amount of linolenic acid in the diet as was the case with the linseed oil diet causes a reduction in the amount of VLCPFA in the lecithin fraction of both liver and heart.

It was also observed that only arachidonic acid of the linoleic series and docosahexaenoic acid of the linolenic series were present in significant amounts when the dietary linoleic or linolenic acids, respectively, were at low levels. This seems to indicate that the other metabolites of the 18-carbon acids may serve to some degree as reserve supplies for these two very long chain polyenoic fatty acids when the dietary supply of the precursors is abundant.

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Utilization of Volatile Fatty Acids in Ruminants. V. Purification of Acetyl-Coenzyme A Synthetase from Mitochondria of Lactating Bovine Mammary Gland

Shahida Qureshi and Robert M. Cook*

A procedure for purification of acetyl-CoA synthetase from mitochondria of lactating bovine mammary gland is described. The purification method employs $(NH_4)_2SO_4$ fractionation, and three chromatographic steps using first DE-23 cellulose, DE-52 cellulose, and finally calcium phosphate. A 760-fold purification was achieved. The molecular weight is 63,000 as determined by sucrose density gradient centrifugation and the sedimentation coefficient is 4.4 S. Michaelis-Menten constants for Mg, CoA, ATP, and acetate are 6.51×10^{-4}, 2.92×10^{-4}, 2.24×10^{-4} and 6.10×10^{-4} *M*, respectively. Three bands of enzyme activity could be detected upon polyacrylamide gel electrophoresis. The data indicate that mammary acetyl-CoA synthetase exists as multiple forms.

Procedures for the purification of acetyl-CoA synthetase from bovine heart mitochondria have been reported by Webster (1965). When we used these procedures to attempt to purify the enzyme from bovine heart or mammary gland mitochondria, standard tests used to demonstrate purity (i.e., polyacrylamide electrophoresis and sedimentation equilibrium studies) indicated the presence of more than one protein in the purified preparations. Similarly, Farrar (1970) was unable to obtain a preparation of acetyl-CoA synthetase that showed a single protein band on polyacrylamide gel electrophoresis.

By employing purification procedures that are somewhat different from those reported by Webster (1965) and by using column chromatography on calcium phosphate gel as reported by Huang and Stumpf (1970), we have obtained acetyl-CoA synthetase in a highly purified form from the mitochondria of the lactating bovine mammary gland. Mammary tissue was removed in early lactation. The data indicate that acetyl-CoA synthetase exists in multiple forms. The details of these experiments are reported in this paper.

EXPERIMENTAL SECTION

Materials. Coenzyme A, acetyl-CoA, ATP, and AMP were purchased from Sigma Chemical Company. DEAE-cellulose (DE-23 and DE-52) was from Whatman. Calcium phosphate gel was prepared according to the method of Miller et al. (1965). Ovalbumin was obtained from Pharmacia. The chemicals for polyacrylamide gel electrophoresis were purchased from Canalco. Ammonium sulfate used throughout the experiment was a special enzyme research grade from General Biochemicals.

Enzyme Assay. Acetyl-CoA synthetase activity was assayed by measuring the acetate-dependent disappearance of the free sulfhydryl group of coenzyme A. The procedure was a slight modification of that reported by Mahler et al. (1953). In a total volume of 0.2 ml the complete reaction mixture contained 5 μmol of potassium acetate, 1.1 μmol of dipotassium ATP, 1.5 μmol of $MgCl_2$, 0.17 μmol of CoA-SH, 16 μmol of Tris-HCl (pH 8.6), and from 4 to 25 μg of protein. Blank tubes did not contain coenzyme A. The incubation time was for 3 or 10 min at 37°. The reaction was stopped by adding 2.8 ml of the nitroprusside reagent prepared according to the method of Grunert and

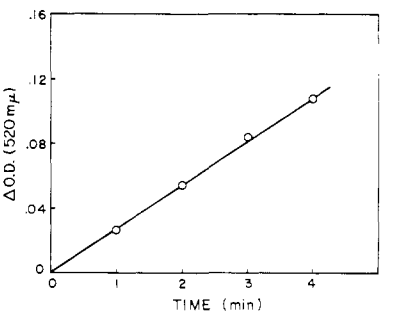

Figure 1. Effect of time on the linearity of the acetyl-CoA synthetase reaction.

