PURIFICATION AND PROPERTIES OF ENZYMES INVOLVED IN THE PROPIONIC ACID FERMENTATION[1]

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ABSTRACT

ALLEN, S. H. G. (Western Reserve University, Cleveland, Ohio), R. W. KELLERMEYER, R. L. STJERNHOLM, AND HARLAND G. WOOD. Purification and properties of enzymes involved in the propionic acid fermentation. J. Bacteriol. 87:171–187. 1964.—Chromatographic procedures are described for the separation and purification of phosphotransacetylase, acetyl kinase, malic dehydrogenase and coenzyme A (CoA) transferase. Purity of the enzymes was judged by homogeneity in an ultracentrifuge and by specific activity. Phosphotransacetylase was obtained 85% pure with a specific activity of 27.1. The preparation of acetyl kinase was a homogeneous protein with a specific activity of 531. The malic dehydrogenase likewise was homogeneous with a specific activity of 938. The CoA transferase, which was about 56% pure with a specific activity of 42.6, is the purest preparation of this enzyme yet described. The pH optimum was 6.5 to 7.8, and the K_m for succinyl-CoA in the transfer of CoA to acetate was found to be 1.3×10^{-4} M; for acetate, in the same transfer, the K_m was 7.0×10^{-3} M; for succinyl-CoA to propionate it was 6.8×10^{-5} M, and for propionate, in the same reaction, 6.2×10^{-4} M. Methods are described for the enzymatic production of methylmalonyl-CoA, malonyl-CoA, propionyl-CoA, acetyl-CoA, and succinyl-CoA. The role of these enzymes in the propionic acid fermentation as well as the possible mechanism responsible for the high yields of adenosine triphosphate from glucose are considered.

The formation of propionate by propionibacteria has been extensively investigated, and the pathway is known to involve a number of enzymes which have been purified and studied. These include methylmalonyl-oxaloacetic transcarboxylase (Swick and Wood, 1960; Wood and Stjernholm, 1961; Wood et al., 1963), methylmalonyl isomerase (Swick and Wood, 1960; Stadtman et al., 1960; Eggerer et al., 1960; Stjernholm and Wood, 1961; Overath et al., 1962b; Phares, Long, and Carson, 1962; Hegre, Miller, and Lane, 1962; Kellermeyer et al., J. Biol. Chem. *in press*), and methylmalonyl racemase (Mazumder et al., 1961; Allen et al., 1962; Overath et al., 1962b; Mazumder et al., 1962; Allen et al., 1963). Other enzymes which are present but have not previously been purified from propionibacteria include phosphotransacetylase (Stadtman, 1952), acetyl kinase (Rose et al., 1954), malic dehydrogenase (Ochoa, 1955), and coenzyme A (CoA) transferase (Stadtman, 1953). [The enzyme from propionibacteria has an equal activity on propionate and acetate, and we previously have referred to it as propionyl kinase (Kellermeyer and Wood, 1962; Wood et al., 1963). In the fermentation, the enzyme most likely catalyzes the formation of acetate (see Fig. 4), and the name acetyl kinase seems preferable.]

This paper describes the extensive purification of these enzymes, using in part methods already described for methylmalonyl-oxaloacetic transcarboxylase, methylmalonyl isomerase, and methylmalonyl racemase. By coupling purified preparations of CoA transferase, methylmalonyl isomerase, methylmalonyl racemase, methylmalonyl-oxaloacetic transcarboxylase, and malic dehydrogenase, a partial reconstitution of the propionate fermentation was achieved. Synthesis of acyl-CoA esters can be affected by linking various combinations of these enzymes. The yields of CoA esters from free CoA are such as to make their enzymatic synthesis practical.

MATERIALS AND METHODS

Oxaloacetic acid, sodium pyruvate, β-reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide (NAD+) and tris(hydroxymethyl)aminomethane (tris) buffer were from Sigma Chemical Co., St. Louis, Mo.;

reduced monosodium glutathione, pyruvic kinase, lactic dehydrogenase, and phosphoenolpyruvate were from Calbiochem; adenosine triphosphate (ATP) was from Pabst Laboratories; and CoA was from either Pabst Laboratories or Formochimica Cutolo Calosi SPA, Naples, Italy. The 5,6 - dimethylbenzimidazolyl-cobamide (DBC) was a generous gift of Karl Folkers of Merck Sharp and Dohme, Rahway, N.J. The diethylaminoethyl (DEAE) cellulose (type 40, 0.9 meq/g), the triethylaminoethyl (TEAE) cellulose (0.8 meq/g), and the cellulose phosphate (0.8 meq/g) were from the Brown Co., Boston, Mass.; all were washed before use with 0.1 N NaOH, 0.1 N HCl and water, and then were equilibrated with 0.05 M potassium phosphate buffer (pH 6.8). All chromatography was done at 4 C. The ammonium sulfate was the special enzyme grade from Mann Research Laboratories. Unless otherwise noted, protein was concentrated by bringing the solutions to 90% saturation with solid ammonium sulfate, and the precipitated protein was separated by centrifugation at 0 C at approximately 25,000 × g and was stored at −10 C. The concentration of ammonium sulfate in protein solutions was estimated with a purity meter (Barnstead Still and Sterilizer Co., Boston, Mass.), which had been precalibrated with known ammonium sulfate.

The CoA esters were chemically prepared by the method of Simon and Shemin (1953) as modified by Swick and Wood (1960), and were determined by the hydroxamate method of Lipmann and Tuttle (1945) or enzymatically as described below. Protein was estimated by the biuret reaction and by the spectrophotometric method of Layne (1957).

The enzyme assays were made at room temperature (25 C) unless otherwise stated. The specific activities of the enzymes are expressed in micromoles of product formed per minute per milligram of protein, and total units of enzyme are expressed as specific activity times total milligrams of protein in the fraction.

Sedimentation analyses were carried out with a Spinco model E ultracentrifuge at 5 C and at 60,000 rev/min.

Propionibacterium shermanii 52W was grown in a glucose-supplemented medium (Wood and Stjernholm, 1961) for 5 days in five 20-liter bottles, each containing 15 liters. The cells were then harvested in a Sharples centrifuge. Approximately 250 g (wet weight) of cells were obtained from 75 liters of medium.

The preparation of enzymes consisted of the following eight steps.

Step I: preparation of extract. A cell-free extract was prepared by use of glass beads (100 µ in diameter; type 130-5005; Minnesota Mining & Manufacturing Co., St. Paul, Minn.) and an Eppenbach colloid mill as previously described (Wood et al., 1963). The extract (560 ml) contained 15.8 g of protein, and was diluted six times with cold distilled water. Phosphotransacetylase, malic dehydrogenase, acetyl kinase, and CoA transferase activities in the extract were determined (Table 1). The extract also contained methylmalonyl-oxaloacetic transcarboxylase, methylmalonyl isomerase, and racemase; the purification of these enzymes from cell-free extracts has been reported (Wood et al., 1963; Kellermeyer et al., in press; Allen et al., 1963a).

Step II: absorption on DEAE cellulose and batch elution. The diluted cell-free extract described above was mixed with 2,200 ml of gravity-packed DEAE cellulose, and the mixture was stirred at 0 C for 2 hr. A portion was then centrifuged, and the clear supernatant solution was tested for enzyme activity. Very little protein remained in the solution (Table 1), and practically all the CoA transferase, phosphotransacetylase, malic dehydrogenase, and acetyl kinase were absorbed onto the DEAE cellulose. The mixture was then centrifuged in 1-liter plastic bottles at 3,000 × g for 15 min in an International PR-2 centrifuge at 0 C. The supernatant solution was discarded.

