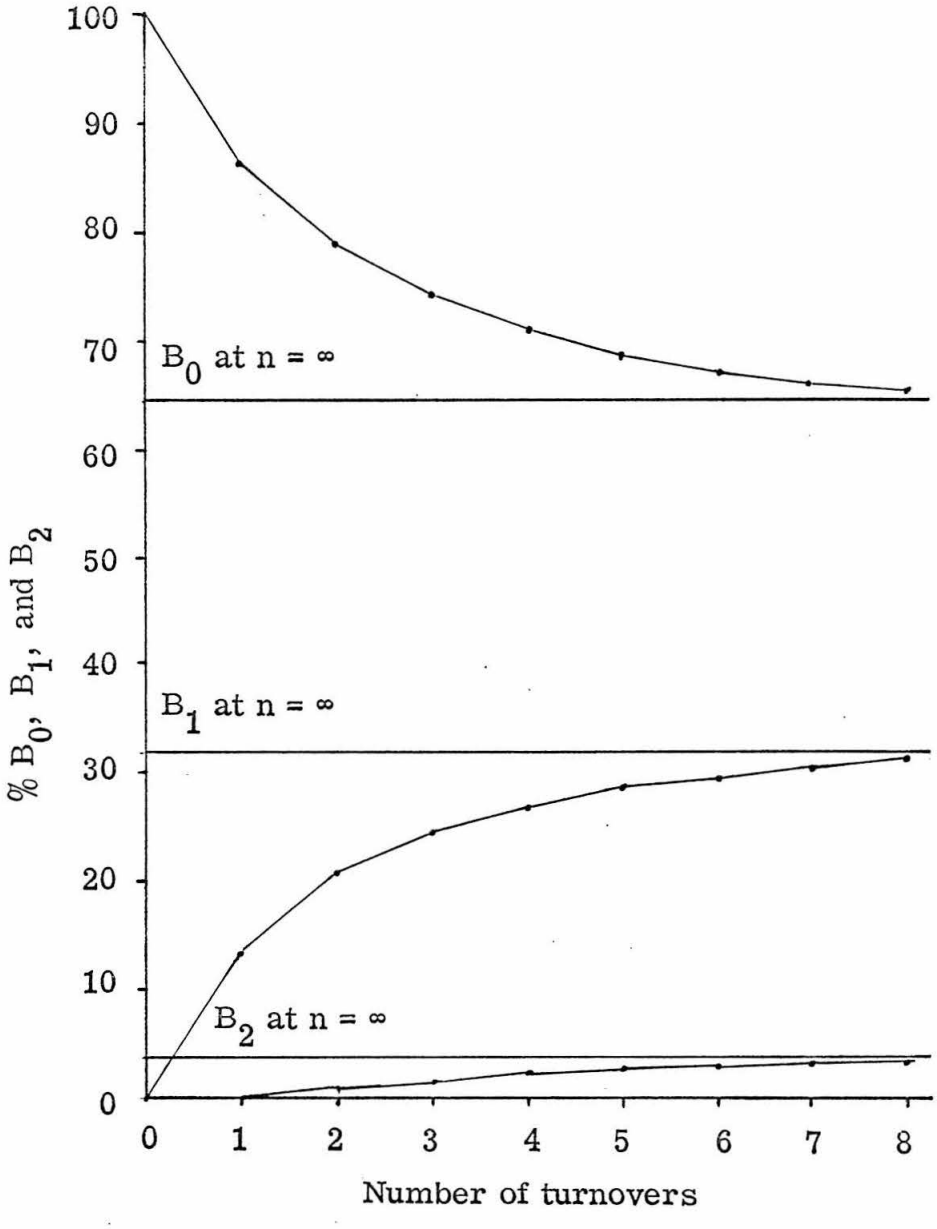


Figure 13

Number of Turnovers vs. Percent of B_0, B_1, and B_2

that this assumption may not be valid. When 5'-^{3}H-adenosyl coenzyme B_{12} was incubated with mutase and succinyl coenzyme A, only 32% of the tritium was recovered in the succinic acid. In a similar experiment, methylmalonyl coenzyme A was used in place of succinyl coenzyme A. In this case, only 16% of the tritium was recovered in the succinic acid product. No explanation is given for the low percent recovery of tritium; however, one possible explanation is that the coenzyme B_{12} is tightly bound to the enzyme and does not rapidly equilibrate with the excess free coenzyme B_{12}. If this were the case, only bound coenzyme B_{12} could exchange tritium with the substrate, leading to a low recovery of tritium based on the total amount of coenzyme B_{12} present.

Other evidence indicates that coenzyme B_{12} is tightly bound to the enzyme (12). The dissociation constant, K_M, for coenzyme B_{12} and methylmalonyl coenzyme A mutase is 3.45×10^{-7}. This value is extremely small compared with the values of 10^{-4} to 10^{-5} usually found for substate binding constants (for example, $K_M = 3.45 \times 10^{-5}$ for succinyl coenzyme A and $K_M = 8 \times 10^{-5}$ for methylmalonyl coenzyme A). Furthermore, after isolating the enzyme (12) (which included three chromatographies, two dialyses and three ammonium sulfate precipitations), 38% of the enzyme was shown to have inactive coenzyme B_{12} still attached to the active site. This last piece of evidence shows that the rate of dissociation must be very slow.

If the bound and free coenzyme B_{12} are not at equilibrium during the course of the reaction, the effective concentration of coenzyme B_{12} is smaller than the 0.016 μmoles added. In this case, eight turnovers would represent a smaller percentage of succinyl coenzyme A formed. The case discussed above, which assumes

participation of all the added coenzyme B_{12} in the isomerization reaction, represents the limiting situation in which the greatest number of turnovers will be necessary before application of the steady state approximation becomes valid. One can, therefore, safely conclude that B_0, B_1, and B_2 have come to at least 97.6% of their relative equilibrium concentrations during the first 0.7% of the reaction.

Conclusions

A. Hydrogen Transfer in the Methylmalonyl Coenzyme A Mutase Reaction

1. Rate determining step and magnitude of the isotope effect

From the calculations presented in the previous section, several conclusions can be made concerning the role of coenzyme B_{12} in the transfer of hydrogen from methylmalonyl coenzyme A to succinyl coenzyme A. All the evidence indicates that the rate determining step for the reaction is the transfer of hydrogen from methylmalonyl coenzyme A to coenzyme B_{12}.

The magnitude of the isotope effect, $k_H/k_D = 4.4$, indicates that the rate determining step must involve the formation and/or rupture of a carbon-hydrogen bond. While this can occur in either of two steps, hydrogen transfer from methylmalonyl coenzyme A to coenzyme B_{12} or from coenzyme B_{12} to succinyl coenzyme A, the lack of appreciable back reaction suggests that rapid exchange does not occur between the hydrogens at C-4 of methylmalonyl coenzyme A and C-5' of the adenosyl group of coenzyme B_{12}. Thus, the rate of hydrogen transfer from coenzyme B_{12} to succinyl coenzyme A must be fast compared to the rate of transfer from methylmalonyl coenzyme A to coenzyme B_{12}.

Of all the models considered, only two come close to predicting the observed deuterium distribution in the product. Both of these models incorporate an isotope effect for the hydrogen transfer from methylmalonyl coenzyme A to coenzyme B_{12}. Using the same models, but incorporating the isotope effect into the transfer of hydrogen from coenzyme B_{12} to succinyl coenzyme A

gives a predicted deuterium distribution in the product quite different from the observed distribution (see Table 18). Thus, one can conclude that the rate determining step is the transfer of a hydrogen from C-4 of methylmalonyl coenzyme A to C-5' of the adenosyl group of coenzyme B_{12}.

2. Mechanism

Several mechanisms which have been proposed in the past can now be eliminated on the basis of the observed deuterium distribution found in the succinic acid. The mechanism proposed by Whitlock (Figure 5), and discussed in Part I, could give no hydrogen scrambling. The only possible mechanism for scrambling would be for the intermediate acrylic acid to become detached from the coenzyme B_{12} during the reaction. However, it is known that no exchange occurs when free 1-^{14}C-acrylic acid is added to the reaction (14). Therefore, this mechanism can be effectively removed as a possible pathway for the conversion of methylmalonyl coenzyme A to succinyl coenzyme A.

