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*METHYLMALONYL ISOMERASE, II. PURIFICATION AND PROPERTIES OF THE ENZYME FROM PROPIONIBACTERIA**

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Evidence has been presented[1] that the formation of propionic acid from pyruvate by the propionibacteria occurs by the following reactions:

$$\text{pyruvate} + \text{methylmalonyl CoA} \rightleftarrows \text{oxaloacetate} + \text{propionyl CoA} \quad (1)$$

$$\text{oxaloacetate} + 4\ \text{H} \rightarrow \text{succinate} \quad (2)$$

$$\text{succinate} + \text{propionyl CoA} \rightleftarrows \text{succinyl CoA} + \text{propionate} \quad (3)$$

$$\text{succinyl CoA} \rightleftarrows \text{methylmalonyl CoA} \quad (4)$$

$$\text{Sum:} \quad \text{pyruvate} + 4\ \text{H} \rightarrow \text{propionate}$$

Reaction (1) is catalyzed by methylmalonyl-oxaloacetic transcarboxylase; reaction (2) involves the reduction of oxaloacetate to malate, conversion to fumarate, and reduction to succinate and is coupled with the oxidation of pyruvate to acetate and CO_2; reaction (3) is catalyzed by propionyl CoA transferase[2] and reaction (4) by methylmalonyl isomerase. The methylmalonyl CoA is regenerated in this sequence and is recycled. These reactions are very similar to those described by Flavin *et al.*[3] in their studies of the reverse process, i.e., the utilization of propionate by animal tissue. This conversion occurs as illustrated below:

$$\text{propionyl CoA} + CO_2 + \text{ATP} \rightleftarrows \text{methylmalonyl CoA} + \text{ADP} + P_i \quad (5)$$

$$\text{methylmalonyl CoA} \rightleftarrows \text{succinyl CoA} \quad (4)$$

$$\text{succinyl CoA} + \text{propionate} \rightleftarrows \text{succinate} + \text{propionyl CoA} \quad (3)$$

$$\text{succinate} \rightleftarrows \text{oxaloacetate} + 4\ \text{H} \quad (2)$$

$$\text{oxaloacetate} \rightleftarrows \text{pyruvate} + CO_2 \quad (6)$$

$$\text{Sum:} \quad \text{propionate} + \text{ATP} \rightarrow \text{pyruvate} + 4\ \text{H} + \text{ADP} + P_i$$

The main difference of this sequence from the previous sequence is the fixation of

CO_2 in reaction (5) instead of transcarboxylation in reaction (1). Even these differences may not exist, since it is quite likely that reaction (5) also occurs in bacteria and reaction (1) in animals.[1]

Interest in methylmalonyl isomerase was greatly stimulated when it was found that coenzymes containing vitamin B_{12} derivatives are required for this reaction. The "B_{12}-coenzymes" were first discovered by Barker and co-workers[4] during their studies of glutamate, β-methylaspartate isomerization by *Clostridium tetanomorphum*. Smith and Monty[5] then observed that methylmalonyl isomerase activity was lower in livers from B_{12}-deficient rats than in livers from normal rats, and Gurnani *et al.*[6] demonstrated that the "B_{12}-coenzyme" (dimethylbenzimidazolylcobamide) of Barker would restore the isomerase activity of liver extracts from the deficient animals. Stern and Friedman[7] and Stadtman *et al.*[8] also demonstrated the activating effect of this coenzyme; the former used an ammonium sulfate fraction of ox-liver extract and the latter a charcoal-treated extract of propionibacteria. Barker and co-workers[4] previously had found that the "B_{12}-coenzyme" could be removed from their haloenzyme by charcoal treatment. The methylmalonyl isomerase from animals apparently is not as easily freed of its coenzyme as is the bacterial enzyme. Lengyel *et al.*[9] were unable to resolve the isomerase from kidney by charcoal treatment but found that the coenzyme could be dissociated from the enzyme by acidification in the presence of ammonium sulfate solution.

The present work deals with the purification and properties of the methylmalonyl isomerase obtained from propionibacteria. The preparation is about 200 times more active than those previously described.

Materials and Methods.—The methods and compounds used were the same as those described by Wood and Stjernholm.[10] The dimethylbenzimidazolylcobamide was a gift of Dr. Karl Folkers of the Merck Institute. A fresh solution (10^{-4} *M*) was prepared each week and it was protected from light. The propionyl carboxylase was a gift of Dr. S. Ochoa, New York University.