The DEAE cellulose was suspended in 3,000 ml of 0.05 M phosphate buffer (pH 6.8) and stirred for 20 min at 0 C. The mixture was centrifuged as before, and the supernatant solution, which contained little protein, was discarded. The DEAE cellulose was next treated for 20 min with 3,000 ml of 0.1 M phosphate buffer (pH 6.8), and the supernatant solution was found to contain 1,500 units of malic dehydrogenase and 2,125 units of phosphotransacetylase (Table 1, II DEAE 1st 0.1 M). The cellulose was next stirred for 8 hr in 3,000 ml of 0.1 M phosphate buffer (pH 6.8), and the supernatant solution was found to contain 3,900 units of malic dehydrogenase and 5,440 units of phosphotransacetylase. The protein in the two 0.1 M phosphate solutions which contained 31% of the phosphotransacetyl-

TABLE 1. *Absorption and batch elution of enzymes from DEAE cellulose with phosphate buffer (pH 6.8)*

Sample	Vol	Protein		Phosphotrans-acetylase		Malic dehydrogenase		Acetyl kinase		CoA transfer	
		Concn	Amt	SA*	Units*	SA*	Units*	SA*	Units*	SA*	Units*
	ml	*mg/ml*	*mg*								
I Cell-free extract	3,360	4.7	15,800	1.5	24,200	22.0	348,000	0.86	13,620	1.31	20,800
II DEAE supernatant solution	4,120	0.32	1,320	0	0	0.12	158	0.04	53	0	0
II DEAE 0.05 M	3,120	0.24	1,749	0	0	0.06	47	0.13	95	0	0
II DEAE 1st 0.1 M	3,050	0.48	1,465	1.45	2,125	1.03	1,500	0	0	0	0
II DEAE 2nd 0.1 M	3,020	0.41	1,244	4.40	5,440	3.11	3,900	0	0	0	0
II DEAE 1st 0.3 M	3,890	1.43	5,570	1.35	7,540	38.0	212,000	1.58	8,800	2.20	12,300
II DEAE 2nd 0.3 M	3,240	0.67	2,180	0.61	1,340	29.5	64,400	1.05	2,300	2.32	5,040
II DEAE 0.5 M	2,450	0.53	1,300	0.40	517	14.4	18,700	0.57	737	1.79	2,340
Total recovery, units			13,828		16,962		300,705		11,985		19,680
Total recovery, %			88		70		87		88		95

* Specific activity (SA) of the enzymes is expressed in μmoles per min per mg of protein, and total units of enzyme as specific activity times total mg of protein in the fraction.

ase was concentrated by precipitation with ammonium sulfate and designated as II DEAE 0.1 M.

The remainder of the four enzymes, as well as the isomerase, racemase, and transcarboxylase, were removed from the DEAE cellulose by use of 0.3 M phosphate buffer. Two successive elutions at 0 C were employed. In the first, the DEAE cellulose was stirred in 3,900 ml of buffer for 8 hr; in the second, for 2 hr with 3,200 ml of buffer. The protein in these eluates was precipitated and designated DEAE 1st and 2nd 0.3 M (Table 1). It contained 38% of the phosphotransacetylase, 80% of the malic dehydrogenase, 81% of the acetyl kinase, and 83% of the CoA transferase.

The DEAE cellulose was then treated with 2,400 ml of 0.5 M phosphate buffer for 20 min at 0 C. A small amount of phosphotransacetylase, malic dehydrogenase, and acetylkinase, and a significant amount (11%) of the CoA transferase were in this fraction. This protein was designated II DEAE 0.5 M.

Step III: chromatography of the protein of fraction II DEAE 0.1 M on TEAE cellulose. The protein was dissolved in 43 ml of 0.05 M phosphate buffer (pH 7.3) and was dialyzed against 1,000 ml of 0.1 M phosphate buffer for 2 hr. Phosphotransacetylase activity was markedly reduced by prolonged dialysis. The concentration of ammonium sulfate was found to be 0.4 M after dialysis and was lowered by dilution to 0.05 M with cold distilled water. The protein (4.6 g in 496 ml) was absorbed on a column of TEAE cellulose (5.2 by 21 cm). The protein remained absorbed on the column during a brief washing with 0.05 M phosphate buffer. The elution was begun with 0.1 M phosphate buffer (pH 6.8) and ~30-ml fractions were collected every 20 min. Phosphotransacetylase (Table 2, III TEAE 0.1 M) appeared in fractions 18 to 33 (415 ml), which contained 697 mg of protein and 3,620 units of phosphotransacetylase (specific activity, 5.2). No methylmalonyl racemase and only a small amount of malic dehydrogenase was present in these fractions. Phosphate buffer (0.15 M, pH 6.8) was then added to the column, and the next 545 ml (fractions 34 to 53), which contained 974 units of phosphotransacetylase and 295 mg of protein, was labeled III TEAE 0.15 M (Table 2). This fraction also contained some methylmalonyl racemase (324 units; specific activity, 1.1) and a small amount of malic dehydrogenase. The major portion of the malic dehydrogenase was eluted with the 0.2 M phosphate buffer (pH 6.8). Fractions 79 to 90 (337 ml), which contained 113 mg of protein and 2,820 units of malic dehydrogenase were designated III TEAE 0.2 M (Table 2). The protein of all combined fractions was concentrated by use of 0.9% saturated ammonium sulfate.

Step IV: chromatography of protein of fraction II DEAE 0.3 M on cellulose phosphate. A tightly packed column of cellulose phosphate (5.3 by 26

TABLE 2. *Step III: partial separation of enzymes obtained in fraction II DEAE 0.1 M by chromatography on TEAE cellulose with phosphate buffer (pH 6.8)*

Fraction	Pooled fractions	Phosphotrans-acetylase		Methylmalonyl racemase		Malic dehydrogenase	
		SA*	Units	SA	Units	SA	Units
III TEAE 0.10 M	18–33	5.2	3,620	0	0	0.07	45
III TEAE 0.15 M	34–53	3.3	974	1.1	324	0.11	32
III TEAE 0.20 M	79–90	0.9	102	0.25	28	25	2,820

* Specific activity.

TABLE 3. *Step V: partial separation of enzymes obtained in fraction IV C-PO₄ 0.05 M by chromatography on TEAE cellulose with phosphate buffer (pH 6.8)*

Fraction	Pooled fractions	Phosphotrans-acetylase		Malic dehydrogenase		Acetyl kinase		CoA transferase	
		SA*	Units	SA	Units	SA	Units	SA	Units
V TEAE 0.1 M	35–50	13.0	2,244	0	0	0	0	0	0
V TEAE 0.10 M	30–34⎱	5.6	3,098	0.03	15	0	0	0	0
V TEAE 0.15 M	51–79⎰								
V TEAE 0.20 M	106–144	0.21	222	142	149,180	3.1	3,241	0.45	454
V TEAE 0.25 M	145–194	0.01	6	11.9	7,360	3.28	2,050	6.5	4,065

* Specific activity.

cm) was prepared and washed with 0.05 M phosphate buffer. The 7.7 g of protein in fraction II DEAE 0.3 M (Table 1) were dissolved in 0.05 M phosphate buffer (pH 7.4; final volume, 94 ml). The concentration of sulfate plus phosphate was lowered to 0.08 M by dilution with cold distilled water. The protein solution was adsorbed on the column, which was then washed with 0.05 M phosphate buffer (pH 6.8). The phosphotransacetylase, malic dehydrogenase, acetyl kinase, and CoA transferase were collected in the first 2,900 ml of the 0.05 M phosphate wash, which contained 3.8 g of protein. This material (IV C-PO₄ 0.05 M) was stored at −10 C and was used without further treatment in step V. The protein which remained absorbed onto the cellulose phosphate contained the methylmalonyl-oxaloacetic transcarboxylase and the methylmalonyl racemase. Their purification was described elsewhere (Wood et al., 1963; Allen et al., 1963a).

Step V: chromatography of fraction IV C-PO₄ 0.05 M on TEAE cellulose. The material from the previous step, IV C-PO₄ 0.05 M, was placed on a TEAE-cellulose column (5.3 by 21 cm) and was washed with 0.05 M phosphate buffer (pH 6.8). The effluent was found to contain no protein. A 2-liter amount of 0.1 M phosphate buffer was next passed through the column. Protein was obtained in fractions 35 to 50, which contained 2,244 units of phosphotransacetylase with an average specific activity of 13.0 (Table 3, V TEAE 0.1 M). Further elution with 2 liters of 0.15 M phosphate buffer (pH 6.8) yielded protein containing phosphotransacetylase in fractions 51 to 79. These fractions were combined with fractions 30 to 34 from the 0.1 M eluate, since both fractions had comparable specific activities. Treatment with 0.2 M phosphate buffer (pH 6.8) gave a large protein peak, which contained practically all the malic dehydrogenase and a great deal of the acetyl kinase (V TEAE 0.2 M, Table 3). This fraction also contained a small amount of CoA transferase. Phosphate buffer (0.25 M, pH 6.8) was next passed through the column, and the protein peak which was designated V TEAE 0.25 M (Table 3) contained 4,065 units of CoA transferase, along with some malic dehydrogenase and considerable acetyl kinase.