Recently, Hogenkamp (15) proposed a mechanism in which the 4'-adenosyl hydrogen of coenzyme B_{12} is removed by a basic group (presumably on the enzyme) to form a carbanion. This carbanion can then abstract a hydrogen from the methyl group of methylmalonyl coenzyme A. After rearrangement to succinyl coenzyme A carbanion, the substrate abstracts a hydrogen from the 4'-adenosyl position of coenzyme A to give succinyl coenzyme A and the starting coenzyme B_{12} carbanion. The complete sequence is shown in Figure 14. Again, this mechanism cannot account for the deuterium scrambling found in the succinic acid. Since there

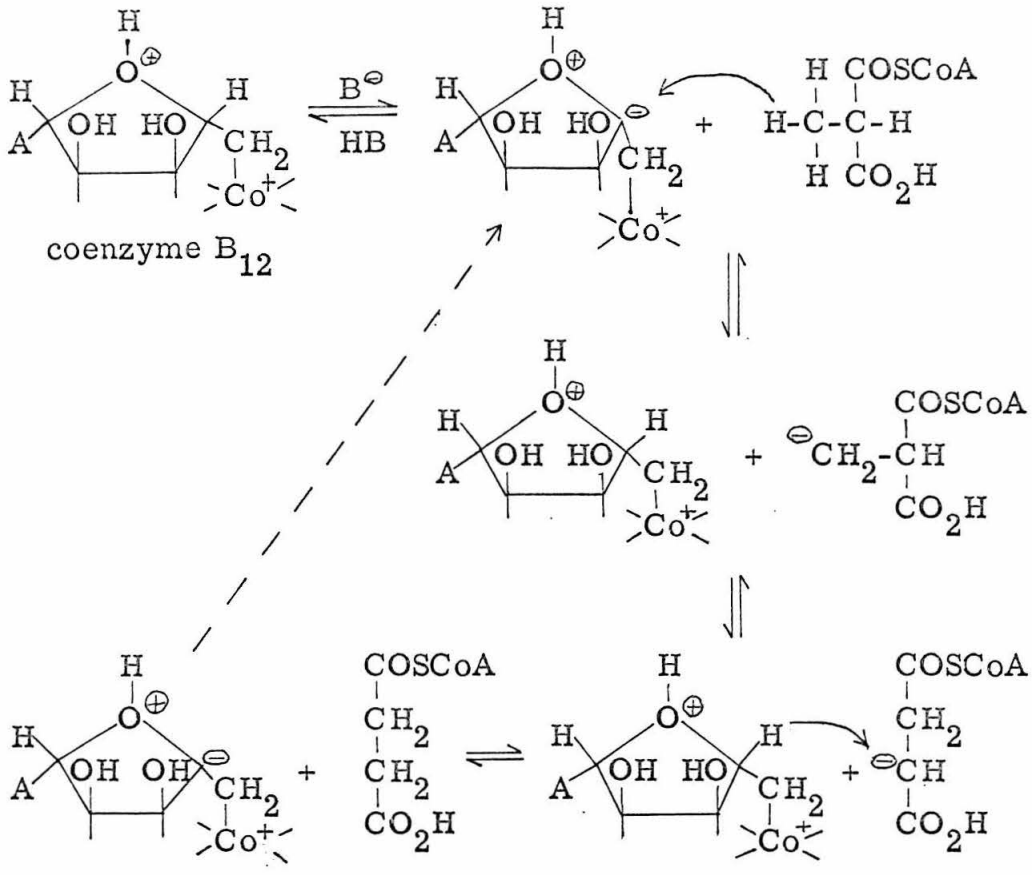


Figure 14

Mechanism of Isomerization Proposed by H. P. C. Hogenkamp

is only one 4'-adenosyl hydrogen, no randomization can occur due to equilibration with other hydrogens on coenzyme B_{12}. Scrambling could only occur by separation of the substrate carbanion and the coenzyme B_{12} carbonium ion, which is highly unlikely.

Three models for the isomerization of methylmalonyl coenzyme A to succinyl coenzyme A were considered, which involve equilibration of the hydrogens in the 5'-adenosyl position of coenzyme B_{12}. The calculated deuterium distribution in succinyl coenzyme A for the one hydrogen equilibrium model did not agree with the observed distribution. However, both the two hydrogen equilibrium and two hydrogen "merry-go-round" models gave values which agreed reasonably well with the observed deuterium distribution (Table 18). A more rigorous derivation, taking into account the small percentage of back reaction, was calculated for these two models (Table 19).

In order to determine which model seems most likely, let us examine the percentage of each species separately. Table 21 presents the calculated percentages for each species and δ, their difference from the observed percentages. Comparing the δs for each species shows that for the equilibrium model, two calculated values (S_0 and S_2) lie within the 95% confidence limit (2σ) of the observed value, while for the "merry-go-round" model, no value lies within the 95% confidence limit.

A better idea of the closeness of fit can be obtained by looking at the percentage deviation from the observed value for each species. This value is computed as $100\ \delta_n/S_n$, and is given in Table 22. For comparison, the percent deviation for the observed values are computed as $100\ \sigma_n/S_n$ (see Appendix for quantitative error analyses and significance test).

Table 21

Percentage of S_0, S_1, S_2, and S_3 for the Two Most Likely Models

Species	Two Hydrogen Equilibrium (%)	[1] $\delta_{eq.}$	Two Hydrogen, "Merry-go-round" (%)	[2] $\delta_{mgo.}$	Observed (%)
S_0	67.5	1.7	63.4	2.4	65.8 ± 1.0
S_1	12.5	2.3	16.4	1.6	14.8 ± 0.3
S_2	11.1	0.6	16.3	4.6	11.7 ± 0.6
S_3	8.9	1.2	3.9	3.8	7.7 ± 0.4

[1] $\delta_{eq.}$ = |calculated value-observed value| for equilibrium model.

[2] $\delta_{mgo.}$ = |calculated value-observed value| for "merry-go-round" model.

Table 22

Percent Deviation from the Observed Deuterium Distribution for the Two Most Likely Models

Species	% Deviation for Equilibrium Model	% Deviation for "Merry-go-round" Model	% Deviation for Observed Values
S_0	2.6	3.7	1.5
S_1	15.5	10.8	2.1
S_2	5.1	39.3	5.1
S_3	15.6	49.4	5.2

A comparison of the percent deviation for the two models shows that in three of the four species (S_0, S_2, and S_3) the deviation is smaller for the equilibrium model. The ratio between the percentages vary from 1.5 for S_0, to 8 for S_2. Although the percent deviation is greater for the equilibrium model in S_1, the ratio of the percentages is less than 1.5.

A comparison of the calculated value of S_3 is useful since it is present in small but significant quantities. Since the calculated percentage of S_3 is small, it is influenced less by deviations in the values used for its calculation. The deviation of S_3 for the equilibrium model is 15.6% or three standard deviations from the observed value, while the deviation of S_3 for the "merry-go-round" model is nearly 50%.

Although the fit with the observed values is not perfect, all evidence tends to favor the two hydrogen equilibrium mechanism as the mode of hydrogen transfer in the methylmalonyl coenzyme A mutase reaction. A schematic drawing of this mechanism is shown in Figure 15. The hydrogen is shown leaving as a proton, for which there is no real evidence (a primary hydrogen isotope effect can range from $k_H/k_D = 1$ to 10 for loss of hydrogen as a proton, radical or hydride ion) (16).

There is some reason to believe that the carbon-cobalt bond is at least partially polarized (17). The complete X ray crystallographic structure of coenzyme B_{12} shows the Co-C-C angle is 130°, rather than 109° expected for a covalent bond. The carbon-cobalt bond may be partially ionized giving rise to the two canonical forms shown in Figure 16. At one extreme the carbon-cobalt bond is covalent, and the cobalt is in the +1 state, while at the other extreme, the carbon-cobalt bond is completely ionic, and the cobalt is in the +2 state.