The methylmalonyl isomerase was purified by the procedures described by Wood and Stjernholm.[10] The cells were ruptured either by sonic disintegration or by grinding in a Waring blender with glass beads. The cell-free extract was treated with calcium phosphate gel and fractionated with ammonium sulfate. The protein precipitating between 40 to 90 per cent saturation was eluted from a DEAE-cellulose column using a series of phosphate buffers of increasing molarity of pH 6.8. The methylmalonyl isomerase was eluted with 0.15 *M* phosphate buffer and was associated with a pink color, which was easily observed in the tubes on the fraction collector. Although the enzyme was eluted from the cellulose column in a broad band (see Fig. 1, Wood and Stjernhom,[10] fractions 105 to 115) it was obtained virtually free of methylmalonyl-oxaloacetic transcarboxylase, lactic dehydrogenase and malic dehydrogenase. The specific activity of the isomerase in these fractions ranged from 0.4 to 3.6 and was quite stable at 4°. The protein content was low, therefore the combined fractions (725 ml) were lyophilized and the dry powder was placed in a cellulose bag and dialyzed for 12 hr against two liters of distilled water, then for 6 hr against one liter of 0.005 *M* Tris-HCl buffer, pH 7.4 containing 10 mg of cysteine. The volume of the dialyzed material was 75 ml. It was made 0.01 *M* with respect to Tris-HCl buffer and then finely ground solid ammonium sulfate was added with stirring to give 90 per cent saturation. The pH was maintained at pH 6.8 by addition of 0.4 *M* Tris base during the addition of the ammonium sulfate. The precipitated protein was recovered by centrifuging for 20 min at 20,000 *g* and was dissolved in 3 ml of distilled water. The final enzyme preparation (12 mg protein per ml) was stored at −12° and used without further purification; it possessed a specific activity of 3.05 and although it was contaminated slightly with malic dehydrogenase (specific activity 0.05), it was free of lactic dehydrogenase and methylmalonyl-oxaloacetic transcarboxylase activities.

The isomerase in fractions 116, 117, and 118 (see Fig. 1, Wood and Stjernholm[10]) was partially separated from transcarboxylase by ammonium sulfate precipitation. The 60 to 80 per cent

fraction contained protein with an isomerase specific activity of 2.0, transcarboxylase of 0.28, malic dehydrogenase 0.10, and lactic dehydrogenase nil (see Table 1 of Wood and Stjernholm[10]). The above results are typical of several fractionations which have been performed with protein extracted from propionibacteria.

Assay of Methylmalonyl Isomerase.—Most investigators have assayed methylmalonyl isomerase by measuring the rate of incorporation of C^{14} from either methylmalonyl CoA or propionyl CoA into succinyl CoA. If propionyl-C^{14} CoA was used, it was converted to methylmalonyl CoA by either propionyl carboxylase or transcarboxylase, which was either added or was present in the enzyme preparation. The isomerase also has been assayed by determining the rate of formation of succinyl CoA from unlabeled methylmalonyl CoA. Beck *et al.*[11] hydrolyzed the succinyl CoA and then determined the succinate with succinoxidase. Stadtman *et al.*[8] utilized the marked difference in the heat stability of the two CoA esters, methylmalonyl CoA being stable at 100° for 2 minutes at pH 6.0–7.0, whereas succinyl CoA is completely destroyed by this treatment.

The present assay consists of a coupled reaction catalyzed by the enzymes, methylmalonyl isomerase, methylmalonyl-oxaloacetic transcarboxylase, and malic dehydrogenase. Succinyl CoA, pyruvate and DPNH are the substrates, and the reactions are as follows:

$$\text{succinyl CoA} \rightleftarrows \text{methylmalonyl CoA}$$

$$\text{methylmalonyl CoA} + \text{pyruvate} \rightleftarrows \text{oxaloacetate} + \text{propionyl CoA}$$

$$\text{oxaloacetate} + \text{DPNH} + \text{H}^+ \rightleftarrows \text{malate} + \text{DPN}^+$$

Under optimum conditions in the presence of excess malic dehydrogenase and transcarboxylase, the rate of the DPNH oxidation is linear with time and proportional to the concentration of methylmalonyl isomerase (see Fig. 1). To obtain the maximum reaction velocity, a relatively high concentration of succinyl CoA (1.5×10^{-3} *M*) is required. The transcarboxylase must be quite free of isomerase and was prepared by selecting from the DEAE cellulose column fractions which possessed high transcarboxylase and low isomerase activity. A 40–60 per cent ammonium sulfate fraction was prepared to separate the transcarboxylase from the isomerase, and occasionally the resulting fraction was purified by a second passage through a DEAE cellulose column.

The above procedure could be used to assay the eluates from the cellulose column, since those fractions which contained methylmalonyl isomerase were free of DPNH oxidase or lactic dehydrogenase activities. When the latter enzymes were present, the assay was performed by coupling the isomerase with the transcarboxylase and the resulting oxaloacetate was determined with malic dehydrogenase after deproteinization. The reaction mixture contained 10 μmoles of pyruvate, 1.5 μmoles of succinyl CoA, 30 μmoles of Tris-HCl buffer (pH 7.4), 2.5 μmoles of glutathione, transcarboxylase (0,1 units), the isomerase preparation, and water to give a final volume of 0.5 ml. After incubating at 30° for 15 min, the reaction was terminated by the addition of 0.4 ml of 10 per cent trichloroacetic acid. The mixture was held in an ice bath for 10 min and then centrifuged to remove the precipitated protein. The oxaloacetate formed in the reaction was determined by adding 0.5 ml of the trichloroacetic acid extract to 160 μmoles of Tris base, 100 μmoles of Tris-HCl buffer,

pH 7.4, and 0.35 μmoles of DPNH contained in a cuvette possessing a 1-cm light path. The reaction was initiated by the addition of an excess of malic dehydrogenase (0.5 units). The total volume in the cuvette was 3.0 ml. Since most of the crude enzyme preparations contained transcarboxylase, it usually was not necessary to add the purified transcarboxylase.

