cytidylic acid, and uridylic acid. The molar ratios were 1.17 ± 0.03, 1.00, 1.21 ± 0.06, and ± 0.07, respectively.

The sugar present was determined by chromatographing the above HCl hydrolysate and a control mixture of sugars on Whatman #1 paper using a mixture of butanol, ethanol, and ammonia.[11] The sugar had an Rf characteristic of ribose.

Summary.—The evidence presented indicates that the bacteriophage f2 contains RNA and not DNA as its nucleic acid. The evidence is based primarily on the analysis of purified material but also on the distribution of the two types of nucleic acid synthesized after infection. Although f2 is an extremely small phage, there is a compensating large yield per bacterium (about 10,000 P.F.U.). Therefore, the synthesis of phage materials can be followed and the phage itself readily purified. In its general features such as adsorption and intracellular growth, f2 resembles the DNA bacteriophages. Further studies on the biology and chemistry of f2 are in progress.

We gratefully acknowledge our indebtedness to Dr. M. Jesaitis for advice and aid in the preparation and analysis of large quantities of f2. We also thank Miss Doris Degen, Mr. S. Cooper, and Mr. M. Estrin for technical aid.

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*TRANSCARBOXYLASE, II. PURIFICATION AND PROPERTIES OF METHYLMALONYL-OXALOACETIC TRANSCARBOXYLASE**

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Swick and Wood[1] recently have demonstrated a new type of biochemical reaction in which one compound, a carboxyl donor, is decarboxylated and a second compound, a carboxyl acceptor, is carboxylated. Thus, it is possible to accomplish a direct carboxylation without intervention of CO_2 or the expenditure of energy to activate the CO_2. The conversion is illustrated in reaction (1).

$$\begin{array}{l} \mathrm{COO^-} \\ | \\ \mathrm{CH_3{-}CH{-}COSCoA} \end{array} + \mathrm{CH_3{-}CO{-}COO^- \rightleftarrows CH_3{-}CH_2{-}COSCoA} + \mathrm{COO^-{-}CH_2{-}CO{-}COO^-} \quad (1)$$

methylmalonyl CoA — pyruvate — propionyl CoA — oxaloacetate

This reaction permits net synthesis of oxaloacetate from methylmalonyl CoA and pyruvate or of methylmalonyl CoA from oxaloacetate and propionyl CoA, and therefore differs from exchange reactions in which there is carboxyl transfer but no net synthesis. Lynen *et al.*[2] observed this type of exchange with β-methylcrotonyl carboxylase from *Mycobacterium* using β-methylglutaconyl-CoA-1,3,5-C^{14} and unlabeled β-methylcrotonyl CoA. This carboxyl transfer has been proposed to occur as illustrated in reaction (2).

$$\begin{array}{l} \mathrm{CH_2{-}C^{14}OO^-} \\ | \\ \mathrm{CH_3{-}C^{14}{=}CH{-}C^{14}OSCoA} \end{array} + \text{E-biotin} \rightleftarrows \begin{array}{l} \mathrm{CH_3} \\ | \\ \mathrm{CH_3{-}C^{14}{=}CH{-}C^{14}OSCoA} \end{array} + \text{E-biotin}_{\tau}\mathrm{C^{14}O_2} \quad (2)$$

β methylglutaconyl CoA — β methylcrotonyl CoA

$$\begin{array}{l} \mathrm{CH_3} \\ | \\ \mathrm{CH_3{-}C{=}CH{-}COSCoA} \end{array} + \text{E-biotin}\mathrm{{-}C^{14}O_2} \rightleftarrows \begin{array}{l} \mathrm{CH_2C^{14}OO^-} \\ | \\ \mathrm{CH_3{-}C{=}CH{-}COSCoA} \end{array} + \text{E-biotin}$$

$$\text{Sum: } \begin{array}{l} \mathrm{CH_2{-}C^{14}OO^-} \\ \diagup \quad \diagdown \\ \mathrm{CH_3{-}C^{14}{=}CH{-}C^{14}OSCoA} \end{array} + \begin{array}{l} \mathrm{CH_3} \\ | \\ \mathrm{CH_3{-}C{=}CH{-}COSCoA} \end{array} \rightleftarrows$$

$$\begin{array}{l} \mathrm{CH_3} \\ | \\ \mathrm{CH_3{-}C^{14}{=}CH{-}C^{14}OSCoA} \end{array} + \begin{array}{l} \mathrm{CH_2C^{14}OO^-} \\ | \\ \mathrm{CH_3{-}C{=}CH{-}COSCoA} \end{array}$$

A similar carboxyl transfer has been demonstrated by Lane and Halenz.[3] However, in this case there was net synthesis, but it probably occurred because the propionyl carboxylase activated homologous types of CoA compounds. The reaction is illustrated in equation (3).

$$\begin{array}{l} \mathrm{C^{14}OO^-} \\ | \\ \mathrm{CH_3{-}CH{-}COSCoA} \end{array} + \mathrm{CH_3{-}CH_2{-}CH_2{-}COSCoA \rightleftarrows CH_3CH_2COSCoA} + \begin{array}{l} \mathrm{C^{14}OO^-} \\ | \\ \mathrm{CH_3{-}CH_2{-}CH{-}COSCoA} \end{array} \quad (3)$$

methylmalonyl CoA — butyryl CoA — propionyl CoA — ethylmalonyl CoA

The unique feature of methylmalonyl-oxaloacetic transcarboxylase is that it catalyzes a reaction which involves compounds from different pathways of metabolism, i.e., the CoA esters of fatty acid metabolism and the α-keto acids of carbohydrate

metabolism. It thus can shuttle carboxyl groups from one metabolic pathway to another and therefore has great potential in synthetic reactions.

The studies of Swick and Wood[1] were performed using unfractionated protein extracts prepared from *Propionibacterium shermanii*. The results reported here deal with the purification and properties of the enzyme. Although there is still a possibility that the transcarboxylase reaction may be catalyzed by more than one enzyme, the results from the present study are in accord with the view that it is a single enzyme. The enzyme will be referred to as methylmalonyl-oxaloacetic transcarboxylase to indicate its dual activity on CoA derivatives and keto acids. The activity of the enzyme appears to be restricted to the keto acids pyruvate and oxaloacetate but has a broader specificity for CoA compounds.