Step VI: further purification of phosphotransacetylase from fractions III TEAE 0.1 M, III TEAE 0.15 M, V TEAE 0.1 M, and V TEAE 0.15 M. The combined fractions which were stored as the (NH₄)₂SO₄ precipitate were dissolved in 0.05 M tris-HCl buffer (pH 7.4) and adjusted to a con-
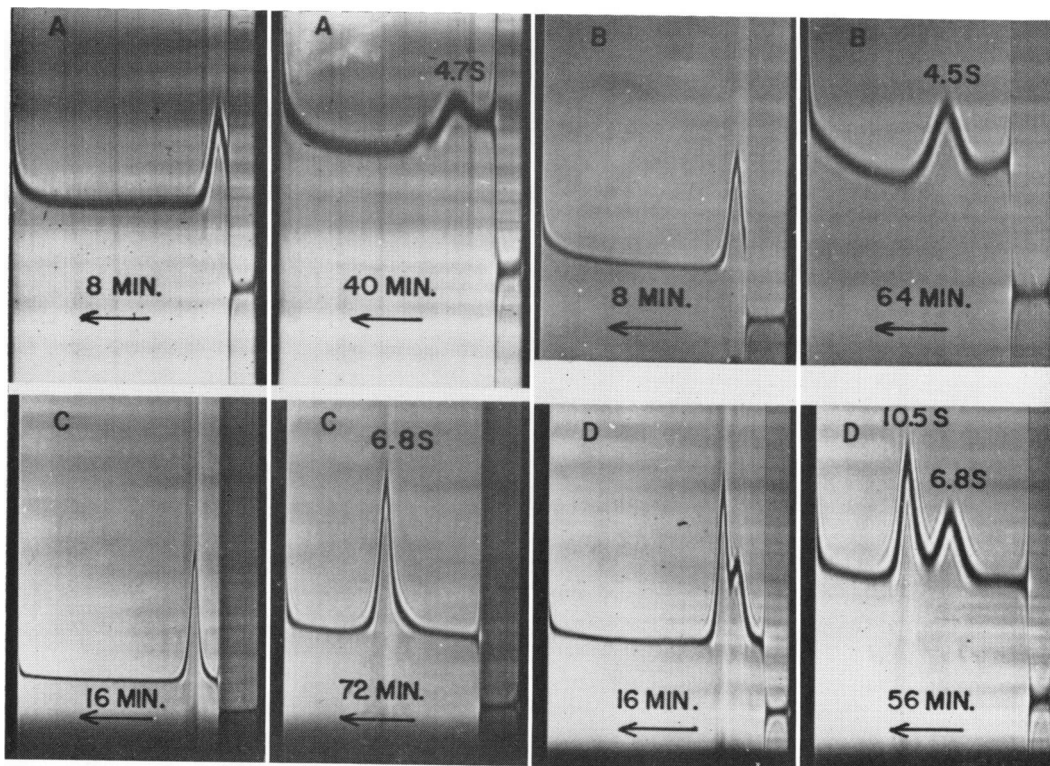
FIG. 1. *Sedimentation patterns from a Spinco model E ultracentrifuge at 5 C and 60,000 rev/min. (A) Phosphotransacetylase: the preparation had a specific activity of 27.1; the protein concentration was 2.9 mg/ml in 0.1 M potassium phosphate buffer (pH 7.2); the schlieren photographs were at 8 and 40 min at phase plate angles of 45° and 35°, respectively; and the S_{20} value of the major component was 4.7 S. (B) Malic dehydrogenase: the preparation had a specific activity of 938 and the protein concentration was 10 mg/ml in 0.1 M potassium phosphate buffer (pH 7.2); the photographs were at 8 and 64 min at phase plate angles of 55° and 35°, respectively; and the S_{20} was 4.5 S. (C) Acetyl kinase: the kinase had a specific activity of 531 and the protein concentration was 8.2 mg/ml in 0.1 M tris-HCl buffer (pH 7.4); the photographs were at 16 and 72 min at phase plate angles of 70° and 50°, respectively; and the S_{20} value was 6.8 S. (D) CoA transferase: the CoA transferase had a specific activity of 42.6, and the concentration was 7 mg/ml in 0.1 M potassium phosphate buffer (pH 7.2); the photographs were at 16 and 56 min at phase plate angles of 50° and 35°, respectively; and the S_{20} values for the two protein components were 6.8 and 10.5 S. The faster sedimenting peak ($S_{20} = 10.5$) is the CoA transferase, since it had become relatively larger than the other component ($S_{20} = 6.8$) during purification of the enzymes.*

centration of 10 mg per ml. The ammonium sulfate concentration was estimated with Nessler's reagent; the solution was adjusted to 45% saturation at 4 C by addition of saturated ammonium sulfate and was centrifuged. The ammonium sulfate fractionation was continued, and the precipitate obtained between 45 and 55% saturation contained the bulk of the phosphotransacetylase with a specific activity of 27.1. Figure 1A shows the sedimentation patterns obtained with this preparation of phosphotransacetylase. The bulk of the protein (85%) was located in one peak with

an S_{20} of 4.7 S. The preparation also contained a smaller component peak with a higher S_{20} value. It was assumed that the larger peak was phosphotransacetylase, but further purification was not attempted.

Step VII: further purification of malic dehydrogenase and acetyl kinase from Fraction V TEAE 0.2 M. A large quantity of malic dehydrogenase is present in propionic acid bacteria, and the enzyme is easily purified because it precipitates between 80 and 90% saturation of ammonium sulfate (Table 4). Combined fractions correspond-

TABLE 4. *Step VII: purification of malic dehydrogenase and acetyl kinase by ammonium sulfate fractionation*

Sample	Total protein	Malic dehydrogenase		Acetyl kinase	
		SA*	Units	SA	Units
	mg				
Original material	1,586	117	217,500	24.8	39,200
Ammonium sulfate					
0–30†	41.8	6.05	253	0.85	35
30–60	592	8.4	4,960	2.35	1,390
60–70	130	36.9	4,800	110.0	14,400
70–80	205	112	23,000	34.9	7,150
80–90	192	938	180,000	0.15	40
Total	1,161		213,013		23,015
Per cent recovery	73		98		59

* Specific activity.
† Per cent saturation.

ing to V TEAE 0.2 M (Table 3), which contained 1,586 mg of protein, were fractionated with ammonium sulfate. A saturated solution of ammonium sulfate at 4 C was used below 70% saturation, and solid ammonium sulfate above 70%. Little malic dehydrogenase activity was recovered below 70% saturation, and a highly purified enzyme was obtained between 80 and 90% saturation. The malic dehydrogenase (specific activity, 938) was homogenous in an ultracentrifuge, with a S_{20} value of 4.5 S (Fig. 1B).

The 60 to 70% ammonium sulfate fraction (Table 4) was dialyzed for 2 hr against 1 liter of 0.1 M tris-HCl buffer (pH 7.0) with a change of the buffer at 1 hr, and was placed onto a DEAE-cellulose column (1.8 by 15 cm), which had been previously equilibrated with 0.05 M phosphate buffer (pH 6.8). The acetyl kinase was eluted from the column with the 0.15 M buffer, and the fractions with the highest specific activity were pooled and precipitated with ammonium sulfate (90% saturation). The precipitate was dissolved in 0.01 M tris-HCl buffer (pH 7.4) and fractionated with saturated ammonium sulfate at 4 C. The fraction between 50 and 70% saturation contained acetyl kinase with a specific activity of 531, and a small amount of malic dehydrogenase (specific activity, 4.0). After dialysis against 1 liter of 0.01 M tris-HCl buffer (pH 7.4) for 6 hr, the preparation was tested for homogeneity in an ultracentrifuge. The results (Fig. 1C) show a single homogeneous peak with an S_{20} of 6.8 S.
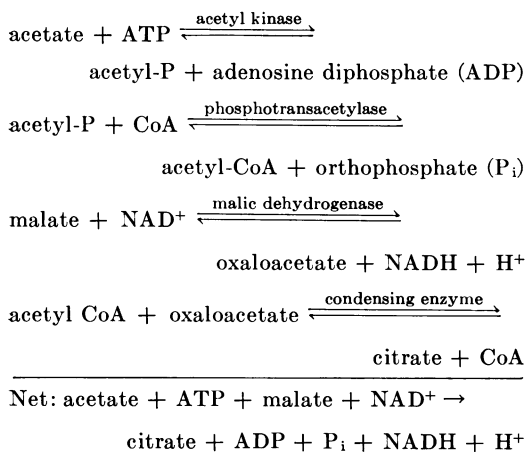
Step VIII: further purification of CoA transferase from fraction V TEAE 0.25 M. An ammonium sulfate fractionation was carried out on the fraction, V TEAE 0.25 M (Table 3), and the protein obtained between 60 and 90% saturation was dialyzed against 0.05 M tris-HCl buffer (pH 7.4) for 10 hr. Approximately 170 mg of protein with a CoA transferase specific activity of 22 were absorbed onto a TEAE-cellulose column (2 by 17 cm) and were eluted by a gradient obtained by addition of 0.5 M phosphate buffer (pH 6.8) to 500 ml of 0.1 M phosphate buffer at the same pH. The CoA transferase was obtained in a single protein peak. The fractions with the highest activity were pooled, and the protein was concentrated by ammonium sulfate precipitation (90% saturation). After dialysis against 0.1 M tris-HCl buffer (pH 7.0) containing 0.001 M reduced glutathione, the protein (57 mg with a specific activity of 29) was placed on a DEAE-cellulose column (2.2 by 15 cm) which had been equilibrated with 0.05 M phosphate buffer (pH 6.8). The protein was eluted by a gradient elution obtained by adding 0.5 M phosphate buffer (pH 6.8) to 500 ml of 0.1 M phosphate buffer (pH 6.8). Little protein was present in the first 144 ml of buffer. The next 77 ml contained the CoA transferase which was concentrated by ammonium sulfate precipitation (90% saturation) and then a 0 to 70% ammonium sulfate fraction was prepared. The precipitate contained transferase having a specific activity of 42.6. An ultracentrifuge analysis of the protein showed two protein peaks with S_{20} values of 6.8 S and 10.5 S (Fig. 1D). The faster sedimenting peak apparently was the CoA transferase, since it had become relatively larger than the other peak during purification of the transferase. It is possible that the

other protein is acetyl kinase, which has an S_{20} value of 6.8 S. Acetyl kinase has been somewhat difficult to completely separate from the transferase.
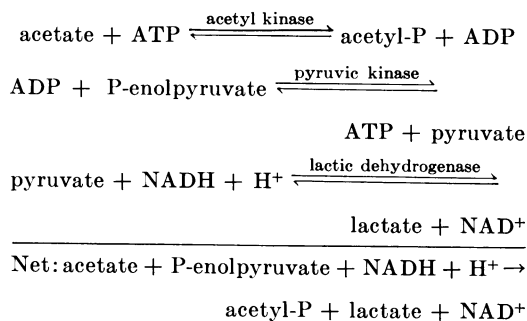
RESULTS