Properties of Methylmalonyl Isomerase.—Stability: The purified isomerase preparation could be stored at −10° for two months without loss of activity, when assayed under the conditions described in Figure 1. The enzyme could be diluted

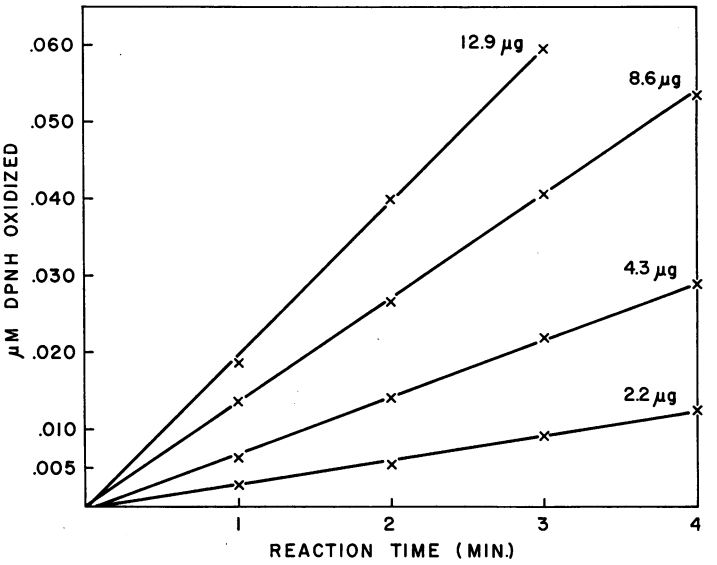


FIG. 1.—Spectrophotometric assay of methylmalonyl isomerase by a coupled reaction with transcarboxylase and malic dehydrogenase. The complete mixture contained 25 μmoles of Tris-HCl buffer (pH 7.0), 6 μmoles of sodium pyruvate, 2.5 μmoles of glutathione, 0.15 μ moles of DPNH, 0.004 μ moles of dimethylbenzimidazolylcobamide, 0.25 units of malic dehydrogenase, 0.25 units of transcarboxylase, and the isomerase as indicated. The reaction was started by the addition of 1.0 μmole of succinyl CoA. The change in optical density at 340 mμ was measured in a glass cuvette (d = 0.5 cm). Temperature 23°. Final volume 0.6 ml. The solutions were held at room temperature for 4 or 5 min in the dark prior to the addition of the succinyl CoA. A correction for isomerase activity of the transcarboxylase was determined by using the above reaction mixture but without addition of isomerase. The ΔOD was 0.007 per minute and was subtracted from the reading obtained with the isomerase present.

either in water or one per cent bovine albumin solution and maintained at 4° for as long as 40 hr without loss of enzymic activity.

pH optimum: The isomerase functions over a broad pH range with only small changes in activity between pH 5.8 and 7.7.

Cobamide requirements: The isomerase obtained from the DEAE column was not completely dissociated from the cobamide coenzyme and possessed some activity without the addition of the coenzyme, whereas the enzyme obtained by precipitation with 90 per cent ammonium sulfate possessed negligible activity in the absence of added coenzyme. The activating effect of the "B_{12} coenzyme" and

the inactivation of the coenzyme by light are illustrated in Figure 2. The isomerase activity was measured by the coupled reaction with transcarboxylase and malic dehydrogenase. It is seen that after illumination there was practically no isomerase activity and the addition of coenzyme (DBC) at 7 min completely restored the isomerase activity. Separate experiments have shown that the resolved protein is not inactivated by light.