Materials.—The CoA esters were synthesized as described by Swick and Wood[1] using modifications of the methods of Simon and Shemin[4] or of Beck *et al.*[5] The ethylmalonyl CoA was a sample given to us by Dr. E. R. Tustanoff, Western Reserve University. The oxaloacetate and DPNH (Sigma), pyruvate and glutathione (Nutritional Biochemical Corp.), phosphoenol pyruvate (California Foundation), lactic dehydrogenase, malic dehydrogenase and pyruvokinase (Boehringer), and avidin (2,500 units per gm, Nutritional Biochemicals Corp.) were commercial products. The propionyl carboxylase was a generous gift from Dr. Severo Ochoa, New York University. It was a three-times crystallized enzyme. The saturated ammonium sulfate was made from ammonium sulfate which had been recrystallized from glass-distilled water, and sufficient concentrated NaOH solution was added during the saturation so that the pH was 7.0 in a sample which was diluted 20 times.

Growth Conditions.—Propionibacterium shermanii (52W) was grown at 30° for 3 to 5 days in 20-liter bottles with cotton stoppers and containing 15 liters of medium of the following composition: 4.4 gm of K_2HPO_4 and 3.4 gm of KH_2PO_4, 5.0 gm of glycerol or glucose, 5.0 gm yeast extract, 1 mg calcium pantothenate, 1.0 mg thiamine hydrochloride, 1.0 mg biotin, and 10 mg of $Co(NO_3)_2 \cdot 6H_2O$ per liter. The cells were harvested in a pre-cooled Sharples centrifuge.

Preparation of Cell Free Extracts.—Two methods were used to extract the enzymes from the cells. Both methods give good yields but the procedure with the Waring blender is more convenient for larger quantities of cells.

Sonic rupture of cells: The cells were suspended in an equal weight of ice-cold 0.3 *M* K_2HPO_4 containing 0.3 mg cysteine per ml and the suspension then was subjected to a Raytheon 10 kc/second disintegrator for 20 min while the temperature was maintained at 0–4° by circulating a refrigerant through the cooling chamber. The extract was clarified by centrifuging in a refrigerated angle-head centrifuge for 20 min at 25,000 *g*. The cloudy supernatant liquid was recentrifuged for 30 min at 30,000 *g* giving a clear brown colored supernatant solution.

Rupture of the cells with a Waring blender: The procedure based on the method described by Lammana and Mallete[6] was as follows: 77 gm of cells, 100 gm of glass beads (Superbrite glass beads, Minnesota Mining and Manufacturing Co., Type 100–5005), and 100 ml of ice-cold 0.3 *M* K_2HPO_4 containing 10 mg of cysteine were ground at top speed in a 1 liter Waring blender at −10°. The blender was stopped at regular intervals to avoid over-heating. After grinding for 10 min, 100 ml of 0.3 *M* K_2HPO_4 were added, and the mixture was ground again in the blender for another 3 min. The process was repeated with a further addition of 100 ml

of 0.3 *M* K_2HPO_4. The temperature was not higher than 15° during the treatment. The mixture was centrifuged first at 6,000 *g* for 10 min to remove the glass beads and cell debris and then at 30,000 *g* for 30 min to yield 230 ml of a clear-brown supernatant solution.

Purification of Methylmalonyl-Oxaloacetic Transcarboxylase.—The cell free extracts obtained from either process usually contained about 25 mg of protein per ml. All fractionations were performed at 0°.

Step 1. Calcium phosphate gel treatment: A 15 per cent calcium chloride solution was added dropwise to the extract which was stirred mechanically in an ice bath while the pH was maintained at pH 7.0 by the dropwise addition of 10 per cent Na_3PO_4 solution. Sufficient $CaCl_2$ was added to give 1 mg of $Ca_3(PO_4)_2$ gel per mg of protein (0.072 ml of 15 per cent $CaCl_2$ per mg of protein). The mixture was stirred for an additional 15 min and then centrifuged at 25,000 *g* for 15 min. The supernatant solution, which usually contained about 15 mg of protein per ml, was dialyzed for 3 hours against 15 volumes of distilled water containing 10 mg of cysteine per liter and then for 10 hours against 15 volumes of 0.05 *M* Tris-HCl buffer at pH 7.4 containing 10 mg cysteine per liter.

Step 2. Ammonium sulfate fractionation: The solution from Step 1 was stirred in an ice bath and finely powdered ammonium sulfate was added slowly to give 40 per cent saturation. The precipitate was removed and discarded and the solution was adjusted to 90 per cent saturation by the further addition of solid ammonium sulfate. The precipitate was isolated by centrifuging at 20,000 *g* and dissolved in sufficient distilled water to yield a solution containing about 25 mg of protein per ml. Excess ammonium sulfate was removed by dialysis for 12 hours against approximately 50 volumes of 0.01 *M* Tris-HCl buffer pH 7.4, 0.001 *M* EDTA, and 0.001 *M* cysteine with a change of dialyzing solution after the first 6 hours.

Step 3. Fractionation on a DEAE-cellulose column: The DEAE-cellulose was prepared for use by a series of washes in the following solutions in the order given: 0.1 *N* NaOH, water, 0.1 *N* HCl, water, 0.1 *M* phosphate buffer, pH 7.0, and 0.01 *M* phosphate buffer pH 7.0. The cellulose was packed to form a column 6.5 cm wide and 30 cm long and equilibrated overnight against 0.01 *M* phosphate buffer, pH 7.0. The protein solution from Step 2 (2.0 to 4.0 gm of protein) was applied to the top of the column and the column was washed with 2,000 ml of the same buffer containing 20 mg of cysteine. The chromatogram was then developed as described in the legend of Figure 1 with the aid of a series of phosphate buffers of increasing molarity at pH 6.8 and containing 10 mg of cysteine per liter.

