Methylmalonyl Isomerase: A Study of the Mechanism of Isomerization

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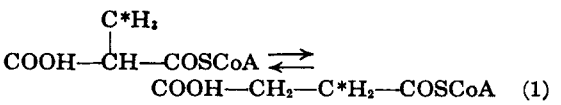
Received July 6, 1962

Methylmalonyl-CoA is isomerized to succinyl-CoA by a B_{12}-coenzyme dependent methylmalonyl isomerase and the rearrangement has been shown to occur with a shift of the CoA-carboxyl unit. However, it has not been determined whether the shift of the CoA moiety occurs by an *intra*molecular rearrangement, *i.e.* within the same molecule, or by an *inter*molecular mechanism. The data from this study show that the rearrangement occurs by an *intra*molecular shift of the CoA-carboxyl group. Intramolecularly doubly labeled methylmalonyl-CoA was synthesized with C^{13} in the CoA-carboxyl and the methyl carbons. Equal amounts of the labeled methylmalonyl-CoA and unlabeled methylmalonyl-CoA were combined and then converted to succinyl-CoA by methylmalonyl isomerase; the succinyl-CoA was then converted to butadiene for mass analysis. The mass analysis showed there was no change in the mass pattern from that of the methylmalonyl-CoA precursor, a finding which could only occur if the CoA-carboxyl group shifted *intra*molecularly.

The cobamide (B_{12}-coenzyme) dependent isomerization of methylmalonyl-CoA to succinyl-CoA is an important reaction in the metabolism of propionate in mammalian tissue and bacteria (Flavin *et al.*, 1955; Swick and Wood, 1960; Stadtman *et al.*, 1960; Stjernholm and Wood, 1961). It has been demonstrated by C^{14} labeling experiments (Eggerer *et al.*, 1960; Swick, 1962; Hegre *et al.*, 1962) that the isomerization occurs by a shift of the CoA-carboxyl unit to the methyl group (reaction 1). However, these studies do not show whether the rearrangement occurs by an *intra*molecular shift or an *inter*molecular shift



(1)

of the CoA-carboxyl group. Eggerer *et al.* (1960) have postulated that the role of the "B_{12}-coenzyme" is to create a free radical of methylmalonyl-CoA through oxidation-reduction of the cobamide cobalt; the CoA-carboxyl then shifts according to the mechanism proposed by Urry and Karasch (1944), presumably by an *intra*molecular rearrangement (Figure 1a). Barker and co-workers (Barker *et al.*, 1958; Weisbach

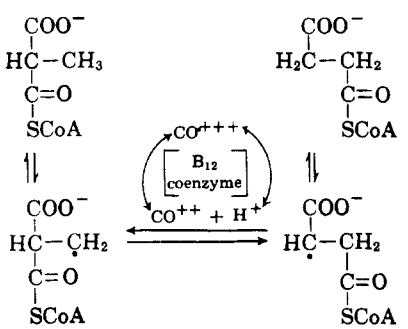


Figure 1a

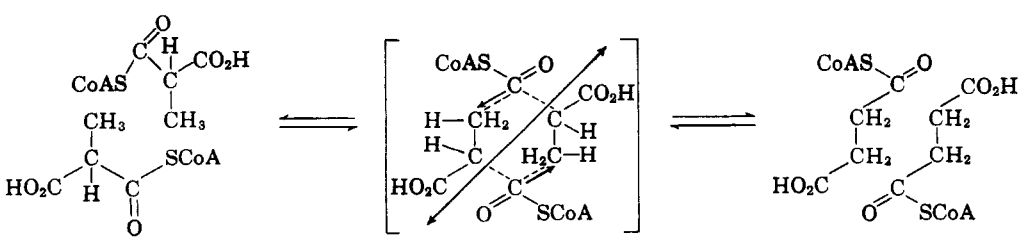


Figure 1b

FIG. 1.—Examples of *intra* and *inter*molecular mechanisms of methylmalonyl-CoA isomerization. Figure 1a depicts the free radical mechanism postulated by Eggerer *et al.* (1960) as an example of one type of *intra*molecular shift of the CoA-carboxyl within the molecule. Figure 1b is an example of one type of *inter*molecular shift of CoA-carboxyl groups (Hegre *et al.*, 1962).