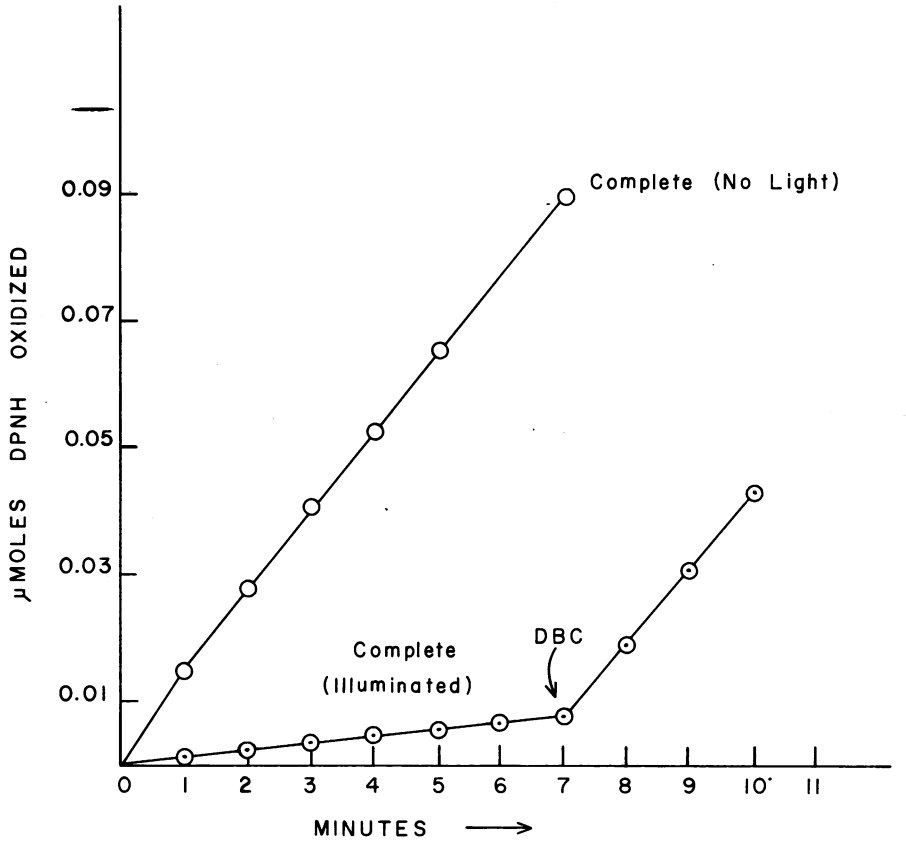


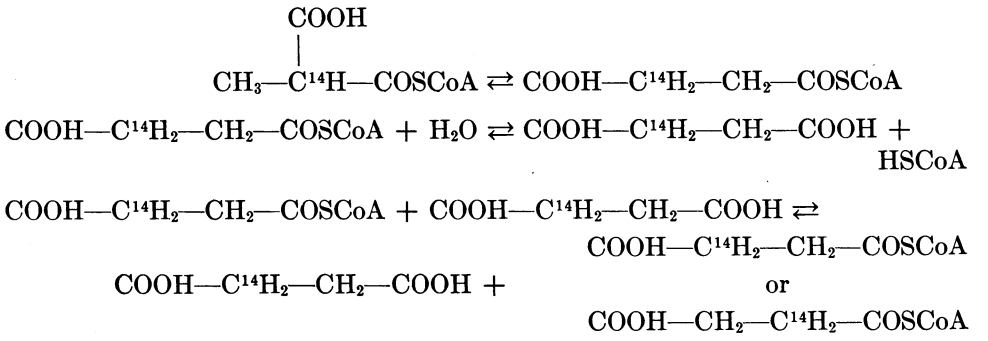
FIG. 2.—The effect of dimethylbenzimidazolylcobamide (DBC) on methylmalonyl isomerase and the inactivation of DBC by light.

Two cuvettes each containing 30 μmoles of Tris buffer (pH 7), 6 μmoles of pyruvate, and 0.002 μmoles of DBC in 0.30 ml of solution were held in an ice bath for 15 min. One was illuminated with a 100-watt tungsten lamp at a distance of about 12 inches. To each was added 3 μmoles of glutathione, 0.15 μmoles of DPNH, 0.25 units of malic dehydrogenase, 0.30 units of transcarboxylase, and 0.012 units of isomerase. The reaction was started by the addition of 0.7 μmoles of succinyl CoA. The final volume was 0.6 ml. Temperature 25°. The optical density was measured at 340 mμ in a glass cuvette (d = 0.5 cm). At 7 min, 0.002 μmoles of DBC was added to the light-treated mixture.

It has been found by Lengyel *et al.*[9] that the kidney isomerase is activated by dimethylbenzimidazolylcobamide ($K_m = 2.1 \times 10^{-8}$ M) and by benzimidazolylcobamide ($K_m = 2.0 \times 10^{-7}$ M) and that the adenylcobamide is inactive. By contrast, all three coenzymes are active with the isomerase from *Propionibacterium shermanii*. Using the enzyme preparation described in this report, Lengyel *et al.*[9] found the K_m values to be 2.4×10^{-8} M for dimethylbenzimidazolylcobamide,

1.3×10^{-7} M for benzimidazolylcobamide and 1.0×10^{-7} for adenylcobamide. Under our conditions of assay, we find in preliminary experiments that the K_m for dimethylbenzimidazolylcobamide is 2.1×10^{-8} M, which is in good agreement with the value found by Lengyel *et al.*[9]

Estimation of Propionyl CoA Transferase and of CoA Deacylase in the Isomerase and Transcarboxylase Preparations.—The propionic acid bacteria contain propionyl CoA transferase, which catalyzes the transfer of CoA between propionyl CoA and succinate,[1, 2] reaction (3). For studies of the mechanism of the isomerization, it is important that the isomerase be free of this transferase activity. Using an enzyme from propionibacteria, Eggerer *et al.*[12] investigated the conversion of methylmalonyl-2-C^{14}-CoA to succinyl CoA and found 80 per cent of the C^{14} in the 3 position and only 20 per cent in the 2 position of the resulting succinyl CoA. They, therefore, have concluded that the isomerase reaction occurs by transfer of the CoA esterified carboxyl since the C^{14} would remain adjacent to the esterified carboxyl if it were not mobilized. The C^{14} which was found in the 2 position probably occurred because the isomerase contained CoA transferase and deacylase which caused randomization of the C^{14} as illustrated below.