Spectrophotometric assay and properties of the enzymes. The assay for phosphotransacetylase consisted of the following series of reactions:

acetate + ATP $\xrightleftharpoons{\text{acetyl kinase}}$

acetyl-P + adenosine diphosphate (ADP)

acetyl-P + CoA $\xrightleftharpoons{\text{phosphotransacetylase}}$

acetyl-CoA + orthophosphate (P_i)

malate + NAD^+ $\xrightleftharpoons{\text{malic dehydrogenase}}$

oxaloacetate + NADH + H^+

acetyl CoA + oxaloacetate $\xrightleftharpoons{\text{condensing enzyme}}$

citrate + CoA

Net: acetate + ATP + malate + NAD^+ →

citrate + ADP + P_i + NADH + H^+

The assay contained (in μmoles/ml): tris-HCl buffer (pH 8.0), 80; L-malate, 8.0; reduced gluta-thione, 2.4; CoA, 0.16; acetate, 242; ATP, 4.0; $MgCl_2$, 4.0; and 0.4 units each of the enzymes acetyl kinase, malate dehydrogenase, and citrate-condensing enzyme. The condensing enzyme was prepared as described by Srere and Kosicki (1961) and had a specific activity of 4.0. The reaction was started with phosphotransacetylase. The enzyme was active on acetyl phosphate and propionyl phosphate; other substrates were not tested. The enzyme is extremely stable and has been stored at −10 C in 80% ammonium sulfate for 2 years with no loss of activity.
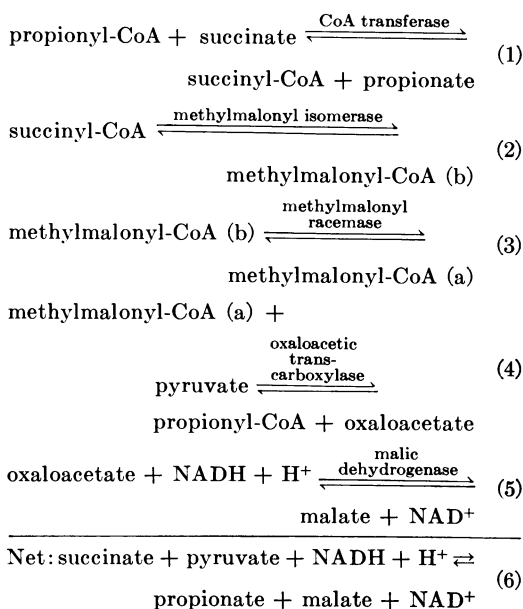
The assay for malic dehydrogenase was a modification of that described by Ochoa (1955), and contained (in μmoles/ml): tris-HCl or phosphate buffer (pH 7.4), 50.0; NADH, 0.25; and oxalo-acetate (pH 6.5), 2.0. The reaction was started by addition of the malic dehydrogenase. The highest specific activity obtained with the ho-mogeneous preparation was about 1,000. The value corresponds to that found for pure malic dehydrogenase from beef heart by Siegel and England (1961). The enzyme is very stable and has been stored in 90% saturation ammonium sulfate for 2 years at −10 C with no loss of ac-tivity.

Acetyl kinase was assayed by linking it to pyruvic kinase and lactic dehydrogenase as il-lustrated in the following reaction:

acetate + ATP $\xrightleftharpoons{\text{acetyl kinase}}$ acetyl-P + ADP

ADP + P-enolpyruvate $\xrightleftharpoons{\text{pyruvic kinase}}$

ATP + pyruvate

pyruvate + NADH + H^+ $\xrightleftharpoons{\text{lactic dehydrogenase}}$

lactate + NAD^+

Net: acetate + P-enolpyruvate + NADH + H^+ →

acetyl-P + lactate + NAD^+

The mixture contained (in μmoles per ml): tris-HCl buffer (pH 7.4), 81; ATP, 4.0; $MgCl_2$, 4.0; phosphoenolpyruvic, 1.6; acetate (or propionate) 81; NADH 0.4; and (in units) pyruvic kinase, 0.4; and lactic dehydrogenase 0.4. The reaction was started by addition of the acetyl kinase. A control without acetate was used to correct for the ADP present in the ATP preparations. The enzyme is extremely stable and has been stored at −10 C in saturated ammonium sulfate for 2 years with no loss of activity. The acetyl kinase utilized propionate and acetate as substrates but was not active on formate, succinate, and methyl-malonate. The activity on propionate was about 15% higher than that on acetate.
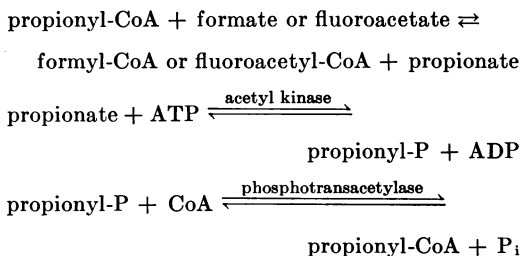
CoA transferase was assayed by the following sequence of reactions:

propionyl-CoA + succinate $\xrightleftharpoons{\text{CoA transferase}}$ (1)

succinyl-CoA + propionate

succinyl-CoA $\xrightleftharpoons{\text{methylmalonyl isomerase}}$ (2)

methylmalonyl-CoA (b)

methylmalonyl-CoA (b) $\xrightleftharpoons{\text{methylmalonyl racemase}}$ (3)

methylmalonyl-CoA (a)

methylmalonyl-CoA (a) +

pyruvate $\xrightleftharpoons{\text{oxaloacetic trans-carboxylase}}$ (4)

propionyl-CoA + oxaloacetate

oxaloacetate + NADH + H^+ $\xrightleftharpoons{\text{malic dehydrogenase}}$ (5)

malate + NAD^+

Net: succinate + pyruvate + NADH + H^+ ⇌

propionate + malate + NAD^+ (6)

The reaction mixture contained in (μmoles/ml): succinate, 6.8; pyruvate, 6.8; reduced glutathione, 3.2; phosphate buffer (pH 7.4), 33; NADH, 0.16, acetyl- or propionyl-CoA, 0.4; DBC, 0.004; and 0.4 units per ml each of the purified enzymes malic dehydrogenase, methylmalonyl isomerase, methylmalonyl racemase, and methylmalonyl-oxaloacetic transcarboxylase. The reaction was started by addition of CoA transferase. A control without CoA transferase was determined at the same time and substracted from values with added enzyme. The rate was proportional to the amount of the transferase, and the assay gave the same results with either acetyl- or propionyl-CoA.

Properties of CoA transferase. The purified CoA transferase catalyzed the reversible transfer of CoA from propionyl-CoA or acetyl-CoA to succinate and had the same activity with either acetyl- or propionyl-CoA, as measured by the coupled assay described above. The transfer from succinyl-CoA to form propionyl-CoA and acetyl-CoA also proceeded at approximately the same rates. The production of propionyl-CoA was assayed with propionyl carboxylase, while the production of acetyl-CoA was assayed with citrate-condensing enzyme as described under K_m determinations (Fig. 3).
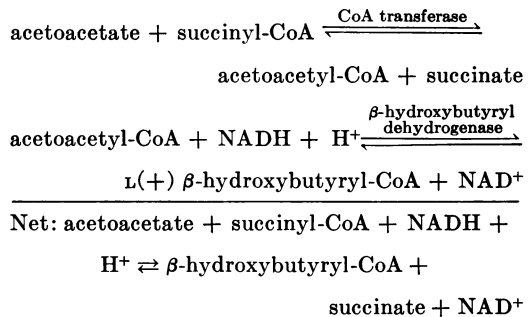
The transferase did not transfer CoA from propionyl-CoA to formate or fluoroacetate. This reaction was tested with an assay consisting of the following coupled reactions:

propionyl-CoA + formate or fluoroacetate $\rightleftarrows$

formyl-CoA or fluoroacetyl-CoA + propionate

$$propionate + ATP \xrightleftharpoons{\text{acetyl kinase}}$$

propionyl-P + ADP

$$propionyl\text{-}P + CoA \xrightleftharpoons{\text{phosphotransacetylase}}$$

propionyl-CoA + P_i

The ADP formed from the second reaction was assayed with pyruvic kinase and lactic dehydrogenase. There was no reaction with either formate or fluoroacetate, and when succinate was added at the termination of the experiment there was an immediate reaction. Succinate did not act as a substrate for the acetyl kinase.