TABLE I

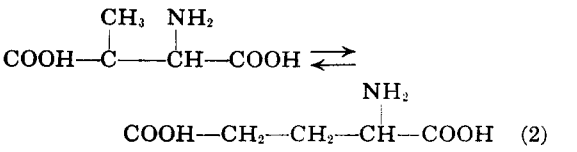
SAMPLE CALCULATION OF A PORTION OF THE THEORETICAL MASS SPECTRUM FOR DOUBLY LABELED C^{13} BUTADIENE

Propylene		Butadiene				Butadiene	
Mass	Proportion of Each Mass in a Single Unit	Propylene 1	Propylene 2	Propylene 3	Propylene 4	Proportion of Each Mass in a Single Unit	Mass
		NO C^{13}					
42	**0.413**	**0.989**	× **0.989**	× **0.422**	× 0.688	0.276	54
		1 C^{13}					
43	**0.574**	0.989	× 0.989	× 0.422	× 0.332	0.521	55
		0.011	× **0.989**	× **0.422**	× 0.668		
		0.989	× **0.011**	× **0.422**	× 0.668		
		0.989	× **0.989**	× **0.578**	× 0.668		
		2 C^{13}					
44	**0.013**	0.011	× 0.989	× 0.422	× 0.332	0.199	56
		0.989	× 0.011	× 0.422	× 0.332		
		0.989	× 0.989	× 0.578	× 0.332		
		0.011	× **0.011**	× **0.422**	× 0.668		
		0.011	× **0.989**	× **0.578**	× 0.668		
		0.989	× **0.011**	× **0.578**	× 0.668		
		3 C^{13}					
45	**0.7×10^{-4}**	0.011	× 0.011	× 0.422	× 0.332	0.42×10^{-2}	57
		0.011	× 0.989	× 0.578	× 0.332		
		0.989	× 0.011	× 0.578	× 0.332		
		0.011	× **0.011**	× **0.578**	× 0.668		
	Sum 1.000					1.000	—

Saying that a unit of butadiene is doubly labeled or singly labeled does not imply that two positions or one position, respectively, of the four carbons will be consistently labeled with C^{13}. Because there is C^{13} in normal carbon (1.1 atoms %) and because the C^{13} enriched sources such as CO_2 are not 100% C^{13}, it is then apparent that the progressive addition of normal or enriched sources of a one-carbon compound to form a two, three or four carbon compound will result in a spectrum of different combinations or mass values. For example the chance combination of normal carbon atoms (1.1% C^{13} and 99.9% C^{12}) to form a two-carbon compound will result in four different types (four possible combinations) of molecules: C^{13}-C^{13}, C^{13}-C^{12}, C^{12}-C^{13}, and C^{12}-C^{12}, the most prominent type being C^{12}-C^{12}. If a source of carbon atoms enriched with C^{13} (57.8% C^{13}, 42.2% C^{12}) is now added to each of the two-carbon species to create a three-carbon unit (arbitrarily referred to as propylene in this example), there will be eight possible types of three-carbon units; the proportion of each type with respect to mass value is obtained by grouping the different possibilities according to mass, successively multiplying the per cent C^{12} or C^{13} of each position within each possibility, and then summing to give the proportion of each mass in a single unit (see boldface type in above table). This process is then continued until the proper number of carbon atoms have been added to make the desired compound.

A sample calculation of the different types and proportions of butadiene which would result from a synthesis involving the addition of a C_1 compound containing 33.2 atoms % C^{13} to a singly labeled propylene with 57.8 atoms % C^{13} is also presented in this table. During the C_1 addition to these 8 types of propylene, the C^{13} atoms and the C^{12} atoms would react with each type of molecule, resulting in twice as many types of butadiene (2^4) as propylene. The mass ratios for the butadiene in this example are the theoretical values for butadiene, which is doubly labeled with 57.8 and 33.2 atoms % C^{13}. The proportion of masses 54–58 in unlabeled butadiene can be calculated by substituting 0.989 for 0.668 and 0.442 as well as 0.011 for 0.332 and 0.578. The mass patterns for the two species of labeled butadiene that would be encountered in the proposed *inter*molecular reaction can be obtained by substituting (1) 0.828 for 0.668 and 0.172 for 0.332 for the doubly labeled species 1.1 1.1 57.8 17.2
(C═C—C═C) and (2) 0.989 for 0.422, 0.011 for 0.578, 0.828 for 0.668 and 0.172 for 0.332 1.1 1.1 1.1 17.2
(C═C—C═C). The results of these calculations are given in Table III.

et al., 1960) have studied an analogous cobamide dependent reaction in which methyl aspartic acid is isomerized to glutamic acid (reaction 2), but again the nature of the molecular mechanism is unknown.



As defined here an *inter*molecular reaction would be said to occur if there were a complete separation of the CoA-carboxyl from the residual propionic acid moiety and if there were random recombination of the CoA-carboxyl and the three-carbon fragment. Hegre *et al.* have suggested that this type of *inter*molecular CoA-carboxyl transfer might occur by a concerted mechanism as shown in Figure 1b. By the use of C^{13} and mass analysis similar to that used by Wood (1952) and Pomerantz (1958), it is possible to demonstrate whether the molecular rearrangement occurs by an *intra*molecular (Fig. 1a) or an *inter*molecular mechanism (Fig. 1b).