We have established that the isomerase purified by the present procedures is free of CoA transferase and deacylase. The propionyl CoA transferase was assayed spectrophotometrically by using the following coupled reactions:

$$\text{propionyl CoA} + \text{succinate} \rightleftarrows \text{succinyl CoA} + \text{propionate}$$

$$\text{succinyl CoA} \rightleftarrows \text{methylmalonyl CoA}$$

$$\text{methylmalonyl CoA} + \text{pyruvate} \rightleftarrows \text{oxaloacetate} + \text{propionyl CoA}$$

$$\text{oxaloacetate} + \text{DPNH} \rightleftarrows \text{malate} + \text{DPN}$$

Table 1 shows the results of such an assay. It is seen that the CoA transferase has a very low affinity for succinate and the rate of reaction increased with increased concentrations of succinate up to 0.03 M. It is noted that there was very little reaction when the isomerase and transcarboxylase were tested in the presence of heat-inactivated CoA transferase, and therefore it is concluded that both the purified transcarboxylase and isomerase were practically free of propionyl CoA transferase.

No deacylase activity for propionyl CoA, methylmalonyl CoA, or succinyl CoA was observed in the purified isomerase and transcarboxylase preparations using the

TABLE 1

ASSAY OF PROPIONYL CoA TRANSFERASE AND THE EFFECT OF THE CONCENTRATION OF SUCCINATE ON THE ASSAY

Conditions	Succinate mMolar	ΔOD_{340} per Minute	Estimated specific activity of CoA Transferase
Complete	0	0.007	—
Complete	1.5	0.045	0.236
Complete	3.1	0.063	0.330
Complete	7.7	0.098	0.512
Complete	11	0.103	0.537
Complete	15	0.111	0.580
Complete	30	0.114	0.605
Complete, CoA transferase inactivated by heat	7.7	0.006	—
No succinate or CoA transferase	0	0.002	—
No pyruvate	7.7	0.006	—
No propionyl CoA	7.7	0.004	—

The complete reaction mixture contained in μmoles: propionyl CoA 0.5, DPNH 0.25, sodium pyruvate 6.0, glutathione 3.0, dimethylbenzimidazolylcobamide 0.002, Tris-HCl buffer pH 7.0 30.0, transcarboxylase 122 μg (0.46 units), methylmalonylisomerase 130 μg (0.13 units), malic dehydrogenase 0.2 units, partially purified CoA transferase 40 μg (.024 units), and succinate as shown. The final volume of the reaction mixture was 0.65 ml. The reaction was started by the addition of sodium succinate and the optical density measured at 340 mμ. The rate was linear after the first 3 min, and ΔOD is the average taken over the next 4 min.

The propionyl CoA transferase was prepared from an extract of propionibacteria obtained by sonic disintegration of the cells which were grown with glycerol as the substrate. The protein of the extract was precipitated with 90% ammonium sulfate and was then dissolved in water and dialyzed 11 hr against 5×10^{-3} *M* Tris-HCl pH 7.0 containing 1×10^{-3} *M* EDTA and 1×10^{-3} *M* cysteine. The dialyzed solution was brought to 40% saturation with ammonium sulfate, and the precipitate was discarded. The supernatant solution which contained the propionyl CoA transferase was dialyzed for 12 hr as described above.

spectrophotometric method of Stadtman.[13] The reaction mixture contained 15 μmoles of Tris-HCl buffer (pH 7), 1.6 μmoles of propionyl CoA, 1.7 μmoles of succinyl CoA, 1.0 μmole of methylmalonyl CoA, 0.26 units of transcarboxylase (Sp. Act. = 6.0), and 0.68 units of isomerase (Sp. Act. = 3.0) in a volume of 1.6 ml in a 0.5 cm cuvette. Under these conditions, it was observed that there was an optical density change of only 0.027 in 30 min at room temperature at 232 mμ. Additional evidence for the absence of deacylase activity was shown by demonstrating that no free thiol groups were formed in the above reaction mixture as measured by the nitroprusside reaction.[13]

Equilibrium of the Methylmalonyl Isomerase Reaction.—There is only one preliminary report[14] concerning the equilibrium of the isomerase reaction in which it is stated that the equilibrium lies in favor of succinyl CoA by a factor of 50 to 100 times. In our hands, the ratio of succinyl CoA to methylmalonyl CoA at equilibrium has been found to be 10.5 ± 1. We have determined the methylmalonyl CoA spectrophotometrically in the presence of succinyl CoA using methylmalonyl-oxaloacetic transcarboxylase and malic dehydrogenase (see legend of Table 3).

For this determination, it is necessary to use a transcarboxylase preparation which contains little or no isomerase activity. Even with the best transcarboxylase, it was found desirable to treat the deproteinized solution from the equilibrium reaction with light to destroy the "B_{12} coenzyme" and to thus further restrict the residual isomerase reaction. If the light treatment is used, an excess of transcarboxylase can be employed and the determination is complete in a few minutes. However, this was true only when the natural isomer of methylmalonyl CoA was being assayed; with chemically synthesized methylmalonyl CoA, the initial high reaction velocity rapidly decreased and continued for some time at a reduced rate. The explanation for this phenomenon is not certain but it seems plausible that the unnatural isomer of methylmalonyl CoA obtained by chemical synthesis is a com-

petitive inhibitor of the transcarboxylase and the inhibition would increase as the amount of the natural form decreases relative to the amount of unnatural form present.