The fractions containing the transcarboxylase were located by a colorimetric test for oxaloacetate. The reaction was performed on a white porcelain spot plate using 0.08 ml of a solution containing 3 μmoles of pyruvate, 0.02 μmoles of methylmalonyl CoA, and 5 μmoles of Tris-HCl buffer pH 7.0 to which was added 0.02 ml of the eluate. The mixture was incubated for 5 min at 30°, then 0.2 ml of 1 *M* acetate buffer pH 5.2 and 0.3 ml of the diazo reagent of Kalnitsky and Tapley[10] were added to the mixture. A brown color develops in a few minutes if the eluate contains transcarboxylase. The reagent blank without enzyme is yellow in color.

It is seen in Figure 1 that part of the methylmalonyl isomerase was eluted from the column prior to the addition of the 0.2 *M* phosphate buffer but it was not completely separated from the transcarboxylase. The isomerase usually is associated

with a pink protein. Malic dehydrogenase was eluted somewhat later than the transcarboxylase peak but the separation of these two enzymes was not complete. The malic dehydrogenase had a high specific activity, 597 in fraction 122. The maximum activity of the transcarboxylase was 6.65 and appeared in fraction 117. The protein of fraction 117 had a specific activity of 0.11 with respect to malic dehydrogenase and 1.03 with respect to methylmalonyl isomerase.

The transcarboxylase is quite stable in the eluate either at 0° or frozen. A solution which was stored frozen but frequently thawed for assay lost about 25 per cent of its activity in 2 months.

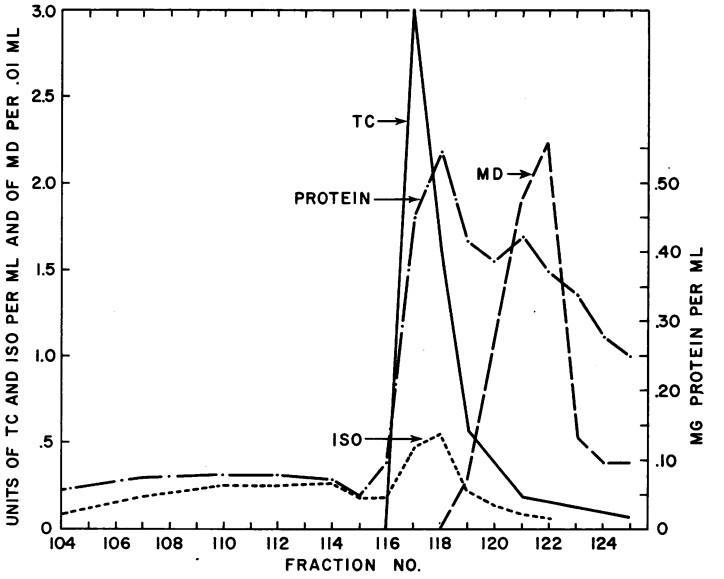

FIG. 1.—Protein content and enzyme activities of the eluate from a typical DEAE cellulose column. 2.04 gm. of protein (Step 3) was placed on a 6.5 × 30 cm cellulose column. Approximately 65 ml fractions were collected at 60 to 90 min intervals. The concentration of phosphate buffer, pH 6.8 was increased stepwise from 0.05 *M* at fraction 16 to 0.1 *M* at fraction 48, to 0.15 *M* at fraction 49, to 0.20 *M* at fraction 110. The protein was determined by light absorption at 280 mμ with a correction for nucleic acid content based on the 260 mμ absorption.[7] The transcarboxylase (TC) was determined as described in Figure 2, the methylmalonyl isomerase (Iso) as described by Stjernholm and Wood,[8] and the malic dehydrogenase (MD) as described by Ochoa.[9] The maximum specific activity of the isomerase was 3.68 in fraction 114, transcarboxylase 6.65 in fraction 117, and malic dehydrogenase 597 in fraction 122.

Those fractions from the DEAE cellulose column which contained the transcarboxylase and very little malic dehydrogenase were combined (116, 117, 118 from the column of Fig. 1) and the protein was precipitated by addition of sufficient solid recrystallized ammonium sulfate to give a 90 per cent saturated solution. The precipitate was dissolved in 3 ml of distilled water and dialyzed 1 hour against 1 liter of 0.0025 *M* glutathione, 0 005 *M* Tris-HCl buffer pH 7.4, and 0.001 *M* EDTA. The dialysis was repeated for an additional hour against fresh solution.

Step 4. Second ammonium sulfate fractionation: The solution from Step 3

was diluted to give a protein content of 10 mg per ml and made 0.01 *M* with respect to Tris-HCl buffer pH 7.4 and 0.001 *M* with respect to glutathione. Saturated ammonium sulfate was added dropwise during mechanical stirring in an ice bath to make the solution 35 per cent saturated. After 10 min additional stirring the mixture was centrifuged in a refrigerated centrifuge for 15 min at 30,000 *g*. The precipitate was discarded. Succeeding ammonium sulfate fractions were obtained between 35–48, 48–60, and 60–80 per cent saturation. In each case the precipitate was taken up in 2 ml of distilled water and was precipitated again by addition of sufficient saturated ammonium sulfate to bring the concentration to 48, 60, and 80 per cent respectively. The transcarboxylase has been found to be stable when held as a suspension at 0° in ammonium sulfate but in solution it loses activity when stored frozen at −12° or unfrozen at 0°. The suspension usually was dissolved in 0.05 *M* glutathione and was used without removal of the ammonium sulfate.

It is seen (Table 1) that the 48–60 ammonium sulfate fractionation contained most of the transcarboxylase and that the isomerase was largely in the 60 to 80 fraction. The malic dehydrogenase also was concentrated in the 60 to 80 fraction (data not shown).