The enzyme did not catalyze transfer of CoA from acetyl-CoA to methyl malonate. This was determined by a direct spectrophotometric assay of methylmalonyl-CoA by use of reactions 4 and 5

of the transferase assay. Neither did the transferase catalyze the formation of acetoacetyl-CoA from succinyl-CoA as measured by β-hydroxybutyryl dehydrogenase (Stern, 1957) by use of the following reactions:

$$acetoacetate + succinyl\text{-}CoA \xrightleftharpoons{\text{CoA transferase}}$$

acetoacetyl-CoA + succinate

$$acetoacetyl\text{-}CoA + NADH + H^+ \xrightleftharpoons{\substack{\beta\text{-hydroxybutyryl} \\ \text{dehydrogenase}}}$$

$$L(+)\ \beta\text{-hydroxybutyryl-CoA} + NAD^+$$

Net: acetoacetate + succinyl-CoA + NADH +

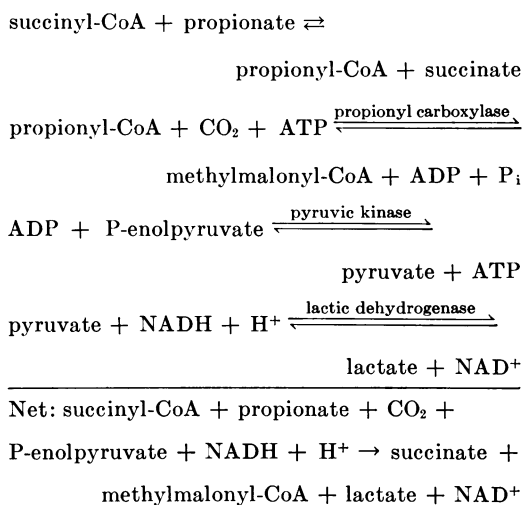H$^+$ $\rightleftarrows$ β-hydroxybutyryl-CoA +

succinate + NAD$^+$

Furthermore, the transferase was unable to catalyze the formation of succinyl-CoA from acetoacetyl-CoA and succinate, as assayed by steps 2, 3, 4, and 5 of the CoA transferase assay, nor did it catalyze the transfer of CoA from β-hydroxy-β-methylglutaryl-CoA (HMGCoA) to acetate. The formation of acetyl-CoA was measured with citrate-condensing enzyme as described under K_m determinations (Fig. 3). The enzyme is very stable and has been stored at -10 C in 60% ammonium sulfate for 2 years with little loss of activity.

Optimal pH. Since the pH optimum for the CoA transferase had not been reported in the literature, this property was tested under conditions described for its assay with a highly purified preparation of the enzyme (specific activity, 21.6). The pH of each mixture was determined in a cuvette at the end of the assay with microelectrodes on a Radiometer model 22 pH meter. The results (Fig. 2) show a pH optimum of between 6.5 and 7.8. Since the pH optima of the three other enzymes in the assay are quite broad (pH 6 to 8) and these enzymes are present in excess, the effect measured was assumed to be on the CoA transferase. To confirm this, a two-step assay was used in which only the pH of the CoA transferase reaction was varied. The reaction mixture contained (in μmoles): buffer, 15; succinate, 10; acetyl-CoA, 0.7; and 0.17 units of CoA transferase, in a total volume of 0.4 ml. Acetate, tris-maleate, and tris-HCl buffers were used. Incubation was at 30 C. The reaction was stopped by addition of 0.05 ml of 4 N perchloric acid, which was neutralized after 15 min at 0 C by 0.08 ml of 2 N

KOH. The precipitated protein was removed by centrifugation, and 0.3 ml of the solution was assayed for succinyl-CoA content enzymatically with methylmalonyl isomerase, transcarboxylase, and malic dehydrogenase as previously described (Stjernholm and Wood, 1961). The optimal pH was again found to be between 6.5 and 7.8 as in the coupled assay.

K_m *determinations.* The K_m for succinyl-CoA in the transfer to propionate was 6.8×10^{-5} M, and for propionate in the same reaction was 6.25×10^{-4} M (Fig. 3). The reaction involved the following sequence:

succinyl-CoA + propionate $\rightleftarrows$

propionyl-CoA + succinate

propionyl-CoA + CO_2 + ATP $\xrightarrow{\text{propionyl carboxylase}}$

methylmalonyl-CoA + ADP + P_i

ADP + P-enolpyruvate $\xrightarrow{\text{pyruvic kinase}}$

pyruvate + ATP

pyruvate + NADH + H^+ $\xrightarrow{\text{lactic dehydrogenase}}$

lactate + NAD^+

Net: succinyl-CoA + propionate + CO_2 +

P-enolpyruvate + NADH + H^+ → succinate +

methylmalonyl-CoA + lactate + NAD^+

A control without succinyl-CoA was used to correct for the ADP in the reagents.

The K_m for succinyl-CoA in the transfer to acetate was 1.3×10^{-4} M, and for acetate in the same reaction was 7.0×10^{-3} M (Fig. 3). With acetate as the CoA acceptor, the acetyl-CoA formed was assayed with citrate-condensing enzyme and malic dehydrogenase according to the following sequence:

succinyl-CoA + acetate $\rightleftarrows$

succinate + acetyl-CoA

malate + NAD^+ $\xrightarrow{\text{malic dehydrogenase}}$

oxaloacetate + NADH + H^+

acetyl-CoA + oxaloacetate $\xrightarrow{\text{condensing enzyme}}$

citrate + CoA

Net: succinyl-CoA + acetate + malate +

NAD^+ $\rightleftarrows$ succinate + citrate + NADH + H^+
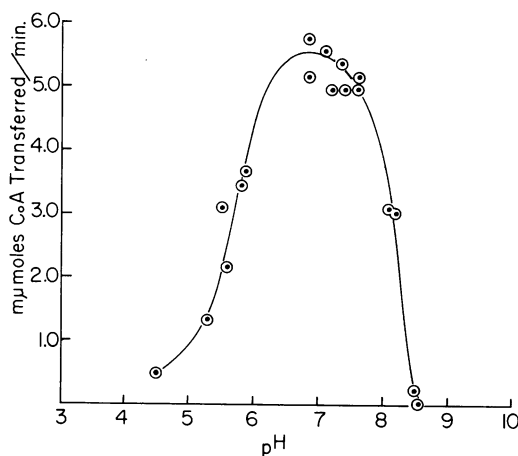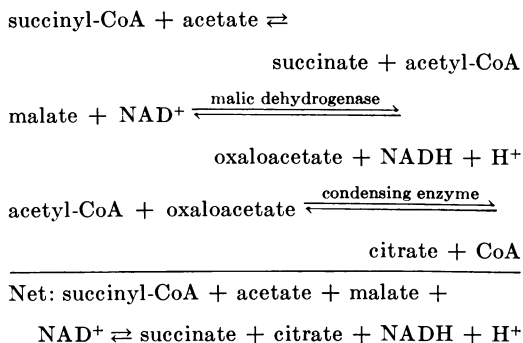
FIG. 2. *Effect of pH on the CoA transferase reaction. The mixture contained the same components as the assay of the CoA transferase in the text. The type of buffer was changed for each pH range, and 0.007 units of the transferase were used in 0.66 ml. The reaction was started by the addition of 0.47 μmoles of acetyl-CoA.*

The Lineweaver-Burke plots for these determinations are presented in Fig. 3A and B.

Enzymatic preparation of acyl-CoA esters. Malonyl-CoA, methylmalonyl-CoA, succinyl-CoA, and propionyl-CoA, as well as other acyl-CoA esters, can be produced with the battery of enzymes described above coupled with other enzymes of the propionic acid cycle.