Theoretical Approach to Problem.—*Intra*molecularly doubly labeled methylmalonyl-CoA was synthesized with C^{13} in both the CoA-carboxyl and the methyl positions. Equal quantities of labeled and unlabeled methylmalonyl-CoA were then mixed and converted to succinyl-CoA by methylmalonyl isomerase, a conversion whose equilibrium is 10 to 1 in favor of succinyl-CoA (Stjernholm and Wood, 1961). After termination of the reaction, the succinyl-CoA was isolated as succinic acid; the succinic acid was then converted to butadiene in order to remove the oxygen with its various isotopes and to obtain a gas which is convenient for mass analysis. The feasibility of this type of experiment is dependent on the differentiation of two mass patterns, one for an *intra*molecular reaction and another for an *inter*molecular reaction. The type of butadiene mass patterns for each of the proposed mechanisms using *intra*molecularly doubly labeled (C^{13}) methylmalonyl-CoA can be calculated, thus enabling one to predict if the labeled compounds utilized will be adequate for the experimental design. An example of such a calculation for a butadiene containing 57.8 and 33.2 atoms % C^{13} in the two labeled positions and the normal complement of C^{13} (1.1 atoms %) in each of the other two carbons is represented in Table I. The proportions of butadiene with different possible masses are shown on the right side of Table I. These are the theoretical values for a unit of butadiene formed by a doubly labeling process with 57.8 and 33.2 atoms % respectively.

Since the theoretical basis for this experiment is dependent on whether the three-carbon propionyl moiety separates from the CoA-carboxyl group, the sample calculation in Table I has been developed to show how the mass proportions of the three-carbon unit can be derived; for purposes of discussion this three-carbon unit may be considered as propylene.

If the CoA-carboxyl group is never separated from the propionyl unit of methylmalonyl-CoA during the isomerization (*intra*molecular) then the mass pattern of butadiene derived from an equal mixture of unlabeled methylmalonyl-CoA and methylmalonyl-CoA prepared by a doubly labeling process (33.2 atoms % C^{13} in the CoA-carboxyl and 57.8 atoms % C^{13} in one position of the propionyl group) would be equal to the average of the respective mass proportions for each species (Table III). The calculated mass proportions for the doubly labeled and unlabeled species of butadiene are given in Table II.

If the methylmalonyl-CoA were converted to succinyl-CoA by an *inter*molecular reaction as depicted in Figure 1b, the specific activity of the C^{13} in the CoA-carboxyl group of the doubly labeled molecules would be diminished by 50%. In effect this would decrease the absolute number of doubly labeled molecules and increase the number of singly labeled molecules, thus creating a mixture of succinyl-CoA (as butadiene) which has an entirely different but predictable mass spectrum from that found for an *intra*molecular reaction. This mixture of butadiene would be comparable to equal quantities of (1) a doubly labeled butadiene species with 57.8 atoms % C^{13} in one position of the propylene unit and 17.2 atoms % C^{13} (1.1 + 33.2/2 = 17.2) in the carbon corresponding to the CoA-carboxyl group, and (2) a singly labeled species with 17.2 atoms % C^{13} in the carbon corresponding to the CoA-group. The mass proportion for each of these two species was calculated according to the example in Table I and the results are given in Table II. The predicted mass spectrum for this type of mechanism would be equal to the average of the respective mass proportions obtained when equal amounts of each of the latter two species are mixed (Table III). It is seen that the values calculated for an *inter*molecular mechanism differs from those for the *intra*molecular mechanism. Therefore, the two types of mechanisms can be differentiated by mass analysis using labeled compounds comparable to those discussed in these sample calculations.

Experimental Procedures

Synthesis of Doubly Labeled Methylmalonyl-CoA.—Doubly labeled 2-methylmalonyl-CoA was prepared by the following reactions:

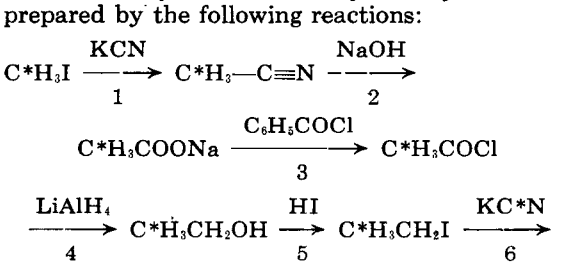


TABLE II

A SUMMARY OF THE PROPORTIONS OF MASSES 54–58 FOR EACH OF THE SPECIES OF BUTADIENE WHICH MIGHT BE ENCOUNTERED IN EITHER AN *Inter*MOLECULAR OR AN *Intra*MOLECULAR REACTION