TABLE 1

PURIFICATION OF METHYLMALONYL-OXALOACETIC TRANSCARBOXYLASE FROM *Propionibacterium shermanii*

	Step	Protein (mg)	Transcarboxylase		Methylmalonyl isomerase	
			Specific activity*	Units	Specific activity*	Units
2	40–90 $(NH_4)_2SO_4$ fraction	2040	0.45	900	0.10	204†
3	DEAE-cellulose, fractions 116, 117, 118 (Fig. 1). 0–90 $(NH_4)_2SO_4$ precipitate	49.5	4.2	208	1.14	56.0‡
4	35–48 $(NH_4)_2SO_4$ fraction	5.0	4.5	23	.16	0.8
	48–60 $(NH_4)_2SO_4$ fraction	19.4	7.0	136	.15	2.9
	60–80 $(NH_4)_2SO_4$ fraction	22.0	0.28	6	2.00	44.0

* Specific activity is in μmoles/min/mg protein.
† Isomerase was determined by the procedure described by Stjernholm and Wood.[8]
‡ Only part of the isomerase which was eluted from the cellulose column was contained in the fractions 116, 117, and 118 (see Fig. 1). The recovery was greater than 56 units.

Assay of Methylmalonyl-Oxaloacetic Transcarboxylase.—The transcarboxylase was assayed by measurement of the rate of Reaction 1 in either direction. If the rate to the right was determined, the formation of oxaloacetate was measured using malic dehydrogenase and DPNH (reaction 5). The reactions were as follows:

$$\text{methylmalonyl CoA} + \text{pyruvate} \rightleftarrows \text{oxaloacetate} + \text{propionyl CoA} \quad (4)$$

$$\text{oxaloacetate} + \text{DPNH} + \text{H}^+ \rightleftarrows \text{malate} + \text{DPN}^+ \quad (5)$$

The optical density change was measured at 340 mμ.

When the rate to the left was used for the assay, the formation of pyruvate was determined with lactic dehydrogenase and DPNH. The reactions were as follows:

$$\text{oxaloacetate} + \text{propionyl CoA} \rightleftarrows \text{pyruvate} + \text{methylmalonyl CoA} \quad (6)$$

$$\text{pyruvate} + \text{DPNH} + \text{H}^+ \rightleftarrows \text{lactate} + \text{DPN}^+ \quad (7)$$

Procedure 1: This procedure is used if the enzyme preparation contains either lactic dehydrogenase or DPNH oxidase. The transcarboxylase reaction is per-

formed first and then the mixture is deproteinized to remove the contaminating enzymes before determining the amount of oxaloacetate. The mixture for the transcarboxylase reaction consists of 10 μmoles of pyruvate, 1.5 to 2.0 μmoles of methylmalonyl CoA, 30 μmoles of Tris-HCl buffer pH 7.4, 2.5 μmoles of glutathione, enzyme, and water to a final volume of 0.5 ml. After incubation at 30° for 5 min, the reaction is terminated by the addition of 0.4 ml of 10 per cent trichloroacetic acid. The mixture is held in an ice bath for 10 min and then centrifuged to remove the precipitated protein. The oxaloacetate formed in the reaction is 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 is started by the addition of an excess of malic dehydrogenase (0.5 units). The total volume in the cuvette is 3.0 ml.

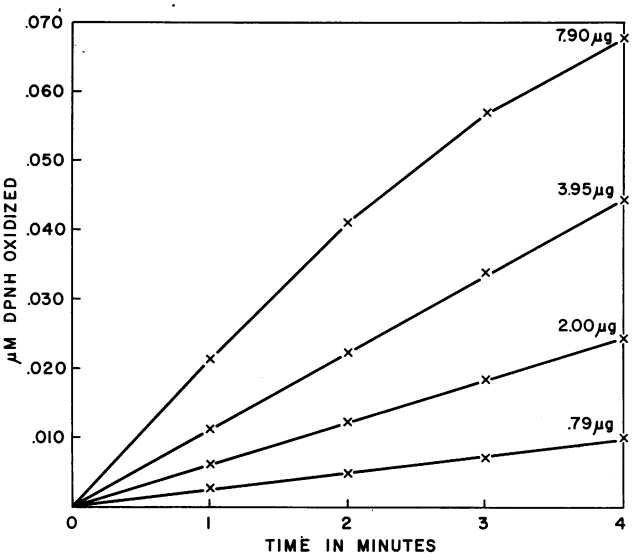

FIG. 2.—Spectrophotometric assay of methylmalonyl-oxaloacetic transcarboxylase by coupling with malic dehydrogenase. The cuvette contained the following expressed in μmoles: Tris-HCl buffer pH 7.0, 15; glutathione, 3.0; DPNH, 0.15; pyruvate, 6.0; methylmalonyl CoA, 0.5; and an excess of malic dehydrogenase (0.2 unit). The reaction was started with the addition of transcarboxylase using the amount as indicated. Final volume 0.6 ml. Temperature 23°. The transcarboxylase had a specific activity of 3.5. An ammonium sulfate suspension of the enzyme was dissolved in 0.05 *M* glutathione.

Procedure 2: This procedure is used if the enzyme preparation is free of lactic dehydrogenase and DPNH oxidase activity. The transcarboxylase reaction is coupled directly with the malic dehydrogenase reaction (reactions 4 and 5). The initial rate of DPNH oxidation is linear and proportional to the concentration of transcarboxylase, (see Fig. 2). The reaction was carried out at 23° in a glass cuvette (d = 0.5 cm) in a Zeiss spectrophotometer. The composition of the reaction mixture is given in the legend of Figure 2. This procedure was used routinely for assay of the enzyme from the cellulose column, Step 3, and all subsequent steps. One unit of enzyme was taken as the amount which catalyzes the formation of 1 μmoles of

oxaloacetate per minute. The specific activity of the enzyme is expressed as units per mg of protein.

Procedure 3: This procedure may be used if the enzyme is free of malic dehydrogenase and DPNH oxidase. This assay is not as accurate as that presented in Figure 2 because there is spontaneous breakdown of the oxaloacetate to pyruvate, and also the transcarboxylase may contain a trace of malic dehydrogenase. The correction for the spontaneous breakdown and for the malic dehydrogenase reaction with the oxaloacetate was determined by measuring the rate of DPNH oxidation in the presence of avidin, which inhibits the transcarboxylase. The rate of the DPNH oxidation obtained in the presence of avidin was subtracted from the DPNH oxidation observed in its absence, the difference was considered to be due to transcarboxylase activity. The reaction is carried out in glass cuvettes (d = 0.5 cm). The mixture contains the transcarboxylase, an excess of lactic dehydrogenase (2 units), 15 μmoles of Tris-HCl buffer, pH 7.0, 3 μmoles of glutathione, 0.3 μmoles of DPNH, and 1.2 μmoles of oxaloacetate. In addition, the control contains 90 μg of avidin. Both are incubated at room temperature for 4 min in order to permit inactivation of the transcarboxylase in the control by the avidin. The reaction is started by the addition of 0.5 μmoles of propionyl CoA to the reaction mixture. The final volume is 0.6 ml and the temperature is 23°.