Production of propionyl-1-C^{14}-CoA. Either propionyl-CoA or acetyl-CoA can be prepared by this procedure. As an example, the production of a specifically labeled propionyl-CoA will be presented. The incubation mixture contained (in μmoles): CoA, 200; propionate-1-C^{14} (215 μc/mole; New England Nuclear Corp., Boston, Mass.), 36.4; ATP, 200; $MgCl_2$, 200, reduced glutathione, 50. The pH of the reaction mixture was adjusted to 7.0 by addition of tris base, and then 40 units each of purified acetyl kinase (specific activity, 531) and phosphotransacetylase (specific activity, 27) were added. The final volume was 10.0 ml, and the mixture was incubated at 30 C. The propionyl-CoA was determined as follows:
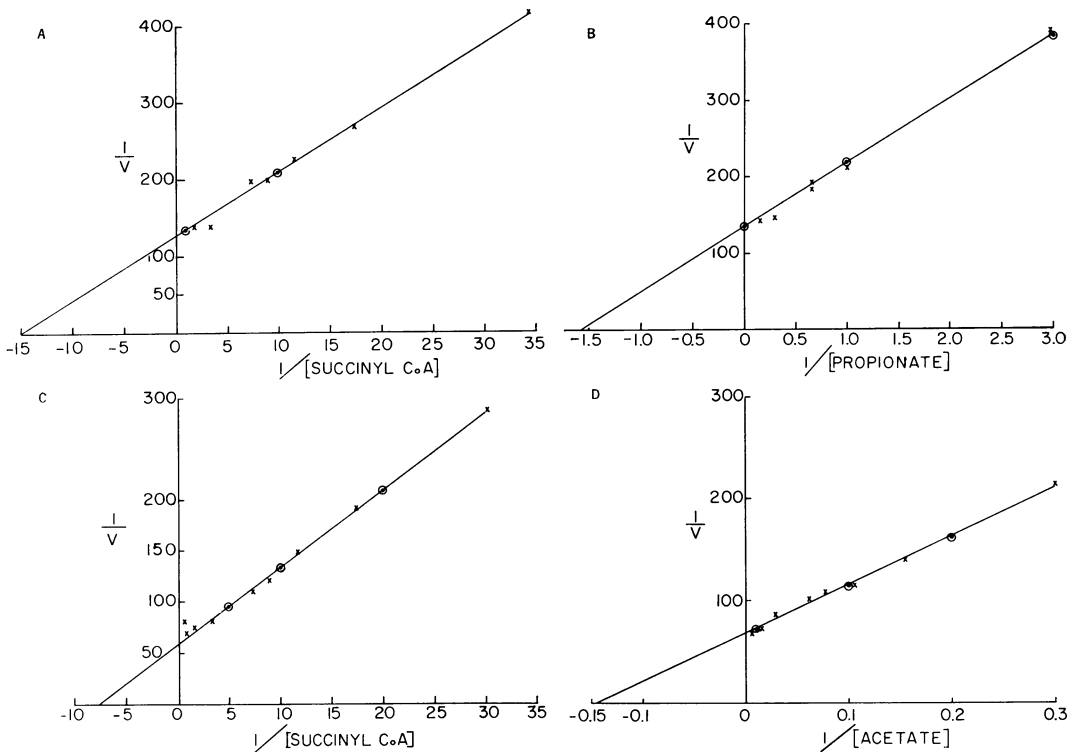
propionyl-CoA + oxaloacetate $\xrightarrow{\text{transcarboxylase}}$

methylmalonyl CoA + pyruvate

pyruvate + NADH + H^+ $\xrightarrow{\text{lactic dehydrogenase}}$

lactate + NAD^+
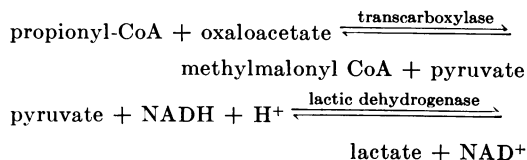
FIG. 3. *Lineweaver-Burke plots of the reaction velocities of CoA transferase with succinyl-CoA and acetate or propionate. (A) Succinyl-CoA with propionate as the CoA acceptor. The reaction at 25 C contained (in µmoles per ml): tris-HCl buffer (pH 7.8), 38.9; reduced glutathione, 3.9; KHCO₃, 84; phosphoenolpyruvate, 1.7; ATP, 5.6; MgCl₂, 11.2; NADH, 0.140; propionate, 6.5; KCl, 56; succinyl-CoA, 0.029 to 0.58; and (in units per ml) lactic dehydrogenase, 0.3; propionyl carboxylase, 0.3; pyruvic kinase, 0.3; and CoA transferase (specific activity = 6.6), 0.0088. The apparent K_m for CoA transfer to propionate is 6.8 × 10⁻⁵M. (B) K_m for propionate with succinyl-CoA. The reaction mixture was identical with A except that the succinyl-CoA concentration was 0.29 µmoles/ml, and the propionate concentrations were from 0.33 to 133 µmoles per ml. The K_m for propionate is 6.25 × 10⁻⁴ M. (C) K_m for succinyl-CoA with acetate as the CoA acceptor. The reaction at 25 C contained (in µmoles per ml): tris-HCl buffer (pH 8.0), 96.5; potassium acetate, 64.5; sodium malate, 6.45; reduced glutathione, 3.2; NAD⁺ 0.97; succinyl CoA, 0.029 to 2.32; and (in units per ml) citrate-condensing enzyme, 0.3; malic dehydrogenase, 0.3; and CoA transferase (specific activity = 10.7, 0.004). The apparent K_m for CoA transfer to acetate is 1.3 × 10⁻⁴ M. (D) K_m for acetate with succinyl-CoA. The reaction mixture was identical with C except that the succinyl-CoA concentration was 0.87 µmoles/ml, and the acetate concentrations were from 0.65 to 129 µmoles/ml. The K_m for acetate is 7.0 × 10⁻³ M. The succinyl-CoA was prepared chemically as described by Simon and Shemin (1953) and assayed enzymatically. Both reactions were linear with CoA transferase concentration when succinyl-CoA was present in excess. The circled dots in this figure represent straight line coordinates derived with the method of least squares to represent the data obtained experimentally.*

The cuvette contained 0.01 ml of the reaction mixture and (in µmoles): oxaloacetate 0.4; tris-HCl buffer (pH 7.4), 10.0; NADH, 0.075; and 0.1 unit each of lactic dehydrogenase and the transcarboxylase in 0.25 ml. The reaction was started with the transcarboxylase, and a control was performed at the same time to correct for spontaneous decarboxylation of oxaloacetate. The formation of propionyl-1-C¹⁴-CoA was complete in 60 min. The reaction was then stopped by addition of 2.5 ml of 2 N perchloric acid and was chilled at 0 C for 15 min. It was then neutralized to pH 7.0 with KOH, and the precipitate was removed by centrifugation, after which the pH was adjusted to 5.5. The average of two assays gave 40.4 µmoles of

propionyl-CoA, which is a recovery of 20% on the basis of the free CoA and 100% on the basis of propionate-1-C^{14}. Since propionyl-CoA was the only CoA ester formed, no further purification was carried out.

Production of methylmalonyl-CoA. Enzymatic synthesis of methylmalonyl-CoA was accomplished with the same reactions described for the production of propionyl-CoA, except that propionyl-CoA carboxylase (a kind gift of Y. Kaziro and S. Ochoa) was used in the terminal step to form methylmalonyl-CoA. In this synthesis, an ATP-regenerating system, phosphoenolpyruvate and pyruvate kinase, was also added to the reaction mixture. The incubation mixture contained (in mmoles): reduced glutathione, 0.22; potassium bicarbonate, 27.6; ATP, 0.9; MgCl$_2$, 0.97; CoA, 0.75; propionate, 11.0; phosphoenolpyruvate, 2.0; and (in units) pyruvic kinase, 180; phosphotransacetylase, 110; acetyl kinase, 110; and propionyl carboxylase, 20. The final volume was 44 ml, the pH was 7.5, and the incubation was at 30 C. The amount of methylmalonyl-CoA was determined in a portion at 30, 60, 120, 150, and 180 min, by use of transcarboxylase and malic dehydrogenase with pyruvate as the carboxyl acceptor. The cuvette contained (in μmoles/ml): pyruvate, 7.5; NADH, 0.09; tris-HCl (pH 7.5), 37.5; and reduced glutathione, 3.8; as well as malic dehydrogenase, 0.3 units per ml. The reaction was started by the addition of 0.3 units per ml of methylmalonyl-oxaloacetic transcarboxylase. As judged by the enzymatic assay for methylmalonyl-CoA, the reaction was complete in 150 min; it was terminated at 180 min with 2 N perchloric acid. The mixture was chilled at 0 C, and the pH of the solution was then adjusted to 6.8 with 10 N KOH. The mixture was filtered at 4 C, yielding a clear solution (106 ml). The amount of methylmalonyl-CoA formed was 615 μmoles, which represented a yield of 82% of the added CoA.

If all the enzyme preparations in the synthesis are free from methylmalonyl racemase (Allen et al., 1963a) production of only one isomer of methylmalonyl-CoA is accomplished. This isomer, designated as methylmalonyl-CoA (a) is the proper form of the substrate for both propionyl carboxylase and methylmalonyl-oxaloacetic transcarboxylase.