Mass	Proportion					Sum
	54	55	56	57	58	
Unlabeled butadiene 1.1 1.1 1.1 1.1[a] C═══C——C═══C	0.957	0.042	0.0007	5×10^{-6}	1×10^{-8}	1.0
Doubly labeled butadiene 1.1 1.1 57.8 33.2 C═══C——C═══C	0.276	0.521	0.191	4.2×10^{-3}	0.2×10^{-4}	1.0
Singly labeled butadiene 1.1 1.1 1.1 17.2 C═══C——C═══C	0.801	0.193	0.6×10^{-2}	0.8×10^{-4}	0.2×10^{-6}	1.0
Doubly labeled butadiene 1.1 1.1 57.8 17.2 C═══C——C═══C	0.342	0.547	0.109	0.2×10^{-2}	0.1×10^{-4}	1.0

[a] Numbers over the carbon atoms denote atoms % C^{13} at that position.

These data are obtained according to the sample calculation in Table I: the values for doubly labeled butadiene (57.8 atoms % C^{13} and 33.2 atoms % C^{13}) were taken directly from Table I.

$$C^*H_3CH_2—C^*\equiv N \xrightarrow[7]{NaOH} C^*H_3CH_2—C^*OONa$$

$$\xrightarrow[\text{propionyl kinase}]{\text{ATP, } Mg^{++}} C^*H_3—CH_2—C^*OPO_3H_2$$
8

$$\xrightarrow[\text{phosphotransacetylase}]{\text{CoASH}} C^*H_3—CH_2—C^*OSCoA$$
9

$$\xrightarrow[\text{propionyl carboxylase}]{\text{ATP, } Mg^{++}} C^*H_3—\underset{\displaystyle COOH}{\underset{|}{CH}}—C^*OSCoA$$

where * = positions enriched with C^{13}.

Methyl iodide (30 mmoles) containing 56.7 atoms % excess C^{13} was reacted with potassium cyanide and converted to sodium acetate (Little and Bloch, 1950; Sakami, 1955). One-half of the sodium acetate (12 mmoles) was fused in a stream of dry nitrogen in the presence of 30 ml benzoyl chloride; the sodium acetate was converted to acetyl chloride and distilled into a trap cooled by cellosolve and solid CO_2 (Sakami, 1955). The acetyl chloride was mixed with 10 ml cold redistilled dry diethyl carbitol. An excess of lithium aluminium hydride (0.6 g) was dissolved in 18 ml of diethyl carbitol and added slowly to the acetyl chloride at 4°. The reaction flask was allowed to come to room temperature and redistilled *n*-butyl carbitol was added. The flask was connected to an air-cooled distillation column which in turn was connected to a trap cooled in solid CO_2 and cellosolve. The system was intermittently flushed with nitrogen and heated to boiling several times. The trap containing the ethanol was placed into another train; the alcohol was volatilized and carried with nitrogen gas through boiling 50% HI. HI fumes were removed by a bead tower containing 20% $CdCl_2$ and 20% $BaCl_2$ and the ethyl iodide was collected in a dry ice trap. An aliquot of the ethyl iodide was converted to ethanol by refluxing with Ag(OH) suspension; the alcohol was separated by distillation and oxidized to acetic acid with a chromic acid solution (Van Slyke and Folch, 1940) and purified by steam distillation and chromatography on a Celite column (Swim and Krampitz, 1950). The sodium acetate was degraded and the C^{13} content in each carbon atom was determined. The methyl position was found to contain 32.2 atoms % excess. The remaining ethyl iodide was reacted with KCN (10% excess) containing 32.2 atoms % excess C^{13} (Little and Bloch, 1950; Sakami, 1955). The propionyl nitrile was hydrolyzed and the sodium propionate was purified by steam distillation and Celite column chromatography (Swim and Krampitz, 1950). The sodium propionate 1,3-C^{13} was then converted to methylmalonyl CoA without isolation of intermediates by a series of reactions (reactions 8, 9, 10) catalyzed by purified enzymes. The reaction mixture contained 1.44 mmoles of sodium propionate, 200 units phosphotransacetylase, 45 units of propionyl carboxylase, 20 mmoles ATP, 2.0 mmoles $MgCl_2$, 5 mmoles $KHCO_3$, 0.4 mmole reduced glutathione, 1.35 mmoles CoASH, 0.8 mmole Tris-HCl buffer *p*H 7.4, and sufficient H_2O to bring the volume to 80 ml. A unit of enzyme is equivalent to an amount which converts 1 μmole of substrate per minute. Pyruvic kinase (45 units) and 1.5 mmoles phosphoenolpyruvate were added to serve as an ATP-generating system for the propionyl carboxylase to favor the formation of methylmalonyl CoA. The final *p*H was 7.2. After 120 minutes of incubation at 30°, 0.86 mmole of methylmalonyl CoA was formed. The reaction mixture was treated with $HClO_4$ (6%, v/v), and held at 4° for 3 hours, after which time the *p*H was adjusted to 6.8 with 10 N KOH. The $KClO_4$ was