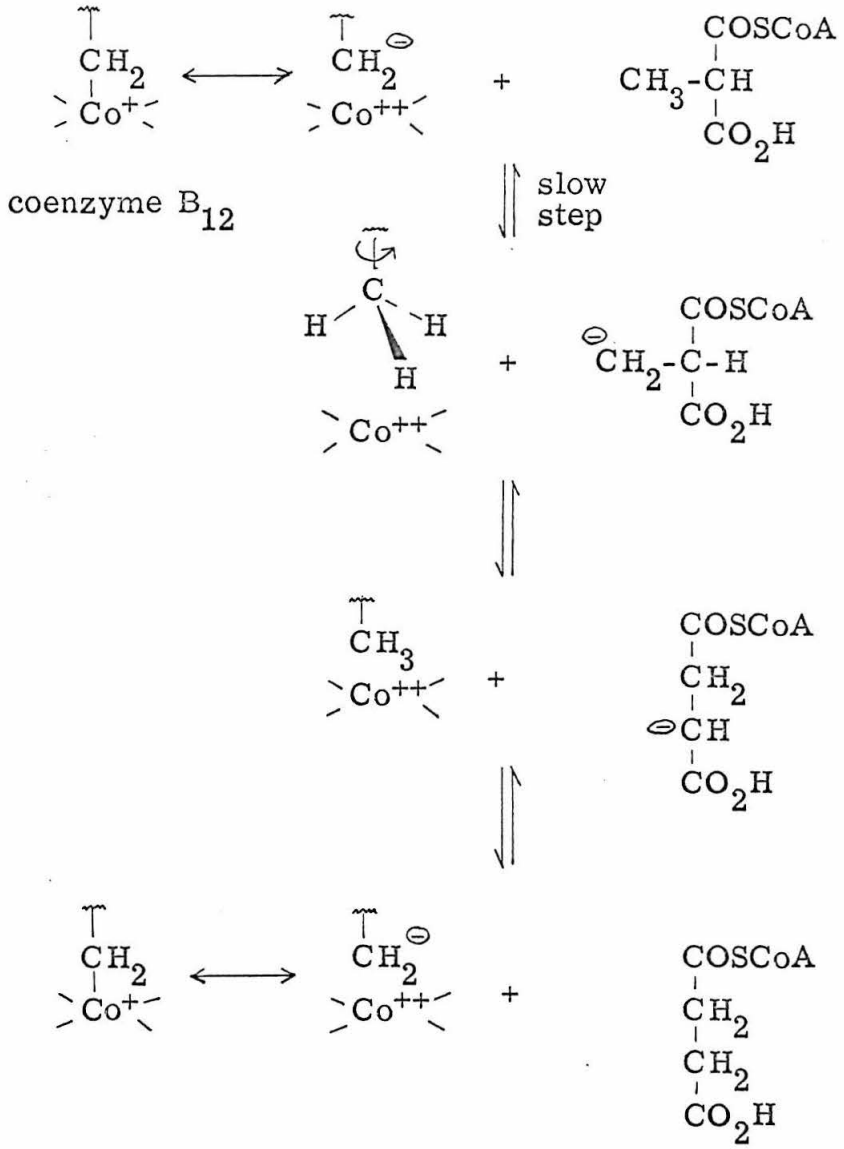


Figure 15

Two Hydrogen Equilibrium Mechanism

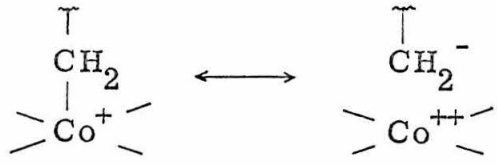


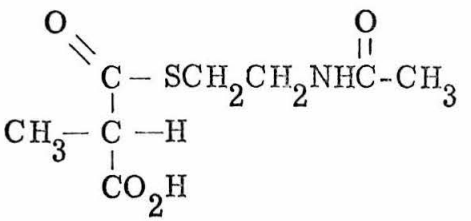
Figure 16

Coenzyme B_{12} Polarization

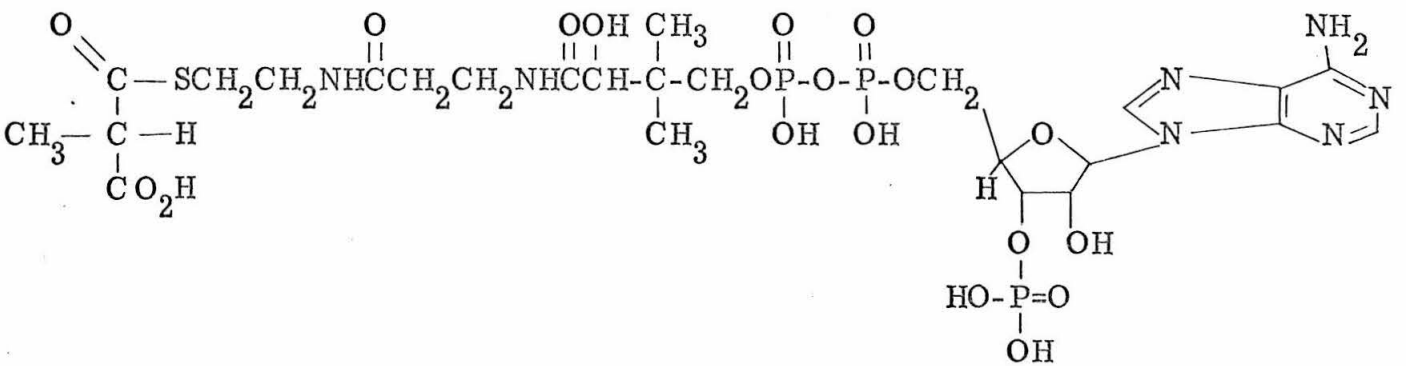
Whether or not the carbon-cobalt bond is actually broken during the reaction is not known. A number of experiments designed to determine if separation occurs are discussed in Proposition II.

B. Discussion of N-Acetyl-S-Methylmalonycysteamine

The purpose of making N-acetyl-S-methylmalonylcysteamine was to try and find a substrate for methylmalonyl coenzyme A mutase which was inexpensive and easily purified, and which could be obtained in gram quantities. N-acetyl-S-methylmalonylcysteamine has a structure identical to methylmalonyl coenzyme A, except that it is missing about two-thirds of the coenzyme A part of the molecule (see Figure 17). The results, presented in Table 9, show that N-acetyl-S-methylmalonylcysteamine is not isomerized to N-succinyl-S-methylmalonylcysteamine by methylmalonyl coenzyme A mutase. There could be several reasons why N-acetyl-S-methylmalonyl-cysteamine is not a substrate; it may not bind to the enzyme, or it may bind at a point other than the active site of the enzyme, or it may bind at the active site in a configuration which does not allow isomerization.



N-Acetyl-S-methylmalonylcysteamine



Methylmalonyl coenzyme A

Figure 17

To determine if N-acetyl-S-methylmalonylcysteamine binds at all to the enzyme, a semi-quantitative inhibition study was undertaken. The results, presented in Table 10, show that N-acetyl-S-methylmalonylcysteamine does inhibit the isomerization of methylmalonyl coenzyme A. A 10 molar excess of inhibitor over substrate lowers the rate of isomerization by about 43%. This indicates that the compound is a weak inhibitor.

Whether the inhibitor binds at the active site or at some other part of the enzyme molecule is not known. However, since the structure of the inhibitor mimics that of the substrate, it is more likely to bind to the active site of the enzyme rather than at some other position. A detailed inhibition study of this compound might be able to distinguish where the N-acetyl-S-methylmalonylcysteamine binds on the enzyme.

The fact that N-acetyl-S-methylmalonylcysteamine is not a substrate leads one to ask what is required before a compound can be a substrate for methylmalonyl coenzyme A mutase. The part of coenzyme A that is missing contains all the ionizable phosphate and amine groups. One can speculate that the substrate is ionically bonded to the enzyme. The bonding could occur through the phosphates, which would be partially ionized at pH 7.2, or through one or more of the amine groups on the adenosine. This hypothesis could be tested by synthesizing possible substrates which contain some, but not all, of the ionic group present in coenzyme A.

Experimental Section

The elemental analysis was conducted by Sprang Microanalytical Laboratory, Ann Arbor, Michigan.

All melting points and boiling points reported herein are uncorrected.

A. Materials

1. Cystamine dihydrochloride was obtained from the Aldrich Chemical Co. and used without further purification.
2. Acetic anhydride was obtained from J. T. Baker Chemical Company and used without further purification.
3. Anhydrous ammonia was obtained from Matheson Co. and purified by passing through sodium hydroxide pellets.
4. Benzene was distilled from phosphorus pentoxide (b. p. 79.5 - 79.7°).
5. Methylmalonic acid was obtained from General Biochemicals and used without further purification except where otherwise noted.
6. Coenzyme A was obtained from Calbiochem Co. as a lyophilized powder containing 75 to 82% reduced coenzyme A.
7. 1,1,1-tri-^{2}H-methyl iodide was obtained from Merck, Sharp, Dohme of Canada (99.5% deuterium).
8. 1-^{3}H-methyl iodide (sp. act. 5 mc.) was obtained from Volk Radiochemical Co. and diluted with 5 g. of freshly distilled methyl iodide (b. p. 42.0 - 42.3°).

9. Methylmalonyl coenzyme A mutase (sp. act. 0.31 μmole/min./mg. protein) was kindly supplied by Dr. R. W. Kellermeyer, Department of Medicine, Western Reserve University, Cleveland, Ohio.

10. Dimethylbenzimidazolylcobamide coenzyme (coenzyme B_{12}) Coenzyme B_{12} was generously supplied by Dr. H. A. Barker, Department of Biochemistry, University of California, Berkeley, Calif., (0.2 μmole) for the earlier assays, and later by Dr. Perlman, Squibb Institute for Medical Research, New Brunswick, N. J., (10 mg.). Five mg. of the coenzyme B_{12} was further purified by chromatography on Dowex 50W, 200-400 mesh, pH 3.0, according to the method of H. A. Barker and coworkers (24).

The purified coenzyme B_{12} had the following absorbancy ratio:

	A_{350}/A_{310}
reported for coenzyme B_{12} (25)	0.88
reported for light inactivated coenzyme B_{12} (25)	3.43
found for chromatographed coenzyme B_{12}	0.88 .

B. Syntheses and Isolations

1. N,N'-Diacetylcystamine

The acylation of cystamine hydrochloride (4.5 g., 0.02 mol.) was carried by standard methods using acetic anhydride in an aqueous sodium acetate buffer (18). After removal of water, the product was extracted with chloroform and precipitated with ligroin (30 - 60°). Recrystallization from chloroform-ligroin gave 3.6 g., (70% yield) of N,N'-diacetylcystamine with m. p. 88-88.5°. Reported m. p. 87°(19).