Methylmalonyl-CoA or malonyl-CoA can be prepared in a similar manner from propionate or acetate, respectively, with transcarboxylase and with oxaloacetate as the carboxylating agent in the place of propionyl carboxylase and CO$_2$. The transcarboxylase reaction is "pulled" toward synthesis of the methylmalonyl-CoA or malonyl-CoA with lactic dehydrogenase and NADH.

Succinyl-CoA can be prepared enzymatically from succinate with CoA transferase and acetyl-CoA. Since succinyl-CoA is not very stable even when stored at −10 C, it is convenient to generate it as needed in the reaction.

The various CoA esters formed enzymatically can be further purified by chromatography at 4 C. Malonyl-CoA has been separated from acetyl-CoA by ascending paper chromatography (Stadtman, 1957). Kaziro and Ochoa (1961) presented a convenient method, using chromatography on Dowex-50 resin (H$^+$ form), for the purification of methylmalonyl-CoA. Overath et al. (1962a) described the separation of methylmalonyl-CoA and propionyl-CoA by paper electrophoresis.

DISCUSSION

The purification procedures described above present a convenient way to isolate the various enzymes of the propionate fermentation. Table 5 summarizes the degree of purification achieved for the four enzymes described in this paper.

The phosphotransacetylase (Table 5) is similar to that described by Stadtman (1952) from *Clostridium kluyveri*. They assayed the enzyme by the arsenolysis reaction and observed a

TABLE 5. *Purity and activity of the enzymes isolated from Propionibacterium shermanii*

Enzyme	Step in prepn	Ultracentrifuge			Specific activity		Reference
		S_{20}	No. of peaks	Per cent purity	Observed value	Highest reported	
Phosphotransacetylase	VI	4.7	2	~85	27.1	63	Stadtman, 1952
Malic dehydrogenase	VII	4.5	1	~100	938	935–1,493	Siegel and England, 1961
Acetyl kinase	VII	6.8	1	~100	531	300	Rose et al., 1954
CoA transferase	VIII	10.5	2	~56	42.6	0.1	Barker et al., 1955

specific activity twice that of our preparation. Whether or not this reflects a higher degree of purification, a more active enzyme, or a difference in the assay is not known. The enzyme from *P. shermanii* was approximately 85% pure as judged by ultracentrifuge patterns. It appeared to be equally active with either propionyl phosphate or acetyl phosphate.

The malic dehydrogenase of *P. shermanii* is very active, and the original extracts of the organism have a specific activity of approximately 20. Purification of the enzyme can be accomplished quite easily with relatively good yields. In the procedure described, the overall recovery from the crude extract to the homogeneous enzyme was 52%. The enzyme compares in specific activity and S_{20} values with those reported from animal sources (Table 5). Siegel and Englard (1961) reported specific activities of 935 to 1,493 and $S_{20,w}$ values of 5.1 and 4.3 S.

The CoA transferase differs from the pig heart enzyme of Stern et al. (1955), since it does not catalyze the transfer of CoA from succinyl-CoA to acetoacetate. It apparently resembles the transferase found in *C. kluyveri* by Stadtman (1953), in *Micrococcus lactilyticus* by Whitely (1953b), and in *P. pentosaceum* by Delwiche, Phares, and Carson (1956). The present preparation has a much higher activity than those described previously (Table 5), but the ultracentrifuge patterns indicate that the enzyme is still only about 56% pure. The major contamination appears to be acetyl kinase. The pH optimum is 6.5 to 7.8; the apparent K_m for succinyl-CoA in the transfer to acetate was 1.3×10^{-4} M, while for the transfer to propionate it was 6.8×10^{-5} M. The specific activity of the enzyme was 38% higher with acetate than with propionate as the CoA acceptor. The two K_m values for succinyl-CoA agree well, considering that different assays were used. The major difference (tenfold) can be seen between the K_m values for acetate and propionate. A maximal velocity is difficult to obtain with acetate requiring a high acetate concentration. The enzyme did not catalyze the transfer of CoA from acetyl-CoA to methylmalonate, acetoacetate, formate, or fluoroacetate, or from HMGCoA to acetate. Since transferase from *C. kluyveri* is able to use formyl-CoA as a substrate, it differs in this respect from the enzyme described here.

The acetyl kinase was homogeneous in an ultracentrifuge and had a higher specific activity than that reported for the acetyl kinase from *Escherichia coli* by Rose et al. (1954). The latter was ten times more active with acetate than with propionate, while the enzyme from propionibacteria had the same activity on acetate or propionate. It was not active with succinate, methylmalonate, or formate.

The assay for CoA transferase (reactions 1 to 6) with purified oxaloacetic transcarboxylase, methylmalonyl isomerase, and methylmalonyl racemase represents a partial reconstitution of the cyclic pathway of propionate formation (reactions 1 to 6). Comparison with the propionate cycle of Fig. 4 shows that the conversion with purified enzymes includes each step of the cycle except the formation of fumarate from malate and reduction of the fumarate to succinate. The propionate is formed from succinate in the reconstructed system just as is shown in the fermentation scheme (Fig. 4), and pyruvate is converted via transcarboxylase to oxaloacetate (reaction 4) which is then reduced to malate (reaction 5). The cycle is blocked at this point in the reconstructed system because of the lack of fumarase and succinic dehydrogenase and the end products, therefore, include both malate and propionate as shown in reaction 6.

The requirement for propionyl-CoA to start the reaction in the reconstructed system can be removed by addition of acetyl kinase, phosphotransacetylase, propionate, CoA, ATP, and Mg^{++}.

propionate + ATP $\xrightleftharpoons{\text{acetyl kinase}}$

propionyl-P + ADP

propionyl-P + CoA $\xrightleftharpoons{\text{phosphotransacetylase}}$

propionyl-CoA + P_i

Net: propionate + ATP + CoA $\rightleftharpoons$

propionyl-CoA + ADP + P_i
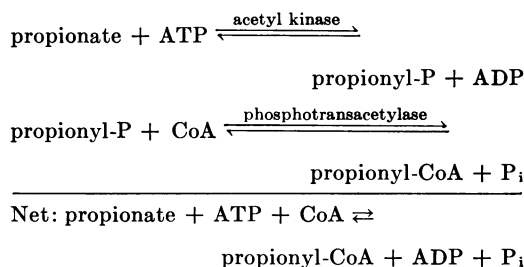
The requirement for each component of this system is shown in Table 6. The significant rates in the absence of malic dehydrogenase or acetyl kinase probably result from cross-contamination of the enzymes, particularly by the malic dehydrogenase, which has a high specific activity, and of CoA transferase by acetyl kinase. In a glucose fermentation, the function of acetyl kinase and phosphotransacetylase is different
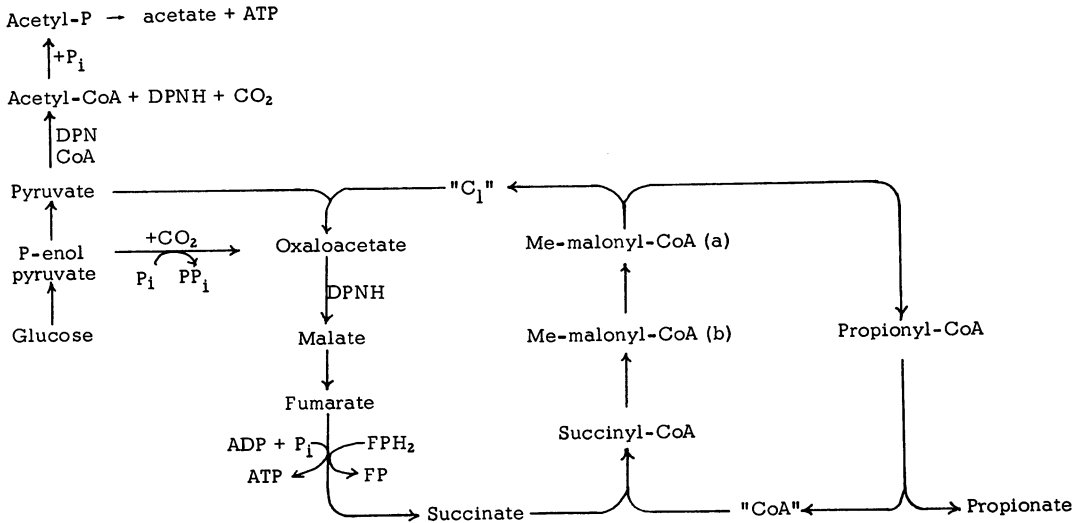
Acetyl-P → acetate + ATP