An attempt also was made to determine the succinyl CoA in the equilibrium mixture using methylmalonyl isomerase, transcarboxylase, and malic dehydrogenase, but this proved to be impractical due to the partial loss of the succinyl CoA during the deproteinization procedure. Typical results are shown in Table 2. In this experiment, analyses are shown in which the succinyl CoA was determined directly without treatment and when perchloric acid was added prior to the isomerase. It is seen that the recovery of the succinyl CoA was only 75 per cent (No. 2) when the isomerase was added subsequent to the perchloric acid. Repeated experiments have shown that there is loss of succinyl CoA during the precipitation of proteins with perchloric acid and that a similar destruction occurs when the solution is deproteinized with trichloroacetic acid.

TABLE 2

THE DESTRUCTION OF SUCCINYL CoA DURING DEPROTEINIZATION WITH PERCHLORIC ACID

No.	Conditions	Incubation at 25° (min)	Succinyl CoA (μmoles)	Succinyl CoA %
1	No perchloric acid or isomerase	0	0.112	—
2	Perchloric acid and then isomerase	0	0.084	75
3	No perchloric acid or isomerase	12	0.105	94
4	Isomerase but no perchloric acid	12	0.105*	94

* Succinyl CoA plus methylmalonyl CoA.

1.5 ml of solution was prepared which contained 37.5 μmoles Tris-HCl buffer (pH 7.0), 0.003 μmoles of dimethylbenzimidazolylcobamide, and 2.35 μmoles of succinyl CoA. From this mixture, 0.2 ml was placed in each of 4 tubes. Tubes 1 and 2 were held in an ice bath, and the following solutions were added in the order given: to tube 1, 0.12 ml of H_2O and 0.10 ml of 0.5 *M* Tris-HCl buffer (pH 6.7), to tube 2, 0.05 ml of 2 *N* perchloric acid, 0.01 ml of isomerase (Sp. Act. = 1.2, 300 γ protein), 0.10 ml of 0.5 *M* Tris-HCl buffer (pH 6.7), and 0.05 ml of 2*N* KOH. 0.02 ml H_2O was added to Tube 3 and 0.01 ml of H_2O plus 0.01 of the isomerase (Sp. Act. = 1.2, 300 γ protein) to Tube 4. Tubes 3 and 4 were held at 25° for 12 min and then 0.1 ml H_2O and 0.1 ml of 0.5 *M* Tris-HCl buffer (pH 6.7) was added to each. 0.15 ml was removed from each of the 4 tubes and added to 0.5 cm glass cuvettes containing the following mixture: glutathione, 4.5 μmoles; DPNH, 0.4 μmoles; dimethylbenzimidazolylcobamide, 0.002 μmoles; pyruvate, 10 μmoles; malic dehydrogenase, 0.3 units; transcarboxylase, 0.98 units; isomerase, 0.24 units. Final volume was 0.9 ml. Succinyl CoA was calculated from the ΔOD at 340 mμ

The amount of succinyl CoA therefore has been calculated by the difference between the original succinyl CoA added to the reaction and the methylmalonyl CoA present after the reaction. For this calculation to be correct, it is necessary that there be no destruction of the succinyl CoA or methylmalonyl CoA during the isomerase reaction. Experiments Nos. 3 and 4 of Table 2 were performed to establish this fact. In No. 3, the succinyl CoA was incubated at 25° for 12 min in the absence of isomerase. The results show that there was little destruction of the succinyl CoA during the 12 min. In No. 4, 0.36 units of isomerase were added to 0.31 μmoles of succinyl CoA, the mixture was incubated for 12 min, and then the total succinyl CoA plus methylmalonyl CoA was determined without deproteinization by the combined action of isomerase, transcarboxylase, and malic dehydrogenase. It is seen that the sum of the two CoA esters was equal to the original succinyl CoA concentration. Clearly there was little if any destruction of succinyl CoA during the isomerase reaction and therefore it appears reasonable to calculate the amount of succinyl CoA present by difference.

Tables 3 and 4 show typical results of the equilibrium studies. Table 3 presents data obtained using chemically synthesized succinyl CoA and methylmalonyl CoA. The natural isomer of methylmalonyl CoA was used in the experiment of Table 4.