2. N-Acetylcysteamine

The reduction of N, N'-diacetylcystamine (6.8 g., 0.03 mol.) was carried out with sodium (2.3 g., 0.10 mol.) in liquid ammonia (20). After evaporation of the ammonia the remaining salts were dissolved in 50 ml. water and acidified with conc. HCl. The solution was saturated with sodium chloride and extracted with three 25 ml. portions of chloroform. After removal of the chloroform, the residue was distilled to give 4 g. N-acetylcysteamine with b. p. 100°/ 0.5 mm. (60% yield). Reported b. p. 138 - 140°/7 mm. (19).

3. Methylmalonyl dichloride

Methylmalonyl dichloride was prepared by the method used in Organic Syntheses for the preparation of malonyl dichloride (21). Eight ml. of product was collected with b. p. 58 - 58.5°/25 mm. representing a yield of 75%. Reported b. p. 41 - 55°/11 mm. (22).

4. N-acetyl-S-methylmalonylcysteamine

A solution of N-acetylcysteamine (1.2 g.,10 mmol.) and pyridine (0.75 g.,9.5 mmol.) in 100 ml. benzene was cooled to 10°. Freshly distilled methylmalonyl dichloride (0.75 g., 4.8 mmol.) in 25 ml. benzene was added dropwise with stirring. After four hours the benzene was decanted from the semi-solid residue. The nitroprusside test for free thiolester (10) showed that all the product was in the residue. The semi-solid was dissolved in .005 N hydrochloric acid and the solution was brought to 80% saturation with sodium chloride. The solution was continuously extracted for 24 hours with ethyl acetate. After removal of the ethyl acetate, the dithiolester

residue was partially hydrolyzed by dissolving in 50 ml. 1 M sodium carbonate and letting the solution stand for one day at room temperature. The aqueous solution was lyophilized giving a white powder containing the sodium salt of the monothiolester, N-acetylcysteamine and inorganic salts.

The powder was extracted with two 50 ml. portions of chloroform to remove the mercaptan. The remaining solid was then dissolved in 50 ml. water, titrated to pH 2.2 (glass electrode), saturated with ammonium sulfate, and extracted with two 25 ml. portions of ethyl acetate. After removal of the ethyl acetate the residual oil was put in the freezer until crystallization occurred. Recrystallization of the N-acetyl-S-methylmalonylcysteamine from ethyl acetate-ligroin (60 - 70°) gave 150 mg. of a white solid with m.p. 107.5 - 109°.

The product gave a negative sulfhydryl test (10) and a positive test for thiolester (10). The IR (Beckman IR-7) showed three carbonyl stretching frequencies at 1740, 1690 and 1630 cm^{-1}.

Anal. Calcd. for $C_8H_{13}NO_4S$: C, 43.82; H, 5.97; N, 6.39; S, 14.63.

Found: C, 43.90; H, 5.92; N, 6.47; S, 14.70.

5. Methylmalonyl coenzyme A

All labelled and unlabelled substrates used were prepared by the method of Trams and Brady (23). The crude methylmalonyl coenzyme A was chromatographed according to the method of J.D. Erfle and co-workers (2). The product was identified by its absorbance at 260 mμ, its positive thiolester test (10) and negative sulfhydryl test (10). In the case of 4-^{3}H-methylmalonyl coenzyme A

the product peak corresponded to the radioactivity peak in a molar ratio of 1:1 as calculated from the known specific activity of the starting methylmalonic acid and the known extinction coefficient for methylmalonyl coenzyme A of ϵ_{260} = 16,800 cm^2/mole (26).

6. Synthesis of a mixture of 4-^{3}H-methylmalonyl coenzyme A and 4,4,4-tri-^{2}H-methylmalonyl coenzyme A

a. Synthesis of 4,4,4-tri-^{2}H-methylmalonic acid and 4-^{3}H-methylmalonic acid

4,4,4-Tri-^{2}H-methylmalonic acid and 4-^{3}H-methylmalonic acid were synthesized from diethyl malonate and the appropriate methyl iodide by the method in Organic Syntheses (27). The crude 4-^{3}H-methylmalonic acid had m. p. 130 - 131° (reported m. p. 131.5 - 132.5° (2)) and was used as a marker to help in the isolation of the products. The crude 4,4,4-tri-^{2}H-methylmalonic acid had m. p. 125 - 126°. The NMR of this acid showed less than 0.5% hydrogen in the methyl position.

b. Chromatography of the methylmalonic acid mixture

A mixture of 200 mg. methylmalonic acid, 200 mg. 4,4,4-tri-^{2}H-methylmalonic acid and 20 mg. 4-^{3}H-methylmalonic acid (sp. act. 43,000 cpm/mg.) was dissolved in 0.5 ml. 0.5 N sulfuric acid. The solution was put on a silica gel - 0.5 N sulfuric acid column (2:1 ratio, 23 x 2 cm.) and eluted with 400 ml. 5% n-butanol in chloroform followed by 10% n-butanol in chloroform. Twenty ml. fractions were taken and analyzed for tritium content. A small amount of dimethylmalonic acid appeared in fractions 9-13 and

methylmalonic acid was eluted in fractions 21-25. The methylmalonic acid solution was taken to dryness and recrystallized from ethyl acetate-ligroin to give 300 mg. of product with m. p. 133. 5 - 134. 5°. The mass spectrum of the product showed that no deuterium scrambling had occurred during the chromatography.

c. Labelled methylmalonyl coenzyme A mixture

A mixture of 4-^{3}H-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 (23). A total of 55 μmoles of methylmalonyl CoA was obtained starting with 200 μmoles of coenzyme A. All of this product was used in the mutase reaction, since it represents a total of only 6 mg. of free acid.

7. Attempted isolation of mutase

Several attempts were made to isolate the enzyme from Propionibacterium shermanii (52W) by the method developed by H. G. Wood and co-workers (28, 29). These isolation attempts followed the published procedure as closely as possible. We did not have an Eppenbach Mill (28) at our disposal with which to grind the cells, so we were forced to use a less efficient Waring Blender. Since no enzyme activity was found in the cell-free extract, the enzyme might possibly reside in a sub-cellular particle which is not broken up by a Waring Blender. After several unsuccessful isolation attempts, a sample of methylmalonyl coenzyme A mutase was generously supplied by Dr. R. W. Kellermeyer (50 mg. with specific activity 0. 31 μmole/min./mg. protein).

C. Enzymatic Isomerization

1. Assay of mutase preparation

The assay was performed by the method of Stadtman and co-workers (30). The procedure was modified by incubating for 20 instead of 30 minutes, and, in later experiments, by replacing the potassium maleate buffer, pH 6.9, with tris-hydrochloride buffer, pH 7.3.

The amount of unreacted thiolester was measured by the hydroxamic acid method (10). Colorimetric measurements were made with a Klett colorimeter using a #50 filter. The procedure was calibrated with a known amount of methylmalonyl monohydroxamic acid prepared by the method in (31).

2. Attempted enzymatic isomerization of N-acetyl-S-methylmalonylcysteamine

The same method used for the assay of methylmalonyl coenzyme A mutase (30) was used for the attempted isomerization except that methylmalonyl coenzyme A was replaced by N-acetyl-S-methylmalonylcysteamine. No isomerization was observed up to a mutase concentration at which isomerization slower by a factor of 100 than methylmalonyl coenzyme A would have been detected (see Table 9).

3. Inhibition of mutase action by N-acetyl-S-methylmalonylcysteamine

The procedure used for the enzyme assay was also used for the inhibition study. Each sample contained 25 μmole tris hydro-

chloride buffer, pH 7.3; 0.08 mg. mutase preparation, sp. act. 0.31 μ mole/min./mg.; 4×10^{-4} μmole coenzyme B_{12}; 1.2 μmole 4-^{3}H-methylmalonyl coenzyme A, sp. act. 3500 cpm/μmole; and a variable amount of N-acetyl-S-methylmalonylcysteamine; total volume 0.50 ml. After incubation for 15 minutes each sample was quenched with 0.50 ml. of 1 N sodium hydroxide containing 20 mg./ml. of succinic acid. Each sample was acidified with 2 N formic acid after 30 minutes and chromatographed on an ion exchange column of Bio-rad AG-1-X2 (1 x 15 cm.). The succinic acid was eluted with 0.2 N formic acid while the methylmalonic acid remained on the column. The succinic acid from each sample was divided in half, weighed and counted for radioactivity using 15 ml. Bray's solution (32) as the scintillating medium. The activity was measured using a Tricarb scintillation counter. The results are tabulated in Table 10.

4. Enzymatic isomerization of the methylmalonyl coenzyme A mixture

The following mixture was incubated for 15 minutes at 37°: 55 μmoles of the above methylmalonyl coenzyme A mixture, 0.016 μmole coenzyme B_{12}, 4.4 mg. of mutase preparation (sp. act. 0.31 μmole/min./mg.), and 1250 μmoles of tris hydrochloride buffer, pH 7.3, in a total volume of 20 ml. The reaction was quenched by the addition of 3 ml. 1N sodium hydroxide. After 30 minutes, the solution was acidified with 3.5 ml. 1N HCl and the volume was reduced to 5 ml. By continuous extraction with ether for 24 hours, the organic acids were removed. After removal of the ether, the acids were dissolved in 0.5 ml. water and chromatographed on a Bio-rad AG-1-X2 ion exchange column (20 x 1.0 cm.) eluting with

a formic acid gradient of 0.2 N to 2.0 N formic acid in a total volume of 300 ml. Ten ml. fractions were collected, and the product acids were located by their radioactivity. Succinic acid (1.30 mg., m.p. 182 - 184°) was eluted in fractions 6-9 and methylmalonic acid (2.66 mg., m.p. 132 - 133.5°) was eluted in fractions 20-23. The reported melting points are 185° and 131.5 - 132.5° respectively (2).