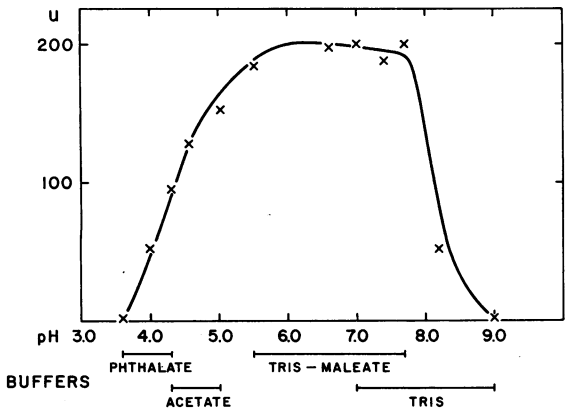

FIG. 3.—Effect of pH on the activity of methylmalonyl-oxaloacetic transcarboxylase. The transcarboxylase activity was measured by Procedure 1 (cf. text) using the following buffers: potassium phthalate, pH 3.6 to 4.3; sodium acetate, pH 4.3 to 5.0; Tris-maleic, pH 5.5 to 7.6; Tris-HCl, pH 7.0 to 8.2. The enzyme was a dialyzed fraction from a DEAE cellulose column and had a specific activity of 0.7. 53 μg of protein were used in the reaction.

Properties of Methylmalonyl-Oxaloacetic Transcarboxylase.—The purified protein was stored at 0° as a suspension in ammonium sulfate. Fresh preparations could be dissolved in water with no loss in activity provided glutathione was used in the reaction mixture, but after storage of the suspension for several weeks, it was necessary to dissolve it directly in 0.05 M glutathione to retain full activity. The glutathione must be freshly prepared, since a solution which was held at 0° and frozen when not in use became inhibitory even though much of the glutathione remained in the reduced form. Additional studies will be required to establish whether the enzyme is an —SH enzyme.

The enzyme has no metal ion or cofactor requirements.

pH optimum: The transcarboylase has a very broad pH range for maximum activity. There is little change in activity between pH 5.5 and 7.8 (see Fig. 3). Assay Procedure 1 was used in this study in order to avoid the adverse effect of pH on the malic dehydrogenase.

Inhibition by avidin: Swick and Wood[1] found that avidin inhibited transcarboxylase activity in partially purified preparations. This inhibition has been con-

firmed with purified enzyme (specific activity, 7.0, Step 4, Table 1). The procedure adopted was similar to that described in the legend of Figure 1 except that the reaction was initiated by the addition of the methylmalonyl CoA. Two cuvettes were prepared with the reaction mixture plus 1.2 μg of enzyme. To one cuvette was added 30 μg of avidin, and both cuvettes were incubated for 4 min prior to the addition of methylmalonyl CoA. Under these conditions, 90 per cent inhibition of the transcarboxylation reaction was observed.

CoA ester specificity: The specificity of the enzyme for CoA esters was determined by using the CoA ester as carboxyl acceptor and oxaloacetate as the carboxyl donor.

oxaloacetate + CoA acceptor $\rightleftarrows$ pyruvate + carboxylated acyl CoA derivative

This procedure eliminated the possible effects of the unnatural isomers which are obtained by chemical synthesis of the CoA ester of the dicarboxylic acid. Furthermore, the homologues of propionyl CoA are more readily prepared than are the homologues of methylmalonyl CoA. The rate of reaction was followed by determining the rate of formation of pyruvate using lactic dehydrogenase and DPNH (Procedure 3). The results of this survey are presented in Table 2. There is considerable oxidation of DPNH in the presence of avidin because the oxaloacetate breaks down spontaneously to pyruvate which is then reduced by lactic dehydrogenase. The transcarboxylase activity is calculated on the basis of the difference in ΔOD. The transcarboxylase clearly has a broad specificity for CoA esters as the carboxyl acceptors. Acetyl CoA is approximately half as effective as propionyl CoA. Butyryl CoA also serves as an acceptor and acetoacetyl CoA appears to act slowly. It would be of interest to determine the product of the latter reaction; it could be either acetomalonyl CoA or β-ketoglutaryl CoA. Because of the low activity of transcarboxylase with acetoacetyl CoA as a substrate, it has not been possible to accumulate sufficient product to permit its identification.

TABLE 2

SUBSTRATE SPECIFICITY OF METHYLMALONYL-OXALOACETIC TRANSCARBOXYLASE WITH CoA ESTERS AS THE CARBOXYL ACCEPTORS AND OXALOACETATE AS THE CARBOXYL DONOR

	Acetyl CoA	Propionyl CoA	Butyryl CoA	Acetoacetyl CoA
ΔOD per min* (no avidin)	0.075	0.060	0.073	0.060
ΔOD per min* (plus avidin)	0.025	0.015	0.035	0.040
Difference in ΔOD per min	0.050	0.045	0.038	0.020
μg of protein	6	3	24	48
Specific activity (μmoles/min/mg protein)	1.6	2.9	0.27	0.08

* Average of first 2 min. The rate was not completely linear in this assay.
Assay by Procedure 3 (see text) 0.6 ml in cuvette (d = 0.5 cm) and 0.5 μmoles of each CoA ester per 0.6 ml. Doubling the concentration of CoA ester did not significantly alter the rate of the reaction. The enzyme was from Step 4 and had a transcarboxylase specific activity of 4.8 when assayed as described in Figure 2. The enzyme contained some malic dehydrogenase (specific activity 0.04) and part of the reading in the presence of avidin is due to its activity.