TABLE III

THEORETICAL RELATIONSHIPS OF MASSES 54–57 EXPECTED FOR SUCCINYL-CoA (AS BUTADIENE) OBTAINED FROM THE C^{13} LABELED METHYLMALONYL-CoA BY EITHER AN *Inter*MOLECULAR OR AN *Intra*MOLECULAR ISOMERIZATION

Butadiene	Mass Proportions 54	55	56	57	Mass Ratios 55/54	56/54	57/54
*Intra*molecular Reaction							
Unlabeled 50% C=C—C=C	0.479	0.021	0.0004	2×10^{-6}			
Doubly labeled 28.4% C(57.8)=C—C(33.2)=C	0.078	0.148	0.057	0.001			
Singly labeled 21.6% C=C—C(33.2)=C	0.137	0.073	0.0023	0.9×10^{-5}			
Sum of Mass Proportions	0.697 +	0.243 +	0.059 +	0.001 = 1.0			
Ratios					0.348	0.085	0.002
*Inter*molecular Reaction							
Doubly labeled 28.4% C(57.8)=C—C(17.2)=C	0.0970	0.155	0.031	0.06×10^{-2}			
Singly labeled 71.6% C=C—C(17.2)=C	0.573	0.138	0.4×10^{-2}	0.6×10^{-4}			
Sum of Mass Proportions	0.670 +	0.293 +	0.035 +	0.07×10^{-2} = 1.0			
Ratios					0.437	0.053	0.001

The doubly labeled methylmalonyl-CoA contained a considerable amount of molecules which was formed by a single labeling process. This occurred because ethyl alcohol was derived from the diethyl carbitol used in the synthesis. The methyl iodide which was used in the synthesis contained 56.7 atoms % excess C^{13}, but the resulting ethyl iodide contained 32.1 atoms % excess in the methyl group. The ethyl iodide therefore consisted of 56.8% (32.1/56.7) molecules which were derived from the methyl iodide and 43.2% which were derived from the diethyl carbitol. The mixture of the two types of methylmalonyl-CoA in experiment A (see text) contained 50% unlabeled molecules and 50% labeled molecules, 21.6% of which were formed by a singly labeling process CH_3 (33.2) (COOH—C—COSCoA) and 28.4% of which were formed by a double labeling process (COOH-CH-COSCoA). The mass pattern of this mixture would be unchanged by an *intra*molecular isomerization, and thus a summation of the fractions of each mass proportion of each species (from Table II) should represent the mass proportions found in the butadiene from experiment A if an *intra*molecular mechanism occurred. If, however, the mechanism occurred by an *inter*molecular mechanism then there would be two species, and 28.4% of the molecules would be comparable to a species formed by a doubly labeling process with 57.8 and 17.2 atoms % C^{13} in the two labeled positions and the rest (71.6%) would be comparable to butadiene formed by a singly labeling process. Summation of the fractions of each mass proportion for each of these latter two species (from Table II) would represent the mass proportions of the butadiene from experiment A if an *inter*molecular mechanism had occurred.

removed by filtration through Whatman No. 5 filter paper and the solution of methylmalonyl CoA was stored at 4°. Unlabeled methylmalonyl CoA was made by the same method starting with unlabeled sodium propionate. The quantity of methylmalonyl CoA produced was assayed with purified transcarboxylase and malic dehydrogenase in a system containing DPNH, pyruvate, and Tris-HCl buffer as described by Wood and Stjernholm (1961).

Conversion of Methylmalonyl CoA to Succinyl-CoA.—The methylmalonyl CoA was incubated with methylmalonyl isomerase (specific activity 7.5) (Stjernholm and Wood, 1961) and methylmalonyl racemase (specific activity 31) (Allen *et al.*, 1962), neither of which contained transcarboxylase or CoA transferase. Specific activity is defined as units of enzyme per mg protein. Three experiments were performed: (A) 275 μmoles of unlabeled methylmalonyl CoA, 275 μmoles of the labeled methylmalonyl CoA, 0.92 μmoles of 5,6-dimethylbenzimidazoylcobamide, 144 units of methylmalonyl isomerase, 141 units methylmalonyl racemase, and 200 μmoles Tris-HCl buffer *p*H 7.4 were combined in a final volume of 139 ml (final *p*H 7.2); (B) 340 μmoles unlabeled methylmalonyl CoA, 0.6 μmole 5,6-dimethylbenzimidazoylcobamide, 93 units methylmalonyl isomerase, 91 units methylmalonyl racemase, and 200 μmoles Tris-HCl buffer *p*H 7.4 were combined in a final volume of 60 ml (final *p*H 7.2); (C) 403 μmoles of the labeled methylmalonyl CoA, 0.72 μmoles 5,6-dimethylbenzimidazoylcobamide, 111 units of methylmalonyl isomerase, 111 units of methylmalonyl racemase, and 200 μmoles of Tris-HCl buffer *p*H 7.4 were