TABLE 3

EQUILIBRIUM OF THE METHYLMALONYL ISOMERASE REACTION

	Experiment 1 — Substrate: Succinyl CoA (Suc CoA)				Experiment 2 — Substrate: Methylmalonyl CoA (MMCoA)			
	Deproteinized solution				Deproteinized solution			
Time (min)	MMCoA (μmoles)	Suc CoA (μmoles)	Suc CoA by difference* (μmoles)	[Suc CoA]/[MMCoA]	MMCoA (μmoles)	Suc CoA (μmoles)	Suc CoA by difference* (μmoles)	[Suc CoA]/[MMCoA]
0	—	3.90	5.28†	—	2.52†	—	—	—
1	0.44	3.51	4.84	10.9	0.53	1.16	1.99	3.8
3	0.46	3.36	4.82	10.4	0.47	1.18	2.08	4.4
5	0.45	3.55	4.83	10.7	0.24	1.49	2.28	9.5
8	0.49	3.36	4.79	9.7	0.22	1.31	2.30	10.5
12	0.42	3.26	4.86	11.5	0.21	—	2.31	11.0

* The Suc CoA by difference was calculated by subtracting the determined value for the methylmalonyl CoA from 5.28 in Experiment 1 and from 2.52 in Experiment 2.

† This value was obtained by enzymatic determination using the original solution of succinyl CoA or methylmalonyl CoA. Colorimetric estimation[14] of the hydroxamic acid derivative gave 8.7 μmoles for the succinyl CoA and 5.0 for the methylmalonyl CoA.

Experiment 1. The mixture for the isomerase reaction contained 75 μmoles of Tris-HCl buffer, pH 7.0; 0.012 μmoles of dimethylbenzimidazolylcobamide and 5.28 μmoles of succinyl CoA in 2.85 ml. For the 0 time determination, 0.38 ml of the mixture was removed and added to 0.1 ml of 2 *N* perchloric acid then 0.02 ml of the isomerase solution (Sp. Act. 3.0, 13 mg protein per ml) was added. To the remaining 2.47 ml of mixture, 0.13 ml of the isomerase solution was added and the mixture was incubated at 25°. 0.4 ml was removed at the indicated times and added to 0.1 ml of 2 *N* perchloric acid. 0.20 ml of 0.5 *M* Tris-HCl buffer, pH 6.7 was added to each tube and 0.12 ml of KOH (equivalent to perchloric acid). The solution was kept in ice for 5 min then centrifuged. Then the solutions were placed in an ice bath under a 100-watt incandescent light bulb for 15 min at a distance of 100 cm.

Experiment 2. Performed the same as Experiment 1 but instead of the succinyl CoA, the reaction mixture contained 5.0 μmoles of methylmalonyl CoA by colorimetric determination of hydroxamic acid derivative.

Determination of methylmalonyl CoA. 0.2 ml of the deproteinized solution was added to a 0.5-cm cuvette containing 70 μmoles of pyruvate, 0.2 μmoles of DPNH, 3.5 μmoles of glutathione, 0.3 units of malic dehydrogenase, and 0.06 units of transcarboxylase (Sp. Act. = 6.0). Total volume = 0.7 ml. The reaction was started by the addition of the transcarboxylase. The 0 time of Experiment 1 gave an optical density change of 0.001 per min. This reading was considered to be the endogenous reading and was subtracted from all the optical density changes observed with the other samples. With the 0 time sample, it required 25 min for the reaction to be complete in Experiment 2.

Determination of succinyl CoA. After the above reaction was complete, 0.15 μmoles of DPNH was added and then the reaction was started by addition of 0.002 μmoles of dimethylimidazolylcobamide, 0.14 units of transcarboxylase (Sp. Act. = 6.0), and 0.008 units of isomerase (Sp. Act. = 3). The 0 time of Experiment 2 gave an optical density change of 0.0005 per min. This reading was considered to be the blank value and was subtracted from the optical density change observed with the other samples.

TABLE 4

EQUILIBRIUM OF THE METHYLMALONYL ISOMERASE REACTION

	Experiment 3 — Substrate: Methylmalonyl CoA (MMCoA)			
	Deproteinized solution			
Time (min)	MMCoA (μmoles)	Suc CoA (μmoles)	Suc CoA by difference (μmoles)	[Suc CoA]/[MMCoA
0	0.924	—	—	—
1	0.307	0.397	0.617	2.0
3	0.107	0.460	0.817	7.6
5	0.089	0.526	0.835	9.4
8	0.093	0.580	0.831	8.9
12	0.087	0.504	0.837	9.6

Preparation of methylmalonyl CoA. The reaction mixture contained 2.0 μmoles of propionyl CoA, 75 μmoles of Tris buffer (pH 7.8), 2.5 μmoles of glutathione, 5 μmoles of ATP, 75 μmoles of $KHCO_3$, 4 μmoles $MgCl_2$, and 0.65 units of propionyl carboxylase in 1.00 ml. Incubation was for 15 min at 30°. The solution was deproteinized with 0.25 ml of 2 *N* perchloric acid and neutralized with 2 *N* KOH. Yield of methylmalonyl CoA by enzymatic determination was 1.25 μmoles.

Isomerase reaction. Performed as described in Table 2 except the volumes were reduced by one-half and the mixture contained 0.97 μmoles of MMCoA.