The products from the other CoA esters were investigated by preparation of the hydroxamic acid derivatives, chromatographing and determining the R_f values. The results are shown in Table 3. It is seen that two hydroxamic derivatives were obtained from each reaction mixture and that one hydroxamate had an R_f corresponding to the hydroxamic acid derivative of the substrate. The second hydroxamate had a lower R_f than that of the substrate. This is to be expected if the

product is a CoA ester of a dicarboxylic acid. The identification of the products is not conclusive but is consistent with the view that the products are malonyl CoA, methylmalonyl CoA, and ethylmalonyl CoA, respectively.

TABLE 3

R_f VALUES OF THE HYDROXAMIC DERIVATIVES FROM TRANSCARBOXYLASE REACTIONS WITH OXALOACETATE AS THE CARBOXYL DONOR AND ACETYL CoA OR PROPIONYL CoA, OR BUTYRYL CoA AS THE ACCEPTORS

Substrate	Acetyl CoA R_f	Propionyl CoA R_f	Butyryl CoA R_f
Hydroxamate from anhydride of substrate	0.25	0.43	0.59
Hydroxamates from transcarboxylase reactions	0.25	0.43	0.59
	0.12	0.25	0.41

The reaction mixture contained in μmoles: oxaloacetate, 4.8; Tris-HCl buffer pH 7, 60.0; glutathione, 12; DPNH, 5.5; acetyl CoA, 2.0 or propionyl CoA 1.8, or butyryl CoA, 3.1; 0.1 units of transcarboxylase (Table 1, Step 4, 48–60), and an excess of lactic dehydrogenase (5 units). Total volume 2.4 ml. Incubated 10 min at 30°C. The reaction was stopped by the addition of 1 ml of hydroxylamine solution (28 per cent H_2NOH-HCl and 3.5*N* NaOH, 1 to 1).

The hydroxamic acid derivatives were prepared by the procedure of Stadtman and Barker.[11] The samples were evaporated to dryness and then taken up in absolute ethanol, centrifuged, and spotted on Whatman No. 1 paper, and chromatographed in isoamyl alcohol, formic acid, and water (3:1:3).[12]

Direct evidence that ethylmalonyl CoA is active in the transcarboxylase reaction was obtained using ethylmalonyl CoA as the carboxyl donor to pyruvate in place of methylmalonyl CoA (procedure of Fig. 2). Ethylmalonyl CoA was utilized at about $^1/_7$ the rate of methylmalonyl CoA.

Keto acid specificity: The next higher homologue of pyruvic acid is α-keto butyrate. If this acid acted as a carboxyl acceptor, one would expect to obtain α-methyl-oxaloacetate (reaction 8) which might be reduced by malic dehydrogenase (reaction 9) as shown in the following hypothetical sequence:

$$\begin{array}{l} \mathrm{CH_3{-}\overset{\displaystyle COO^-}{\overset{|}{C}}H{-}COCoA + CH_3CH_2COCOO^- \rightleftarrows} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \mathrm{CH_3CH_2COCoA + CH_3{-}\overset{\displaystyle COO^-}{\overset{|}{C}}H{-}CO{-}COO^-} \qquad (8) \\ \mathrm{CH_3{-}\overset{\displaystyle COO^-}{\overset{|}{C}}H{-}CO{-}COO^- + DPNH + H^+ \rightleftarrows} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \mathrm{CH_3{-}\overset{\displaystyle COO^-}{\overset{|}{C}}H{-}CHOH{-}COO^- + DPN^+} \qquad (9) \end{array}$$

Attempts to substitute α-ketobutyrate for pyruvate and to link the reaction with malic dehydrogenase have not been successful even with a very large amount of transcarboxylase and malic dehydrogenase In the absence of information on the activity of malic dehydrogenase with α-methyl oxaloacetate, this result is not conclusive.

It will be shown that the equilibrium constant of the transcarboxylase reaction with methylmalonyl CoA, pyruvate, propionyl CoA, and oxaloacetate is approximately 2. Therefore, if the transcarboxylase reaction occurred with α-keto-butyrate, one would expect a substantial formation of propionyl CoA and disap-

pearance of α-ketobutyrate. Since lactic dehydrogenase acts on α-ketobutyrate, it was possible to utilize this enzyme to determine the α-ketobutyrate concentration. Therefore, 0.8 μmoles of methylmalonyl CoA and 0.4 μmoles of α-ketobutyrate were incubated for 30 min with 0.7 units of transcarboxylase in 0.35 ml of 0.05 *M* Tris-HCl buffer, pH 7 containing 0.005 *M* glutathione. A second reaction mixture containing avidin in addition to the above reagents and a third system without the transcarboxylase were also prepared. After the incubation, the mixtures were deproteinized with trichloroacetic acid and the α-ketobutyrate present in the protein-free trichloroacetic acid extract was determined. The α-ketobutyrate concentration was found to be the same in all three samples.

The above reaction was investigated further by preparing the hydroxamic derivatives from the reaction products and isolating them by paper chromatography. Under these conditions, it could be demonstrated that there was formation of propionyl CoA when pyruvate was the carboxyl acceptor, but α-ketobutyrate, α-ketovalerate, β-ketoglutarate, and α-ketoglutarate failed to act as acceptors and thus to promote the formation of propionyl CoA from methylmalonyl CoA. Thus far, it appears that the transcarboxylase is quite specific for pyruvate as a carboxyl acceptor. Presumably, it also is specific for oxaloacetate as a carboxyl donor to the acyl CoA compounds. Because the enzyme is not specific for the acyl CoA compound, an abbreviated name for this enzyme might most properly be oxaloacetic transcarboxylase.

Stoichiometry of the methylmalonyl–oxaloacetic transcarboxylase reaction: The stoichiometry of the transcarboxylase reaction was determined by using propionyl CoA and oxaloacetate as substrates. Prior to this study, it was established that the transcarboxylase (Table 1, Step 4, 48–60) was free of thiolesterase. This was ascertained by demonstrating that there was no change in the absorption at 235 mμ when the enzyme was incubated with methylmalonyl CoA and propionyl CoA.