combined in a final volume of 82 ml (final *p*H 7.2). The mixtures were incubated at 30° and the progress of the reactions was followed by determining the disappearance of methylmalonyl CoA. The equilibrium of the reactions favors the formation of succinyl CoA, and after 100 minutes of incubation there was no further decrease in the methylmalonyl CoA. Each flask was titrated to *p*H 13 with 2 N KOH and heated in a boiling water bath for 30 minutes to deacylate the CoA derivatives. The contents of each were then cooled, the *p*H was adjusted to 1, and the mixture was extracted continuously with ether for 72 hours. The acids in the extracts were titrated and the solution was evaporated to dryness and the succinic acid was isolated by chromatography on Celite columns (20 g) (Swim and Krampitz, 1950). From experiments A, B, and C, 550 μmoles, 286 μmoles, and 341 μmoles of succinate were obtained, respectively. The sodium succinate samples were converted to the free acid by passage through 0.5 × 3 cm columns of Dowex 50X, H^+ resin and were evaporated to dryness. The succinic acid was sublimed as a final purification step and the amounts recovered were (A) 360 μmoles, (B) 254 μmoles, (C) 277 μmoles. A portion of succinic acid from experiment A and a mixture of equal parts of the succinic acid from experiments B and C were converted to butadiene for mass analysis. The butadiene from the combined samples B and C should give the same mass analysis as that which would be obtained if the methylmalonyl CoA of experiment A were converted to succinyl CoA by an *intra*molecular reaction.

Conversion of Succinic Acid to Butadiene.—Succinic acid was converted to butadiene for mass analysis by the following series of reactions:

$$\text{COOH—CH}_2\text{CH}_2\text{—COOH} \xrightarrow{\text{CH}_2\text{N}_2} \text{CO(OCH}_3\text{)—CH}_2\text{—CH}_2\text{—CO(OCH}_3\text{)}$$

$$\xrightarrow{\text{LiAlH}_4} \text{CH}_2\text{OH—CH}_2\text{CH}_2\text{—CH}_2\text{OH}$$

$$\xrightarrow[\Delta]{\text{HI}} \text{CH}_2\text{I —CH}_2\text{—CH}_2\text{—CH}_2\text{I}$$

$$\xrightarrow{\text{N(CH}_3\text{)}_3} \text{I(CH}_3\text{)}_3\text{N—CH}_2\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—N(CH}_3\text{)}_3\text{I}$$

$$\xrightarrow[200°]{\text{AgOH}} \text{CH}_2\text{=CH—CH=CH}_2$$

Diazomethane was prepared from *N*-methyl-*N'*-nitro-*N*-nitrosoquanidine and dissolved in anhydrous diethyl ether. An excess of the anhydrous diethyl ether solution of diazomethane was added to two tubes, one containing 200 μmoles of succinic acid from experiment A and the other 100 μmoles from each of the experiments B and C. The reaction was carried out at 4° with intermittent swirling of the mixture. Methylation was considered to be complete when all the succinic acid had dissolved.

Two ml of a 1 M diethyl ether solution of $LiAlH_4$ was added slowly to each of the two tubes at 4°. The contents were mixed by gentle swirling and held at room temperature for 20 minutes. Ten ml of wet diethyl ether was added to each of the tubes followed by 10 ml of H_2O. The ether layer was removed by an air jet and the contents of each tube passed through a double column of Dowex 50 X 8 (H^+) and Duolite A-4. The eluates were evaporated almost to dryness and then refluxed with 0.5 ml of 50% HI for 3 hours. The reflux condenser was rinsed with H_2O. The diiodobutane separated from solution and the excess HI was decanted. The diiodobutane was washed twice with 2 ml of H_2O and was then converted to tetramethylene-bis(trimethyl-ammonium iodide) by a modification of the method of Fuoss and Chu (1951). Four ml of 25% trimethylamine in methanol was added to each flask and the contents were refluxed for 8–9 hours. The mixture was evaporated to dryness and the residue was dissolved in 1 ml of hot ethyl alcohol (97%) and filtered hot through a fine sintered glass funnel; crystallization occurred immediately after filtration. The compound was isolated and recrystallized from 97% ethyl alcohol. The crystalline bis-quaternary ammonium iodide derivative was mixed with moist freshly prepared AgOH and converted to butadiene by pyrolysis; the flask containing the reactants was placed at the head of a train containing, in succession, a 1 N H_2SO_4 wash tower, a $CaCl_2$ drying tube, and a trap cooled in liquid nitrogen. The train was flushed with CO_2-free helium for 10 minutes and then the reaction vessel was heated for 30 minutes in a 200° oil bath during continuous flushing with helium. The butadiene was identified and found to be pure by gas chromatography and mass spectrometry. The synthetic samples were compared with a known sample of butadiene (Phillips Petroleum Co.).