Methylmalonyl CoA and succinyl CoA determination. Performed on 0.2 ml of the deproteinized solution as described in Table 3. Tho determination of the methylmalonyl CoA was complete in 4 min with the 0 time sample, whereas it required 25 min with the chemically synthesized methylmalonyl CoA in Experiment 2, Table 3

In Experiment 1, it is noted that less succinyl CoA was found in the deproteinized solution at 0 time than was added to the mixture. There was destruction of the succinyl CoA during the deproteinization just as illustrated in Table 2. The succinyl CoA was stable, however, in the neutralized deproteinized solution. No loss of the ester was observed even after 24 hr at 0°. There was little if any destruction of the methylmalonyl CoA during deproteinization. In Experiment 2, 5.0 μmoles of methylmalonyl CoA were found present by the hydroxamic acid reaction[13] and

2.52 μmoles were found by enzymatic determination at 0 time after deproteination. This value is in accord with the conclusion that only one isomer is biologically active. In Experiment 3 in which 0.97 μmoles of the natural methylmalonyl CoA was added, 0.924 μmoles was recovered in the deproteinized solution at 0 time.

The ratios of succinyl CoA to methylmalonyl CoA of Tables 3 and 4 have been calculated using the value of the succinyl CoA as estimated by difference from the amount of added CoA ester. It is seen that very nearly the same ratio was obtained starting with either succinyl CoA or methylmalonyl CoA. The values of the ratio in Table 4 with the natural isomer of methylmalonyl CoA were a little lower than in Table 3, but the determinations were subject to considerable error in the latter case because the quantity of methylmalonyl CoA at equilibrium was quite small. It is noted that equilibrium was attained more rapidly using succinyl CoA as the initial reactant than with methylmalonyl CoA.

Using a value of 10.5 for the equilibrium constant, the ΔF°_{298} for the isomerase reaction is calculated to be -1.4×10^3 calories at pH 7.0.

$$\Delta F^{0} = RT\, 2.303 \log K$$

$$\Delta F^{\circ}_{298} = -1.987 \times 298 \times 2.303 \log 10.5 = -1.4 \times 10^3 \text{ calories}$$

Summary.—Methylmalonyl isomerase from propionibacteria has been purified by chromatography on a cellulose column and by ammonium sulfate fraction. The activity obtained was about 200 times greater than that of previously reported preparations. The isolated enzyme is resolved from "B_{12} coenzyme" and is reactivated by the addition of coenzyme. The enzyme is free of lactic dehydrogenase methylmalonyl-oxaloacetic transcarboxylase, malic dehydogenase, CoA deacylase, and propionyl CoA transferase.

A spectrophotometric assay for the isomerase has been developed in which this enzyme is coupled with methylmalonyl-oxaloacetic transcarboxylase and malic dehydrogenase. Propionyl CoA transferase has been assayed spectrophotometrically by coupling it with isomerase, transcarboxylase, and malic dehydrogenase.

The equilibrium constant, [succinyl CoA]/[methylmalonyl CoA], was found to be 10.5 for the isomerase reaction at pH 7.0, and 25°. The ΔF°_{298} is calculated to be -1.4×10^3 calories.

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* This work was assisted by a grant from the National Institute of Arthritis and Metabolic Diseases (Grant E-1003) of the U.S. Public Health Service. Preceding paper of this series was Swick and Wood.[1] Abbreviations used are: CoA (coenzyme A), ATP (adenosine triphosphate), DPNH (reduced diphosphopyridine nucleotide), Tris (Tris(hydroxymethyl)methylamine), DEAE-cellulose (diethylaminoethyl-cellulose), and EDTA (ethylene diamine tetracetic acid).

Specific activity is expressed in this paper as μmoles of product per minute per mg of protein.

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THE IONIC CENTRIFUGE AND FUSION NUCLEAR POWER

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The Ionic Centrifuge.—By Ionic Centrifuge is meant a central low-voltage arc source of ionization maintained at low voltage at the center of a rather long circular cylinder through which a longitudinal magnetic field passes. The end plates which bound the vacuum space longitudinally are maintained at one voltage; the cylinder which bounds the space circumferentially is maintained at another voltage. The anode of the arc is taken as zero voltage.[1–7] (See Fig. 1.)

The Discharge in the Ionic Centrifuge Violates the Usual Rules of Magnetohydrodynamics.—Let a voltage other than zero be applied to the end plates. Then charged particles of one sign are drawn to the end plates; charged particles of the opposite sign are repelled. A space charge sheath forms adjacent to each end plate. The space charge sheaths have a high electric gradient parallel to the magnetic field, violating the principle that in a hydromagnetic discharge the electric field is *everywhere* perpendicular to the magnetic field.[6, 8]

This high electrical potential gradient parallel to the magnetic field causes the induced current in the gas to have large components parallel to the magnetic field. It causes the derivative of the parallel component of the current density along the direction of the magnetic field to be large. It causes a circulating circumferential component of the magnetic field to exist. It causes a relatively large radial component of the mean velocity of the particles of the gas to exist. It makes large positive or negative radial derivatives in the mean random velocity of the electrons and ions.

Expressed in terms of the components of electric and magnetic fields, the following are no longer true. The mean motion of the gas is no longer given by $[E \times B]/B^2$, where E and B have the usual significance, and the motion of the gas is not circumferential only. Although the intrinsic conductivity of the gas is high, the lines of magnetic force do not travel in any sense with the gas.