The succinic acid was converted to the anhydride by the micro-method developed by G. Popjak and co-workers (9). From 1.30 mg. of succinic acid, 0.5 mg. of succinic anhydride was obtained. The mass spectra of the succinic anhydride, starting methylmalonic acid and product methylmalonic acid were taken and the values reported in the Results.

D. Mass Spectral Analyses

1. Mass spectra of methylmalonic acids

All mass spectra were taken with a Consolidated Electrodynamic Corporation mass spectrometer, model #21-103C. In order to insure that the molar intensities of the methylmalonic acid and 4,4,4-tri-^{2}H-methylmalonic acid are the same, known mixtures of the two acids were analyzed and found to give the correct molar ratios within experimental error. All analyses were done at ionizing voltages of 12.5, 20, and 70 ev. No systematic error in the molar ratio was observed as the energy increased, indicating that no error is introduced due to differences in the ionization potentials of the acids.

Samples of 0.3 to 1.0 mg. of acid were placed in 2 mm. thin-walled capillary tubes which were evacuated and sealed. Each capillary was broken inside the mass spectrometer and the contents expanded into a 2 liter volume at 200°. Under these conditions methylmalonic acid rapidly undergoes decomposition to propionic acid which gives a mass ion peak at m/e 74. The corresponding peak for 4, 4, 4-tri-^{2}H-methylmalonic acid is at m/e 77. From the mass spectra of the deuterated and undeuterated methylmalonic acids, extrapolated spectra for 4, 4-di-^{2}H-methylmalonic acid and 4-^{2}H-methylmalonic acid were obtained. The observed and extrapolated spectra are shown in Tables 11, 12, and 13.

2. Mass spectra of succinic anhydrides

2, 2, 3, 3-Tetra-^{2}H-succinic anhydride was made from ethyl-1, 1, 2, 2-ethane tetracarboxylate and D_2O (9). The spectra of known mixtures of 2, 2, 3, 3-tetra-^{2}H-succinic anhydride and succinic anhydride showed that the correct molar ratios are obtained within experimental error at an ionization potential of 12.5 electron volts.

The analysis of the succinic anhydrides was done at 12.5 ev., because at this low voltage the spectrum is exceedingly simple. The main peaks found are at m/e 28 and 56. The m/e 56 peak is due to loss of CO_2 from succinic anhydride, while the peak at m/e 28 is due to loss of CO_2 and CO. The only other peaks observed were the isotope peaks at m/e 29 and 57, and a peak of relative intensity 0.5% at m/e 55. From this data it is easy to calculate theoretical spectra of the partially deuterated succinic anhydrides. The observed and extrapolated spectra are shown in Table 16.

Appendix

Error Analysis

Since each value put into the equations for the mathematical models has a standard deviation connected with it, it was possible to put error limits on the calculated distributions. The standard deviation of the calculated and observed values for each species can be used as a basis for applying the significance test (t test), which was done for each species for the two hydrogen equilibrium model and the two hydrogen "merry-go-round" model (33).

Propagating the error in k_H/k_D gives the following value for the standard deviation in Δ:

$$\Delta = 4.4 \pm 0.3.$$

The error in Δ was divided equally between k_H and k_D, so $k_H = 4.4 \pm 0.25$ and $k_D = 1.00 \pm 0.06$.

Using the above standard deviations, and the standard deviations for M_0, M_1, M_2, and M_3 (see Equation g), the following standard deviations were calculated for S_0, S_1, S_2, and S_3:

1. For two hydrogen equilibrium,

$$S_0 = 67.5 \pm 6.1\%$$
$$S_1 = 12.5 \pm 1.2\%$$
$$S_2 = 11.1 \pm 1.1\%$$
$$S_3 = 8.9 \pm 0.8\%.$$

2. For two hydrogen "merry-go-round",

$$S_0 = 63.4 \pm 6.1\%$$

$$S_1 = 16.4 \pm 1.5\%$$

$$S_2 = 16.3 \pm 1.5\%$$

$$S_3 = 3.9 \pm 0.4\% \ .$$

The t test was applied to the difference between the calculated and observed value for each species. The results are shown in Table 23. The t test shows that S_2 and S_3 are highly significantly different (99% confidence limit) from the observed values for the "merry-go-round" model, while no value is highly significantly different (99% confidence limit) from the observed values for the equilibrium model. Furthermore, only one value, S_1, is significantly different at the 90% confidence limit for the equilibrium model.

Again, in a more quantitative manner, the statistical analysis of the data supports the conclusion that the two hydrogen equilibrium model most closely fits the experimental data for hydrogen transfer in the methylmalonyl coenzyme A mutase reaction.

Table 23

Values of t for the Two Models

Species	t for Two Hydrogen Equilibrium	t for Two Hydrogen "Merry-go-round"
S_0	0.41	0.57
S_1	2.74	1.58
S_2	0.71	4.23
S_3	1.77	9.87

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Abstract of Propositions

Proposition I

It is proposed that the rate of carbon monoxide exchange be measured between cobalt hydrocarbonyl and ^{14}C-carbon monoxide in an effort to determine the nature of the reactive species in the hydroformylation reaction.

Proposition II

A study of the binding between coenzyme B_{12} and methylmalonyl coenzyme A mutase by the use of coenzyme B_{12} analogues is proposed.

Proposition III

Labelling experiments are proposed to distinguish between different possible mechanisms for the metabolism of hydrogen cyanide by an unidentified basidiomycete.

Proposition IV

A study of the properties of thiepin 1, 1-dioxide is proposed using low temperature photolysis and product analysis of the Diels-Alder adduct formed with tetracyanoethylene.

Proposition V

It is proposed that the Mössbauer spectrum of I^{129} placed in the active sites of liver alcohol dehydrogenase be used to tell if the two sites are conformationally identical.

PROPOSITION I

It is proposed that the rate of carbon monoxide exchange be measured between cobalt hydrotetracarbonyl and carbon monoxide-C^{14} in an effort to determine if the reactive species in the oxo reaction is a transient cobalt hydrotricarbonyl or the intermediate cobalt hydrotetracarbonyl.

It is known that the carbonylation of olefins in the presence of dicobalt octacarbonyl takes place via the intermediate cobalt hydrotetracarbonyl (1). Consequently, many studies of the mechanism of the oxo reaction are done at room temperature using cobalt hydrotetracarbonyl as the catalyst.

Breslow and Heck synthesized CH_3-$Co(CO)_4$, CH_3-CH_2-$Co(CO)_4$ and benzyl-$Co(CO)_4$ and showed that they absorb one mole of carbon monoxide to form the corresponding acyl-cobalt carbonyls (2). Postulating that these compounds are intermediates in the oxo reaction they proposed two mechanisms for the reaction:

Mechanism 1

a. $R\text{-}CH{=}CH_2 + HCo(CO)_4 \longrightarrow R\text{-}CH_2\text{-}CH_2\text{-}Co(CO)_4$

b. $R\text{-}CH_2\text{-}CH_2\text{-}Co(CO)_4 \longrightarrow R\text{-}CH_2\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}Co(CO)_3$

c. $R\text{-}CH_2\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}Co(CO)_3 + CO \longrightarrow R\text{-}CH_2\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}Co(CO)_4$

d. $R\text{-}CH_2\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}Co(CO)_4 + HCo(CO)_4 \longrightarrow R\text{-}CH_2\text{-}CH_2\text{-}CHO + Co_2(CO)_8$

Mechanism 2

a. $HCo(CO)_4 \longrightarrow HCo(CO)_3 + CO$

b. $HCo(CO)_3 + R\text{-}CH{=}CH_2 \longrightarrow R\text{-}CH_2\text{-}CH_2\text{-}Co(CO)_3$