Mass Analysis of Butadiene.—A model 21-130 Consolidated Electrodynamics mass spectrometer was used. The temperature of the inlet system was 20° and an ionizing current of 20 μamperes was used. The pressure of the gas in the inlet system was 25 μ of Hg. The mass spectrum of each gas sample was obtained from mass 14 through 100 by a direct recording of the galvanometric deflections. Ratios of various masses could be obtained from this recording or by use of the isotope ratio accessory unit.

In the ionization chamber of the mass spectrometer, the electron beam not only converts the butadiene to positive ions but also fragments some of the molecules. This fragmentation causes a loss of one or more units of hydrogen and or carbon. Only the loss of hydrogen need be considered in this study, and the positive ions dealt with were as follows:

Non-fragmented molecular species		Fragmented molecular species
58	$H_2C^{13}=C^{13}H-C^{13}H=C^{13}H_2$	$H_2C^{13}=C^{13}H-C^{13}H=C^{13}H_2-1$ H
57	$H_2C^{13}=C^{13}H-C^{13}H=C^{12}H_2$	$H_2C^{13}=C^{13}H-C^{13}H=C^{13}H_2-2$ H
56	$H_2C^{13}=C^{13}H-C^{12}H=C^{12}H_2$	$H_2C^{13}=C^{13}H-C^{13}H=C^{12}H_2-1$ H
55	$H_2C^{13}=C^{12}H-C^{12}H=C^{12}H_2$	$H_2C^{13}=C^{13}H-C^{13}H=C^{13}H_2-3$ H
		$H_2C^{13}=C^{13}H-C^{13}H=C^{12}H_2-2$ H
		$H_2C^{13}=C^{13}H-C^{12}H=C^{12}H_2-1$ H
54	$H_2C^{12}=C^{12}H-C^{12}H=C^{12}H_2$	$H_2C^{13}=C^{13}H-C^{13}H=C^{13}H_2-4$ H
		$H_2C^{13}=C^{13}H-C^{13}H=C^{12}H_2-3$ H
		$H_2C^{13}=C^{13}H-C^{12}H=C^{12}H_2-2$ H
		$H_2C^{13}=C^{12}H-C^{12}H=C^{12}H_2-1$ H

It is necessary to correct the values observed on the mass spectrometer for contributions by loss of hydrogen from $H_2C^{13}=C^{13}H-C^{13}H=C^{12}H_2$, $H_2C^{13}=C^{13}H-C^{12}H=C^{12}H_2$ and $H_2C^{13}=C^{12}H-C^{12}H=C^{12}H_2$ before comparison can be made with the theoretical values as presented in Table II. The relative proportion of masses 54 ($H_2C^{13}=C^{12}H-C^{12}H=C^{12}H_2$), 53 ($H_2C^{12}=C^{12}H-C^{12}H=C^{12}H_2$ – 1 H), 52 ($H_2C^{12}=C^{12}H-C^{12}H=C^{12}H_2$ – 2 H), and 51 ($H_2C^{12}=C^{12}H-C^{12}H=C^{12}H_2$ – 3 H) was determined for unlabeled butadiene, and these values were used to calculate the correction factors for the removal of 1 H, 2 H, or 3 H atoms as described by Wood (1952). The relative amount of mass 58 is too small to be measured accurately.

Enzyme Preparation

Propionyl Carboxylase.—The enzyme preparation used in these experiments had a specific activity of 3 and was a gift of Dr. Kaziro and Dr. Ochoa.

Methylmalonyl Isomerase.—The preparation used in this experiment had a specific activity of 7.5 and was free of transcarboxylase and CoA transferase. It was prepared from *Propionibacterium shermanii* according to the method described by Stjernholm and Wood (1961).

Phosphotransacetylase.—This enzyme was prepared from an extract of *P. shermanii*. It was absorbed on DEAE cellulose and then eluted with 0.1 M phosphate buffer *p*H 6.8. The 45–65% $(NH_4)_2SO_4$ fraction of this eluate was absorbed on a TEAE cellulose column and eluted with a gradient phosphate buffer *p*H 6.8 (0.03 → 0.3 M). A 45–55% $(NH_4)_2SO_4$ fraction of the eluate had a specific activity of 27 and contained no transcarboxylase or CoA transferase.