$\uparrow$ +P$_i$

Acetyl-CoA + DPNH + CO$_2$

FIG. 4. *Reactions of the propionic acid fermentation and the formation of acetate, CO$_2$, propionate, and ATP. Me-malonyl-CoA is methylmalonyl-CoA and (a) and (b) are the two isomers. FP is flavoprotein and FPH$_2$ is reduced flavoprotein.*

a. 1.5 glucose + 3 NAD$^+$ + 3 P$_i$ → 3 P-enolpyruvate + 3 NADH + 3 H$^+$ (Glycolysis)

b. 3 P-enolpyruvate + 3 ADP → 3 pyruvate + 3 ATP (Pyruvokinase)

c. pyruvate + NAD$^+$ + CoA → acetyl-CoA + NADH + H$^+$ + CO$_2$ (Pyruvic dehydrogenase)

d. acetyl-CoA + P$_i$ → acetyl-P + CoA (Phosphotransacetylase)

e. acetyl-P + ADP → acetate + ATP (Acetyl kinase)

f. 2 pyruvate + 2 Me-malonyl-CoA (a) → 2 oxaloacetate + 2 propionyl-CoA (Transcarboxylase)

g. 2 oxaloacetate + 2 NADH + 2 H$^+$ → 2 malate + 2 NAD$^+$ (Malic dehydrogenase)

h. 2 malate → 2 fumarate + 2 H$_2$O (Fumarase)

i. 2 NADH + 2 H$^+$ + 2 P$_i$ + 2 ADP + 2 FP → 2 NAD$^+$ + 2 ATP + FPH$_2$

j. 2 fumarate + FPH$_2$ → 2 succinate + 2 FP (Fumarate reductase)

k. 2 succinate + 2 propionyl-CoA → 2 succinyl-CoA + 2 propionate (CoA transferase)

l. 2 succinyl-CoA → 2 Me-malonyl-CoA (b) (Me-malonyl isomerase)

m. 2 Me-malonyl-CoA (b) → 2 Me-malonyl-CoA (a) (Me-malonyl racemase)

n. 1.5 Glucose + 6 P$_i$ + 6 ADP → 6 ATP + 2 H$_2$O + CO$_2$ + acetate + 2 propionate

than that shown above. They are involved in generating ATP from the acetyl-CoA produced by the oxidation of pyruvate (reactions b, c, and d of Fig. 4).

The presence of fumarase and succinic dehydrogenase has been demonstrated in crude extracts of *P. shermanii* (Allen, *unpublished data*), and Lara (1958) has described the partial purification of a soluble succinic dehydrogenase from *P. pentosaceum.* Pyruvic kinase has also been demonstrated in crude extracts of *P. shermanii* (Allen, *unpublished data*), as have some of the enzymes of the glycolytic pathway (Stjernholm and Wood, 1963). Thus, most of the enzymes necessary for the scheme presented in Fig. 4 have been demonstrated in this bacterium.
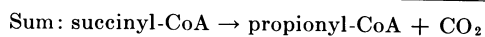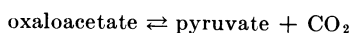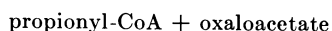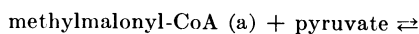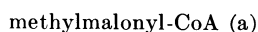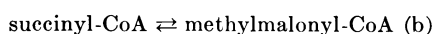
The conversion of succinate to propionate is shown (Fig. 4) to occur via a transcarboxylation, and free CO$_2$ is not a product. However, the propionic acid bacteria do decarboxylate succinate with the formation of CO$_2$ in a biotin-dependent reaction (Delwiche, 1948). This decarboxylation is much slower than that catalyzed by *M. lactilyticus* (Johns, 1951; Whitely, 1953a, b). The possibility exists that succinate is decarboxylated in propionibacteria as

TABLE 6. *Partial reconstruction of the propionate cycle and the requirement of each component*

System	Amt of product formed per min $\times 10^3$	Percentage of complete system
	µmoles	
Complete system*	2.45	100
Complete system minus		
Pyruvate	0.14	5.7
Succinate	0.04	1.6
Propionate	0.135	5.5
ATP and Mg^{++}	0.07	2.9
CoA	0.075	3.1
DBC	0.05	2.0
Transcarboxylase	0.085	3.5
CoA-transferase	0.115	4.7
Isomerase	0.135	5.5
Racemase	0.385	15.7
Malic dehydrogenase	1.50	61.3
Acetyl kinase	0.65	26.5
Phosphotransacetylase	0.25	10.2

* The complete assay mixture contained (in µmoles): potassium phosphate buffer (pH 7.2), 10.0; pyruvate, 2.0; succinate, 2.0; reduced glutathione, 1.0; NADH, 0.05; propionate, 2.0; ATP, 1.0; MgCl₂, 1.0; CoA, 0.1; and dimethylbenzymidazole cobamine (DBC), 0.001; and (in units) malic dehydrogenase, 0.1; CoA-transferase, 0.1; methylmalonyl racemase, 0.1; oxaloacetic transcarboxylase, 0.1; methylmalonyl isomerase, 0.1; phosphotransacetylase, 0.1; and acetyl kinase, 0.1. The total volume was 0.31 ml in a cuvette with a light path, $d_1 = 1.0$ cm. The reaction was measured by following the oxidation of NADH at 340 mµ.

follows:

$$\text{succinyl-CoA} \rightleftarrows \text{methylmalonyl-CoA (b)}$$

$$\text{methylmalonyl-CoA (b)} \rightleftarrows$$

$$\text{methylmalonyl-CoA (a)}$$

$$\text{methylmalonyl-CoA (a)} + \text{pyruvate} \rightleftarrows$$

$$\text{propionyl-CoA} + \text{oxaloacetate}$$

$$\text{oxaloacetate} \rightleftarrows \text{pyruvate} + CO_2$$

Sum: $\text{succinyl-CoA} \rightarrow \text{propionyl-CoA} + CO_2$

On the other hand, there may be a direct decarboxylation of methylmalonyl-CoA such as that described for malonyl-CoA with *Pseudomonas fluorescens* by Wolfe and Rittenberg (1954) and Hayaishi (1955). The mechanism of

the decarboxylation of succinate to propionate and CO_2 in propionibacteria requires further investigation.

An interesting feature of the propionic acid fermentation is the apparent high yield of ATP derived from glucose. Bauchop and Elsden (1960), on the basis of cell yields, considered that approximately 6 moles of ATP are formed per 1.5 moles of glucose fermented. It can be seen that reactions b and e (Fig. 4) would yield 4 moles of ATP per 1.5 moles of glucose. Thus, 2 moles of ATP must, as they suggested, arise from the reactions yielding propionate. One of the unique features of the formation of propionate is that it involves a coupled reduction of fumarate to succinate and oxidation of pyruvate to acetate and CO_2. An electron transport-coupled phosphorylation may occur during this step as indicated in reaction i of Fig. 4, through the reduction of a flavoprotein by NADH and H$^+$. It is seen that this mechanism would yield an additional 2 moles of ATP and, thus, the 6 moles of ATP (see reaction n, Fig. 4). A similar oxidative type of phosphorylation with NADH and H$^+$ and flavoprotein may occur with *C. kluyveri* (Barker, 1956; Gunsalus and Shuster, 1961) in *Ascaris* muscle (Seidman and Entner, 1961; Kmetec and Bueding, 1961) and in beef heart mitochondria (Sanadi and Fluharty, 1963).

When succinate accumulates as an end product of the fermentation, the cycle is broken (Fig. 4), and the oxaloacetate must be generated by fixation of CO_2. The fixation is catalyzed by the enzyme phosphoenolpyruvic carboxytransphosphorylase which was recently described by Siu, Wood, and Stjernholm (1961) and Siu and Wood (1962). The reaction occurs as shown below:

$$CO_2 + \text{phosphoenolpyruvate} + P_i \rightleftarrows$$

$$\text{oxaloacetate} + PP_i$$
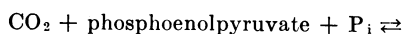
The yield of ATP would then decrease, since some of the phosphoenolpyruvate would not be available from the pyruvokinase reaction to give ATP (reaction b). Under many conditions, succinate is a minor product of the propionic acid fermentation, and this may account for the finding of less than ~6 moles of ATP from 1.5 moles of glucose by the propionic acid bacteria (Fig. 4, reaction n).

It is noted in Fig. 4 (reaction n) that the stoichiometry is 2 moles of propionate to 1 mole

of acetate. It is known that this stoichiometry does not always hold, since Wood and Werkman (1936) found the ratio of propionate to acetate varied from 14 to 2.2 in glucose fermentations. Possible explanations of the high yields of propionate are considered by Stjernholm and Wood (1963).

In the present study, we have presented methods for the enzymatic synthesis of CoA esters. An advantage of this synthesis is the specificity for CoA, thus yielding a purer product than the chemical syntheses which esterify other SH compounds present as contaminants of the CoA. Moreover, if all the enzymes used are free of methylmalonyl racemase, the single stereoisomer of methylmalonyl-CoA can be prepared. The enzymatic synthesis is useful in the production of labeled CoA esters, since the labeled acids are more readily available than the anhydrides used in chemical synthesis.

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