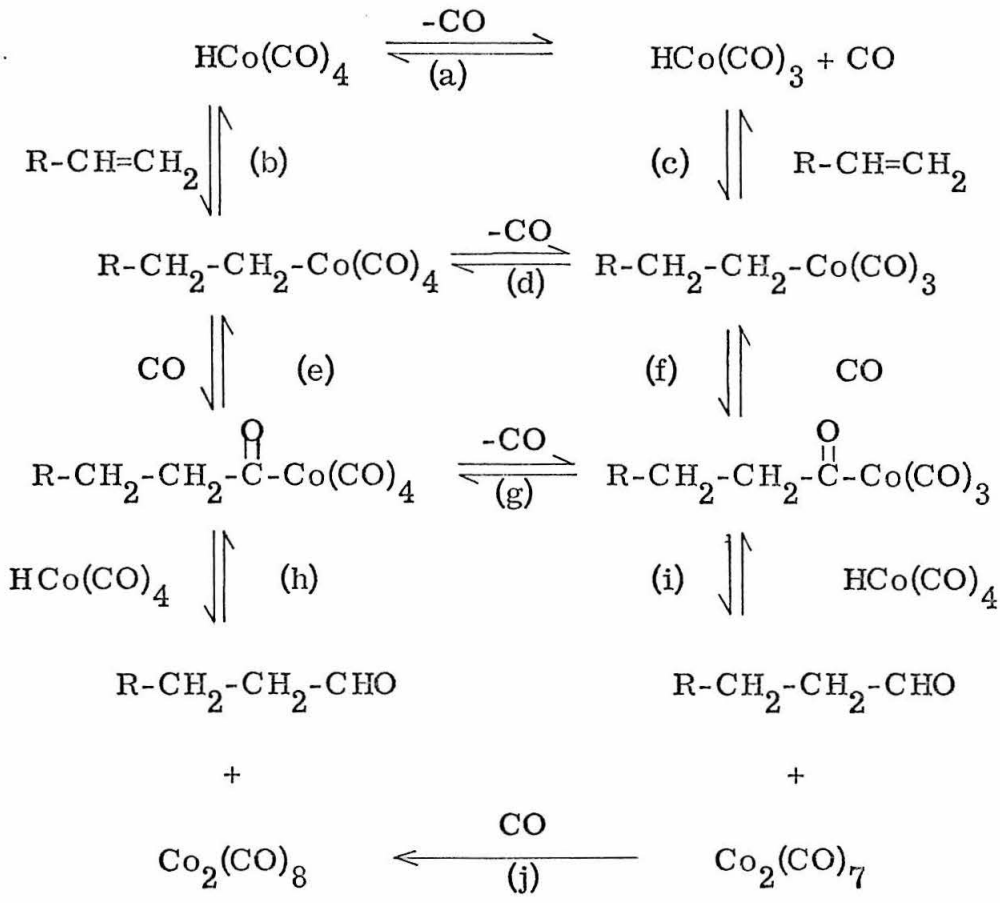
c. $R\text{-}CH_2\text{-}CH_2\text{-}Co(CO)_3 + CO \longrightarrow R\text{-}CH_2\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}(CO)_3$

d. $R\text{-}CH_2\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}Co(CO)_3 + HCo(CO)_4 \rightarrow R\text{-}CH_2\text{-}CH_2\text{-}CHO + Co_2(CO)_7$

e. $Co_2(CO)_7 + CO \longrightarrow Co_2(CO)_8$.

Karapinka and Orchin found that the formation of aldehydes from 1-pentene and cobalt hydrotetracarbonyl is inhibited by carbon monoxide (3). Heck and Breslow repeated this experiment under conditions which favor alkyl-cobalt carbonyl formation (4). They added cobalt hydrotetracarbonyl to a large excess of 1-pentene at 0^o under nitrogen and separately under carbon monoxide. After fifteen minutes the reactions were quenched and the solutions analyzed by infrared spectroscopy. The products formed under nitrogen showed two carbonyl peaks corresponding to an alkyl- or acyl-cobalt carbonyl and an aldehyde, but under a carbon monoxide atmosphere the aldehyde peak was absent while the alkyl- or acyl-cobalt carbonyl peak was reduced by 30%. On the basis of these data they concluded that the addition to olefin involves cobalt hydrotricarbonyl rather than cobalt hydrotetracarbonyl.

A flow diagram which includes both of the previously described mechanisms shows that carbon monoxide inhibition can occur at any one of three steps:

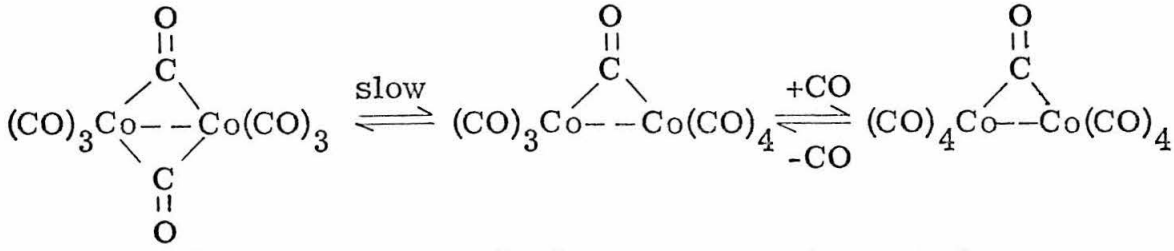


Inhibition at step (a), (d), or (g) is in qualitative agreement with the results of Heck and Breslow, so any one of these steps could be important in the mechanism of the hydroformylation reaction.

The plausibility of cobalt hydrotricarbonyl as an intermediate in the hydroformylation reaction can be tested by measuring the order and rate of exchange of cobalt hydrotetracarbonyl with ^{14}C-carbon monoxide. If cobalt hydrotricarbonyl is an intermediate in the reaction the rate of exchange must be first order and must be faster than the overall rate of the hydroformylation reaction. If the rate of exchange is slower than the overall rate of the hydroformylation reaction then cobalt hydrotricarbonyl cannot be an intermediate in the reaction.

The rate of the hydroformylation reaction would also have to be measured accurately in order to compare the two rates. Kirch and Orchin gave some raw data (1) which fits first order kinetics with respect to carbon monoxide consumption from which a first order rate constant of $k = 3 \times 10^{-3}$ sec^{-1} has been calculated. The temperature is probably 10^{o} - 25^{o} although it is not given.

There are reasons to believe that the rate of carbonyl exchange may not be first fast. Basolo and Wojcicki have measured the rate of exchange between dicobalt octacarbonyl and ^{14}C-carbon monoxide (5). They found that the exchange was first order with a rate constant of $1.3 - 1.9 \times 10^{-3}$ sec^{-1} at 0^{o}. They formulated a mechanism as follows:



They base this mechanism on the fact that the exchange is first order and the rate of exchange of the compound $Co_2(CO)_6(\phi C{=}C\phi)$, which contains no bridge carbonyls, is too slow to measure at 0^{o}($k = 9 \times 10^{-5}$ sec^{-1} at 25^{o}). Although the diphenylacetylene ligand on the above cobalt carbonyl compound is electron withdrawing, making loss of carbon monoxide more difficult, it does indicate that exchange of a non-bridging carbonyl may be slower than exchange of a bridging carbonyl.

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PROPOSITION II

It is known that coenzyme B_{12} is a hydrogen transfer agent in the methylmalonyl coenzyme A mutase reaction, while deaminated cobalamine coenzyme (5'-deoxyinosyl coenzyme B_{12}) does not function as a coenzyme in the reaction (1). The work in this thesis suggests that the 5'-deoxyadenosyl moiety becomes detached from the rest of the coenzyme B_{12} molecule during the reaction. To remain functional as a coenzyme, the two parts of the coenzyme B_{12} intermediate must remain spacially intact throughout the reaction, even if they are not directly bonded to each other.

The enzyme possibly serves as a matrix on which the two parts of coenzyme B_{12} are held together. If the enzyme does serve as a matrix, then the lack of coenzyme activity by 5'-deoxyinosyl coenzyme B_{12} can be explained as a lack of ability to bind to the enzyme during isomerization. In other words, the amine function of 5'-deoxyadenosine is required for bonding to the enzyme.

It is proposed that 4-^{3}H-methylmalonyl coenzyme A be incubated with an excess of 5'-deoxyinosyl coenzyme B_{12} and the mutase enzyme. If the matrix hypothesis is correct, and if the carbon-cobalt bond is actually broken during the reaction, then free 5'-deoxyinosine would be formed. Once hydrogen or tritium is transferred to the 5'-deoxyinosyl position, enzyme bound 5'-deoxyinosine would be formed. If the enzyme-5'-deoxyinosine binding is weak, then free 5'-deoxyinosine could be released. Since there is now no mechanism for transfer of hydrogen from the coenzyme back to the substrate, no isomerization of methylmalonyl coenzyme A to succinyl coenzyme A would take place, which is consistent with experimental observations.

Any 5'-deoxyinosine formed in this manner would contain tritium at the 5'-position. If radioactive 5'-deoxyinosine were detected and isolated as a product of the reaction, it would provide three important pieces of information:

1. When hydrogen (or tritium) is transferred to coenzyme B_{12}, the carbon-cobalt bond is broken to form enzyme-bound 5'-deoxyadenosine.

2. The primary amine group on the 5'-deoxyadenosyl moiety of coenzyme B_{12} is necessary for binding to the enzyme during isomerization (possibly ionically bound to a carboxyl group of aspartic or glutamic acid?).

3. The ratio of the specific activities in the 5'-deoxyinosine and the 4-^{3}H-methylmalonyl coenzyme A would provide a direct measurement of the tritium isotope effect for the hydrogen transfer. This isotope effect would be difficult to obtain by conventional methods.