Propionyl Kinase.—This enzyme was also purified from *P. shermanii* by chromatography on DEAE cellulose and $(NH_4)_2SO_4$ fractionation. The preparation used in this experiment had a specific activity of 320 and sedimented as a homogenous fraction in the analytical ultracentrifuge.

Pyruvic Kinase.—The enzyme was obtained from The California Corporation for Biochemical Research. It was a crystalline preparation with a specific activity of 90.

Results and Discussion

Thus far studies on the mechanism of the methylmalonyl isomerase have demonstrated that the rearrangement occurs by a shift of the CoA-carboxyl unit to the methyl group of methylmalonyl-CoA (Eggerer *et al.*, 1960; Swick, 1962; Hegre *et al.*, 1962). It has not been known whether this group shifts within the same molecule (*intra*molecular, Figure 1a) or becomes separated from the propionate moiety of the methylmalonyl-CoA, and then rejoins a different three-carbon unit to form succinyl-CoA (*inter*molecular shift, Figure 1b). The differentiation of an *inter*molecular versus an *intra*molecular shift of the CoA-carboxyl cannot be accomplished by the usual tracer methods. However, by mass analysis it is possible to differentiate between these two mechanisms. The experimental data are shown in Table IV. Theoretical mass ratios have been calculated that would be expected from either an *intra*molecular reaction or an *inter*molecular reaction. It is seen that the corrected values of experiment A more nearly agree with the theoretical values for the *intra*molecular reaction than they do with those for the *inter*molecular reaction, but the agreement is not complete. The correction of the experimental mass spectrometer values is quite large and could be in error due to a C^{13} isotope effect on the fragmentation of the butadiene. The theoretical ratios are of value in showing the relative magnitude of the masses which might be expected from *intra*- and *inter*molecular reactions, but the theoretical ratios are only approximations of the mass values to be expected experimentally. The comparison between the butadiene of experiment A and the butadiene from the mixture of experiments B and C is the most reliable criterion because the data were derived by the same experimental procedure and no correction of the mass spectrometer reading is required for the comparison. The butadiene derived from a mixture of equal amounts of the two succinyl-CoA samples from experiments B and C may be considered the standard for an *intra*molecular reaction. This is true since this mechanism should give the same result as the separate conversion of the two types of molecules, *i.e.*, no interaction of the two types. The 55/54 ratio of experiment A is somewhat lower (5.7%) than the 55/54 ratio of B and C, whereas judging

TABLE IV

EXPERIMENTAL MASS RATIOS FOR THE BUTADIENE DERIVED FROM SUCCINYL-CoA OBTAINED FROM EXPERIMENTS A, B, AND C

Mass Ratio	Succinyl CoA from Expt. A (Butadiene)		Equal Parts of Succinyl CoA From Expts. B and C		Theoretical Ratios (See Table I)	
	Experimental Value	Corrected Value	Experimental Value	Corrected Value	Intra	Inter
55/54	.335	.358	.355	.385	.348	.437
56/54	.075	.094	.079	.100	.085	.053
57/54	.002	.002	.002	.002	.002	.001

In experiment A succinyl-CoA was obtained by the action of methylmalonyl isomerase and methylmalonyl racemase on a mixture of equal parts of labeled and unlabeled methylmalonyl-CoA. In experiments B and C, the labeled and unlabeled methylmalonyl-CoA samples were converted to succinyl CoA separately and then mixed. If the reaction catalyzed by methylmalonyl isomerase occurs by an *intra*molecular mechanism the mass ratios should be the same from the mixture as from succinyl CoA of experiment A. The theoretical ratios for the *intra*- and *inter*molecular mechanism are shown for comparison.

from the theoretical ratios it should have been higher if an *inter*molecular reaction had occurred. The 56/54 ratio of A is 4.6% lower than B and C but by an *inter*molecular reaction, as estimated from the theoretical value, it should have been 38% lower. The results thus show quite conclusively that the isomerization did not involve an *inter*molecular reaction.

Hegre *et al.* suggested an intermolecular thiolester carbonyl transfer based on a concerted mechanism as shown in Figure 1b, although the data presented by these investigators could be consistent with either an *intra*- or an *inter*molecular shift. The mechanism of Eggerer *et al.* (1960) involving oxidation and reduction of the cobalt of the cobamine is in accord with our results, which indicate an *intra*molecular reaction (see Figure 1a). However, Barker (1961) has suggested two reasons why the cobamide may not function in the way suggested by Eggerer *et al.* (1960): (1) the cobalt in the active coenzyme is in the divalent state and is buried deep within the molecule; and (2) in the glutamate isomerase reaction (reaction 2) there is no incorporation of deuterium from the medium into the glutamate during the rearrangement. The exact role of the B_{12} coenzyme in the isomerization remains to be established, but, whatever the role, the mechanism of isomerization appears to occur by an *intra*molecular reaction.

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