The data shown in Table 4 were obtained by determining the initial and final concentrations of the substrates. Oxaloacetate, pyruvate, and propionyl CoA were measured in one aliquot of the deproteinized reaction mixture using sequentially malic dehydrogenase, lactic dehydrogenase and then propionyl carboxylase, pyruvokinase, and lactic dehydrogenase. The determination of propionyl CoA is based on the assay of Tietz and Ochoa.[13]

The methylmalonyl CoA was determined in a second aliquot of the deproteinized solution after removing the residual oxaloacetate with malic dehydrogenase. This was done by adding transcarboxylase, pyruvate, and DPNH and measuring the oxaloacetate which was generated from the methylmalonyl CoA (see reactions 4 and 5).

It was found advantageous to start from the propionyl CoA side of the reaction because one obtains the natural form of methylmalonyl CoA. It has been found difficult to determine with precision synthetically prepared methylmalonyl CoA by the transcarboxylase reaction, probably because the unnatural isomer is not utilized by the enzyme and may even be a competitive inhibitor.

The stoichiometry of the transcarboxylase reaction is such that the decrease in the propionyl CoA concentration should be equivalent to the decrease in the oxaloacetate concentration and equal to a corresponding increase in the methylmalonyl CoA and pyruvate. Table 4 shows that this requirement is met. The results

support the hypothesis that the transcarboxylation reaction proceeds according to reaction (1).

TABLE 4

STOICHIOMETRY OF THE METHYLMALONYL-OXALOACETIC TRANSCARBOXYLASE REACTION

(propionyl CoA + oxaloacetate ⇄ methylmalonyl CoA + pyruvate)

	Time of incubation (*min*)	Propionyl CoA (*μmole*)	Difference from initial propionyl CoA (*μmole*)	Methyl-malonyl CoA (*μmole*)	Oxalo-acetate (*μmole*)	Difference from initial oxaloacetate (*μmole*)	Pyruvate (*μmole*)
1	0	0.66	—	0.00	0.34	—	0.03
2	5	0.40	0.26	0.22	0.13	0.21	0.23
3*	5	0.95	0.37	0.36	0.31	0.37	0.40

* Initial amounts of substrates were doubled.

The reaction mixture (0.5 ml) contained in μmoles: Tris-HCl buffer, 15; glutathione, 3.0; 0.15 units of transcarboxylase (Table 1, Step 4, 48 to 60) and propionyl CoA and oxaloacetate (pyruvate by spontaneous breakdown) as shown at 0 min. In No. 3, the concentration of substrates was doubled. The reactions were stopped by addition of 0.3 ml 10 per cent TCA and were centrifuged. This was done in No. 1 just prior to the addition of the transcarboxylase. Incubation was at 30° for 5 min.

For determination of the oxaloacetate the reagents were as follows: 0.4 ml of the deproteinized samples of Nos. 1 and 2 and 0.2 ml of No. 3, 30 μmoles Tris-HCl buffer pH 7.0, 0.3 μmoles DPNH, Tris base 0.4 *M* (0.23 ml or 0.12 ml) to neutralize the TCA. The reaction was started with an excess of malic dehydrogenase. Volume = 0.78 ml., 0.5 cm cuvette. After completion of this reaction more DPNH was added and then an excess of lactic dehydrogenase was added for determination of the pyruvate. Volume = 0.81 ml. After this reaction was completed, the following reagents were added for determination of the propionyl CoA. 4 μmoles $MgCl_2$, 0.5 μmole of glutathione, 1 μmole of ATP, 7.5 μmoles of $KHCO_3$, 2 μmoles of phosphoenol pyruvate, 0.5 μmole of DPNH, an excess of pyruvokinase (5 units). The reaction was started with an excess propionyl carboxylase (5 units). Volume = 1.12 ml.

For determination of the methylmalonyl CoA the reagents were as follows: 0.3 ml of deproteinized Nos. 1 and 2 and 0.15 ml of No. 3, 30 μmoles of Tris buffer pH 7.0, 6 μmoles of pyruvate, 1.5 μmoles of glutathione, 0.2 μmole of DPNH, Tris base 0.4 *M* (0.17 ml or 0.085 ml) to neutralize the TCA and an excess of malic dehydrogenase (0.5 units) to remove the oxaloacetate. The reaction was started by addition of 0.3 units of transcarboxylase. Volume = 0.7 ml, 0.5 cm cuvette.

Equilibrium of the methylmalonyl-oxaloacetic transcarboxylase reaction: The equilibrium of the reaction was determined in a similar manner to the study of the stoichiometry except that both the concentrations of the enzyme and the incubation period were increased. These changes were made to insure that an equilibrium would be attained. Approximately the same values were obtained for the equilibrium constant in all three experiments (Table 5). The variation is considered to be within experimental error. That the variation is caused by experimental error and not by failure to attain equilibrium is apparent since the calculated equilibrium is further toward pyruvate and methylmalonyl CoA in experiment 2 than it is in experiment 1. If equilibrium had not been attained, one would have expected the opposite result, since in experiment 2 there was double concentration of substrates and the initial substrates were oxaloacetate and propionyl CoA.

TABLE 5

EQUILIBRIUM OF THE METHYLMALONYL-OXALOACETIC TRANSCARBOXYLASE REACTION

(oxaloacetate + propionyl CoA ⇄ pyruvate + methylmalonyl CoA)

	Time of incubation (*min*)	Oxaloacetate (OA) (*μmole*)	Propionyl CoA (*μmole*)	Pyruvate (*μmole*)	Methylmalonyl CoA (MMCoA) (*μmole*)	$\frac{[Pyruvate][MMCoA]}{[OA][Propionyl\ CoA]}$ K_{eq}
1	10	0.181	0.306	0.362	0.275	1.80
2*	10	0.384	0.585	0.723	0.586	1.89
3*	20	0.356	0.567	0.723	0.568	2.02

* The amounts of substrates were twice those in No. 1.

The reaction mixture in No. 1 contained 15 μmoles of Tris-HCl buffer, 3.0 of glutathione, 0.63† μmoles of propionyl CoA, 0.51† μmoles of oxaloacetate (0.04† μmole of pyruvate) and 0.75 units of transcarboxylase (Sp. Act. 6.6, Step 4 30–60 $(NH_4)_2SO_4$ fraction). The concentrations of propionyl CoA and oxaloacetate were doubled in No. 2 and 3. Volume = 0.6 ml. Temperature of incubation was 30°, pH = 6.5. The reaction was stopped by addition of 0.4 ml of 10 per cent TCA.