The inability to detect free 5'-deoxyinosine in this experiment would indicate that some other factor is responsible for the lack of coenzyme activity with deaminated cobalamine coenzyme. One possible reason for the coenzyme inactivity is that the change from an amine to an oxo function results in a long-range electronic effect on the carbon-cobalt bond. To test this hypothesis, one could synthesize substituted coenzyme B_{12} compounds which still contained the primary adenosyl amine function, but which also contained an electron withdrawing group. By testing 2-fluoroadenosyl cobalamine coenzyme or 8-fluoroadenosyl cobalamine coenzyme as possible

coenzymes in the mutase reaction, one could determine if electron withdrawing groups have an effect on the carbon-cobalt bond during the isomerization of methylmalonyl coenzyme A to succinyl coenzyme A.

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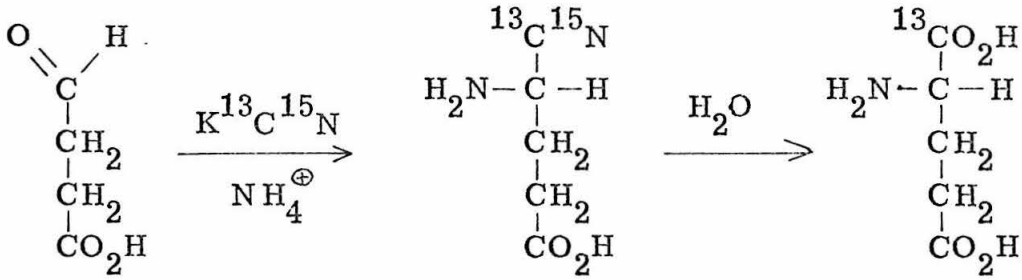
PROPOSITION III

An unidentified basidiomycete is known to metabolize hydrogen cyanide. Labelling experiments have shown that the hydrogen cyanide is incorporated principally into the two amino acids, alanine and glutamic acid (1). The mechanism of incorporation of hydrogen cyanide into glutamic acid involves the addition of ammonia and hydrogen cyanide to succinyl semialdehyde to form an amino nitrile. Subsequent hydrolysis of the nitrile yields the product, glutamic acid (2):

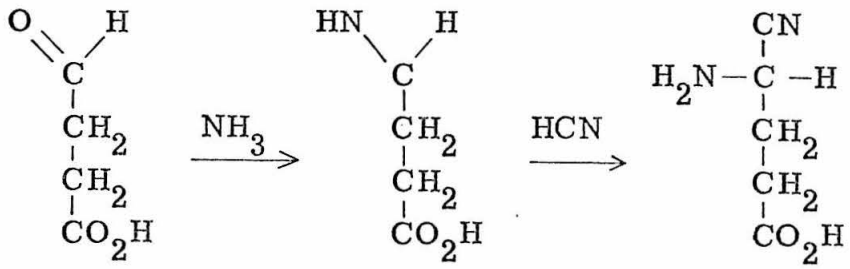


Isolation of labelled 4-amino-4-cyanobutyric acid and labelled glutamic acid have confirmed the above scheme. Use of uniformly labelled ^{14}C-succinyl semialdehyde has shown that succinyl semialdehyde is the precursor to the other four carbon atoms of glutamic acid.

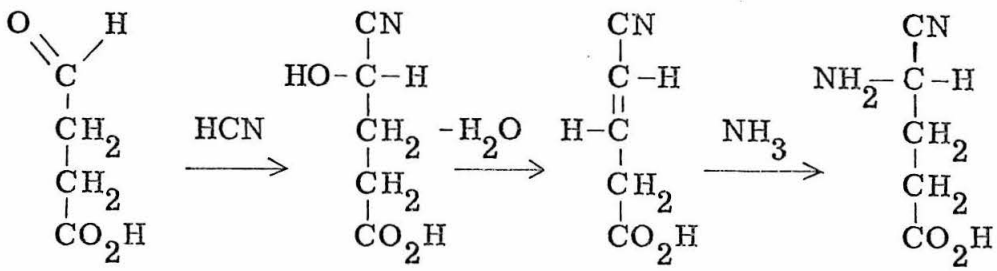
Three possible mechanisms are proposed for the formation of 4-amino-4-cyanobutyric acid, and labelling experiments are proposed in an attempt to distinguish between the mechanisms.

The following mechanisms are all analogous to known organic or enzymatic reactions:

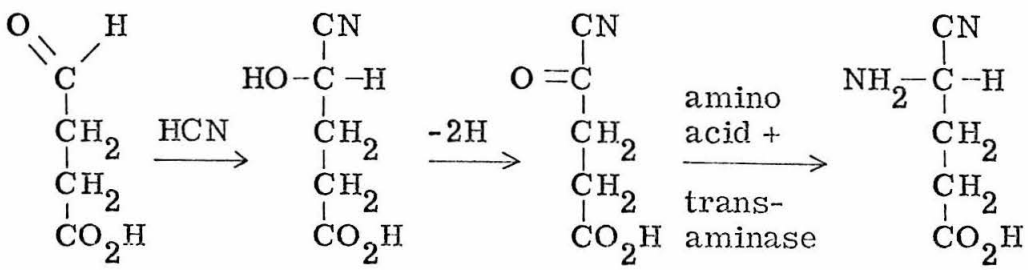
(a)



(b)



(c)



Mechanism (a) is analogous to the Strecker synthesis of amino acids (3), mechanism (b) is similar to the enzymatic synthesis of aspartic acid from fumaric acid (4), and mechanism (c) is similar to the synthesis of numerous amino acids by transamination (5).

If the enzymatic reaction were run separately with 1-^{3}H-succinyl semialdehyde and 2-^{3}H-succinyl semialdehyde, one could distinguish between these three possible mechanisms. If mechanism

(a) were correct, the tritium would be obtained in both reactions; if mechanism (b) were correct, the tritium would be retained starting with 1-^{3}H-succinyl semialdehyde, but one-half the tritium would be lost starting with 2-^{3}H-succinyl semialdehyde; and if mechanism (c) were correct, all the tritium would be lost starting with 1-^{3}H-succinyl semialdehyde, but none would be lost starting with 2-^{3}H-succinyl semialdehyde.

Mechanisms (b) and (c) both have succinyl cyanohydrin as an intermediate. If ^{14}C-labelled succinyl cyanohydrin were used as a substrate, the rate of product formation would tell whether or not the cyanohydrin actually could be an intermediate. For the cyanohydrin to be an intermediate in the reaction, the rate of product formation from cyanohydrin must be at least as fast as the rate of product formation from succinyl semialdehyde, ammonia, and hydrogen cyanide. A slower rate of reaction with the cyanohydrin would eliminate mechanisms (b) and (c) as possible pathways for the reaction.

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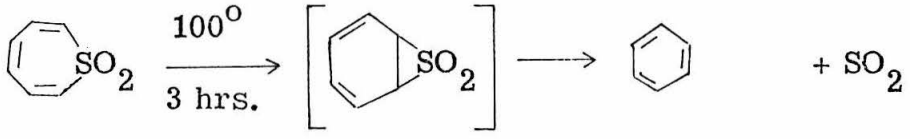
PROPOSITION IV

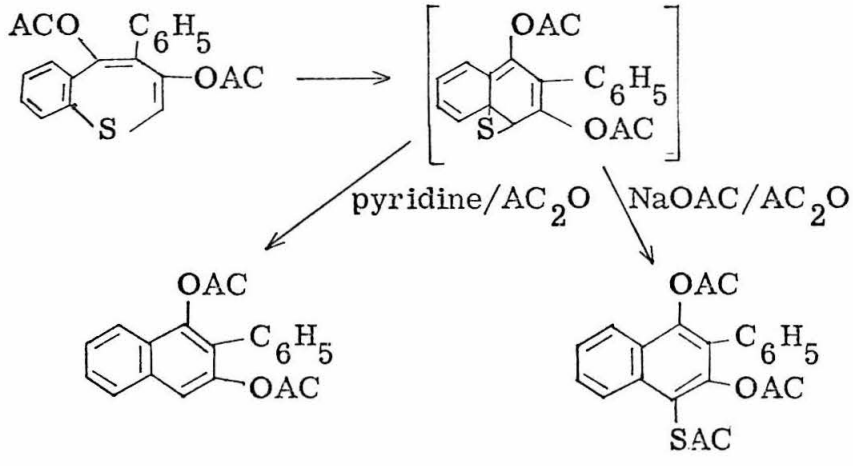
It is proposed that the properties of thiepin 1, 1-dioxide be studied by examination of the products formed by addition of tetracyanoethylene to thiepin 1, 1-dioxide and by low temperature photolysis of thiepin 1, 1-dioxide.

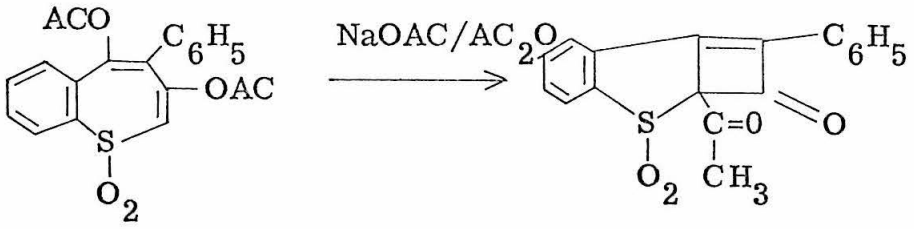
The thermal decomposition of thiepin 1, 1-dioxide to benzene and sulfur dioxide is believed to go through a norcaradiene-type intermediate (1):

(a) 

However, no evidence for the above intermediate has been obtained.

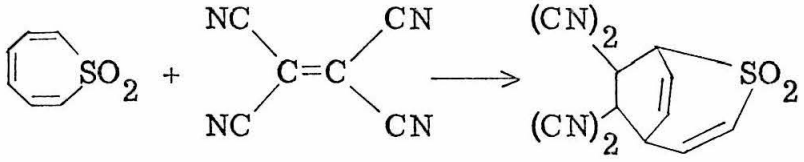
Substituted thiepin 1, 1-dioxide often follows a different reaction path than the corresponding substituted thiepin which probably goes through an episulfide intermediate. For example, 3, 5-diacetoxy-4-phenylbenzo[b]-thiepin and the corresponding 1, 1-dioxide give very different products upon refluxing in acetic anhydride (2):

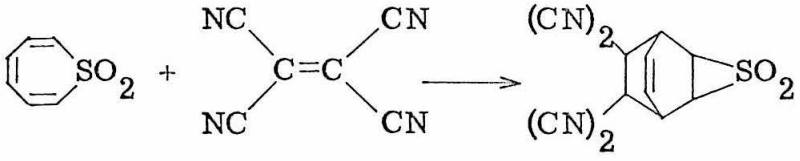
(b) 

(c) 

The product in (c) can be rationalized by assuming that the starting compound cannot form an intermediate episulfone analogous to the episulfide formed in (b).

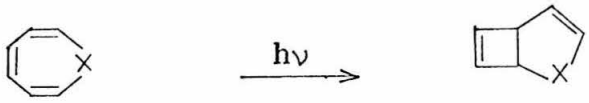
If thiepin 1, 1-dioxide is not in equilibrium with the tautomeric benzene episulfone, then addition of the Diels-Alder adduct, tetracyanoethylene, should give the unsymmetrical product shown in (d) rather than the symmetrical product shown in (e):

(d) 

(e) 

Precedence for both types of addition are known. Oxepin, which is known to be in equilibrium with benzene oxide, gives the symmetrical product (3), while 1-carbomethoxyazepine gives the unsymmetrical addition product (4).

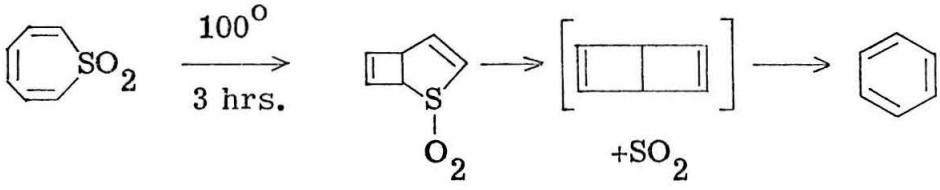
Cycloheptatriene, oxepin and N-substituted azepines are known to cyclyze in the same manner upon irradiation (4, 5):

(f) 

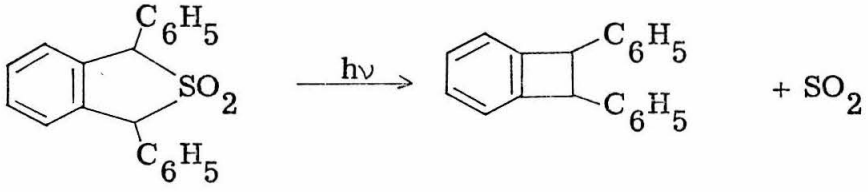
$x = CH_2,\ O,\ N\text{-}COOCH_3$

Photolysis of thiepin 1, 1-dioxide could give a cyclization product similar to the product in (f) with $x = SO_2$. However, it is possible that this product could undergo further reaction to give Dewar benzene and sulfur dioxide. If the photolysis were done at low temperature in a glass, any Dewar benzene formed could be detected.

The possibility of forming Dewar benzene and sulfur dioxide from thiepin 1, 1-dioxide leads to an alternate mechanism for the thermal decomposition of thiepin 1, 1-dioxide, which does not involve the benzene sulfone intermediate shown in (a):

(g) 

The photolytic elimination of sulfur dioxide from a five membered ring is not without precedence. The following sensitized photolysis has been reported in (5):

(h) 

The experiments suggested above would provide evidence for or against the existence of a thiepin 1, 1-dioxide-benzene sulfone equilibrium, and would indirectly give some information on the pathway of thermal decomposition of thiepin 1, 1-dioxide.

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PROPOSITION V

It is proposed that the Mössbauer spectrum of I^{129} placed in the two active sites of liver alcohol dehydrogenase may be able to determine if the two sites are conformationally identical.

Horse liver alcohol dehydrogenase is an enzyme with a molecular weight of about 84,000 (1). It has been shown to contain two zinc atoms/molecule (2) and is known to complex two NAD molecules/enzyme molecule (3). Inhibition studies using o-phenanthroline and other metal chelating agents indicate that the zinc ion is at or near the active site (4). Reaction with p-chloromercuribenzoate indicates that there are 26-28 free sulhydryl groups in the molecule (5).

The evidence indicates that each enzyme molecule contains two independent active sites. Degradation studies have shown this to be true. Iodoacetic acid and iodoacetamide react with only two of the 28 free sulfhydryl groups and lead to complete inactivation. When 1-^{14}C-iodoacetic acid was reacted with the enzyme followed by digestion with succinyltrypsin and separation of the peptides, all of the radioactivity was found in the following octapeptide (6).

$$\begin{array}{l} \qquad\qquad\qquad\quad SCH_2CO_2H \\ \qquad\qquad\qquad\quad | \\ \text{Met-Val-Ala-Thr-Gly-Ileu-Cys-Arg} \end{array} .$$

Thus, there are probably two active sites which have an identical primary sequence in the vicinity of the active site.

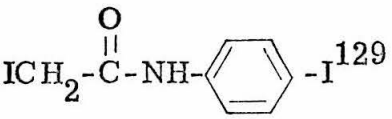
The work done in determining the mechanism of the dehydration of alcohols by alcohol dehydrogenase has always assumed that the active sites are completely identical. However,

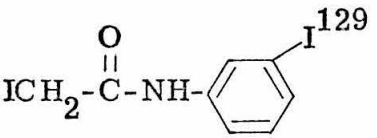
proof that the amino acid sequence at the active sites is identical does not necessarily show that the tertiary structure of the active sites are also identical.

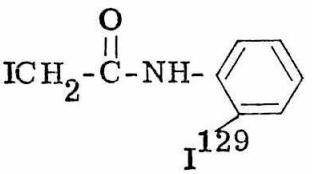
In order to determine if the tertiary structure of the two active sites are indeed identical I^{129} could be added to both of the active sites. The I^{129} Mössbauer spectrum could tell if all the I^{129} atoms are in the same electronic environment. Some of the following compounds may prove useful for incorporating the I^{129} into the active site:

1. 

2. 

3. 

4. 

5. 

Two criteria must be met by these compounds before the experiment can have any meaning. First, the I^{129} must be in the environment of the enzyme rather than sticking out into the solvent. This criteria will probably be met by some, but not all of the above

compounds. Second, the alkylation must not involve extensive denaturation of the protein, for if denaturation occurs then each I^{129} would be in a different environment. It is possible that small conformational changes take place during alkylation, but the fact that the alkylation is so specific, and occurs under physiological conditions (165 min. at 22°, pH 7.5 in 0.1 M phosphate buffer) indicates that major conformational changes are not taking place.

The Mössbauer spectra of either the crystalline, alkylated enzyme or solid solution of the alkylated enzyme containing I^{129} at the active site should show a minimum of eight peaks due to quadrapole splitting (+5/2 to +7/2 transition). If all of the I^{129} atoms are in the same environment there will be only eight peaks. However, if the two active sites have different geometries, then it is likely that there will be I^{129} atoms in two different electronic environments (for example, one may be able to form a hydrogen bond where the other cannot). In this case there will be between eight and sixteen Mössbauer peaks depending on the resolution.

The Mössbauer spectra taken of I^{129} compound have shown that small changes in the electron environment of the I^{129} can lead to measurable chemical shifts (7). For example, the chemical shifts of the alkali iodides are measurably different even though the iodide ion has a closed electron shell which should shield it from perturbations of the inner s-electrons.

Additional information on the nature of the electron environment in the active site near the I^{129} can be obtained by taking the Mössbauer spectra of the alkylating agents in a variety of solvents and comparing them to the Mössbauer spectrum of the alkylated enzyme. If the spectrum of the free alkylating agent is taken in solid solutions of benzene, imidazole, cyclohexane, acetic

acid, methanol and water, the spectrum which fits best with that obtained from the alkylated enzyme will give an indication of the type of functional group which is present near the I^{129} in the enzyme.

The above method can be extended to any enzyme that contains more than one active site and that can be specifically alkylated at the active site.

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