The products were determined as described in the legend of Table 4.

† Values calculated from determination done directly on the solution of the substrate.

Using a value of 1.9 for the equilibrium constant, the ΔF°_{303} for the transcarboxylase reaction is calculated to be -3.9×10^2 calories at pH 6.5.

$$\Delta F^\circ = \text{RT } 2.303 \log \text{K}$$

$$\Delta F^\circ_{303} = -1.987 \times 303 \times 2.303 \log 1.9 = -3.9 \times 10^2 \text{ calories}$$

Discussion.—It has been possible by chromatography on cellulose and by ammonium sulfate fractionation to obtain the enzyme methylmalonyl-oxaloacetic transcarboxylase free from malic dehydrogenase, lactic dehydrogenase, DPNH oxidase, and deacylase. This achievement has permitted the development of an optical assay for transcarboxylase by coupling the reaction with either malic dehydrogenase or lactic dehydrogenase. It also has provided an enzymatic method for the determination of methylmalonyl CoA.

The transcarboxylase appears to have a broad specificity for the CoA esters. This is a very interesting characteristic because it permits the carboxyl group of oxaloacetate arising in the Krebs cycle or elsewhere to be utilized in fatty acid synthesis and vice versa. For example, malonyl CoA is believed to be an intermediate in the synthesis of fatty acids; clearly, the propionic acid bacteria could form malonyl CoA via oxaloacetic transcarboxylation instead of by direct carboxylation with CO_2. It is interesting to consider the possibility that other decarboxylations and carboxylations may be found to be coupled reactions and that methylmalonyl-oxaloacetic transcarboxylase could be but one example of a group of transcarboxylases. At one time, the only type of heterotrophic CO_2 fixation that was known was that of the propionic acid bacteria. Now many types of CO_2 fixation are known. The same development seems possible for transcarboxylation mechanisms. From the point of view of economy and control of cellular reactions, it would seem advantageous to transfer carboxyl groups just as ester phosphates are transferred. Carboxyl groups like phosphate anhydrides could then be generated and utilized in coupled reactions instead of being generated each time that they are required.

The question of whether or not transcarboxylase in propionibacteria is a single enzyme is not answered with certainty. Ultracentrifugation of our best transcarboxylase preparations shows that they contain several proteins. The transcarboxylase activity appears to be associated with a high molecular weight fraction. Even if transcarboxylation does not occur via a single enzyme, the mechanism is of great interest because it would involve the coupling of enzyme reactions in which there was a transfer of carboxyl groups. An understanding of this mechanism of transfer would also be of great theoretical importance. Furthermore, the role of biotin in this transfer is an important consideration. Obviously, in the case of transcarboxylase, if there is an enzyme-biotin-CO_2 complex,[14-16] it is formed without the intervention of ATP.

Preliminary studies with propionyl carboxylase and transcarboxylase have shown that CO_2 is not transferred directly from propionyl carboxylase to transcarboxylase. Nor is there a significant transfer even when a large amount of biotin is added. Only when propionyl CoA is added do the two enzymes couple. In this case, the effect of the two enzymes is to convert pyruvate and CO_2 to oxaloacetate and the propionyl CoA acts catalytically.

$$ATP + CO_2 + \text{propionyl CoA} \rightleftarrows \text{methylmalonyl CoA} + ADP + P_i$$

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

$$\text{Sum:}\quad ATP + CO_2 + \text{pyruvate} \rightleftarrows \text{oxaloacetate} + ADP + P_i$$

Neither the transcarboxylase nor the propionyl carboxylase alone will bring about this conversion. This synthesis of oxaloacetate serves to illustrate how transcarboxylase can serve in promoting synthesis through transfer of carboxyls which arise in other reactions. The combined action of the two enzymes is comparable to that of the enzyme from liver mitochondria which has recently been described by Utter and Keech.[17] This enzyme converts ATP, CO_2, and pyruvate to oxaloacetate, ADP, and P_i, requires a catalytic amount of acetyl CoA (or propionyl CoA), and is inhibited by avidin. It does not exhibit transcarboxylase activity and appears to be a "double-headed" enzyme which gives the same over-all results as propionyl carboxylase and methylmalonyl-oxaloacetic transcarboxylase.

The free energy of the transcarboxylase reaction is -3.9×10^2 calories. Consequently, there can be an easy flow of carboxyl groups in either direction; the direction of the flow depending on which compounds are continuously being removed or regenerated.

Summary.—1. The enzyme system which catalyzes the reversible transfer of carboxyl groups from methylmalonyl CoA to pyruvate to form oxaloacetate and propionyl CoA has been purified from propionibacteria. The reaction is probably catalyzed by a single enzyme which is referred to as methylmalonyl-oxaloacetic transcarboxylase. The enzyme may contain biotin, since it is inhibited by avidin. No added cofactors are required for the reaction.

2. The enzyme has a broad specificity for the CoA component. With oxaloacetate as the carboxyl donor acetyl CoA, propionyl CoA, butyryl CoA, or acetoacetyl CoA will serve as acceptor. The specificity for the keto acid component is narrow. Pyruvate was the only keto acid found capable of acting as a carboxyl acceptor from methylmalonyl CoA.

3. The equilibrium constant of the reaction was found to be 1.9 at pH 6.5 and 30° and the $\Delta F°_{303}$ is calculated to be -3.9×10^2 calories.

4. The possible role of transcarboxylase in promoting synthesis by transferring carboxyl groups between different pathways of metabolism is discussed.

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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 publication of this series was by Swick and Wood.[1] Abbreviations used are: CoA (coenzyme A), ATP (adenosine triphosphate), DPNH (reduced diphosphopyridine nucleotide), Tris (Tris(hydroxymethyl)methylamine), E (enzyme), DEAE-cellulose (diethylaminoethylcellulose), EDTA (ethylene diamine tetraacetic acid), TCA (trichloroacetic acid), and OD (optical density at 340 mμ).

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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