Department of Dairy Science, Michigan State University, East Lansing, Michigan 48824.

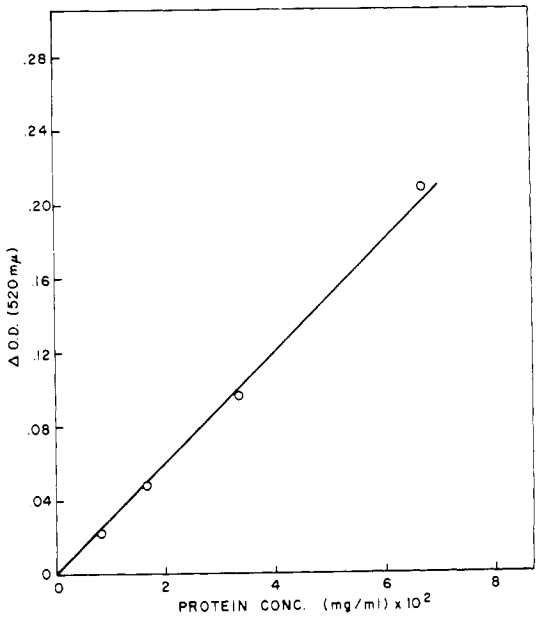

Figure 2. Effect of protein concentration on the linearity of the acetyl-CoA synthetase reaction.

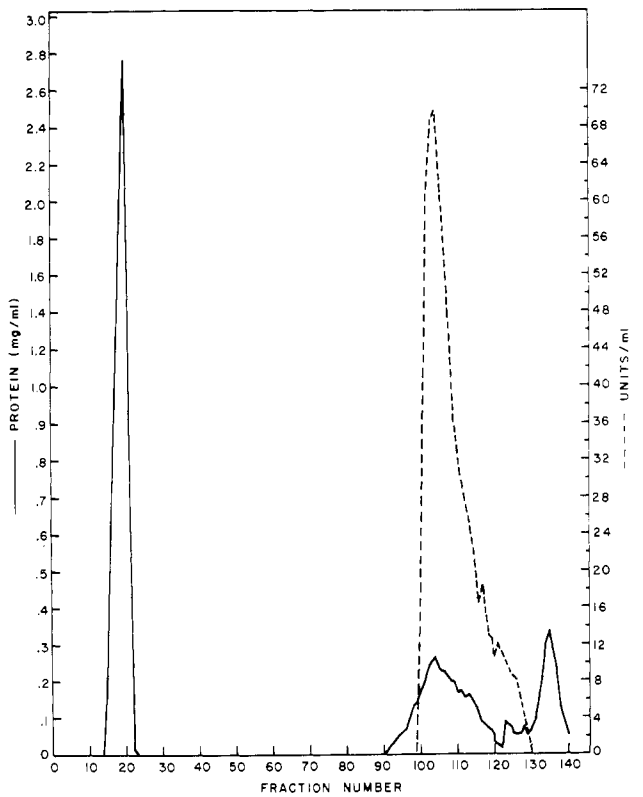

Figure 3. Chromatography of acetyl-CoA synthetase on DE-23 cellulose: (—) protein, milligrams/milliliter; (- - -) enzyme activity, units/milliliter.

Table I. Purification of Acetyl-CoA Synthetase from 128 g (Wet Weight) of Mitochondria Extracted from 1.75 kg of Mammary Gland Tissue

Fraction	Vol, ml	Protein, mg	Act., units	Sp act., units/mg	Recovery, %	Purification, - fold
Mitochondria (after 3rd thaw)	1300	38,000	20,000	0.5	100	1.0
Mitochondrial extract	1145	2,679	17,949	6.7	89	13.4
$(NH_4)_2SO_4$ ppt	32	852	18,573	21.8	93	43.6
DE-23 cellulose	46	64.4	6,750	104.9	34	209.8
DE-52 cellulose	18	27.6	2,938	111.0	10	222.0
$CaPO_4$ gel	36	3.1	1,115	360.0	5.5	720.0

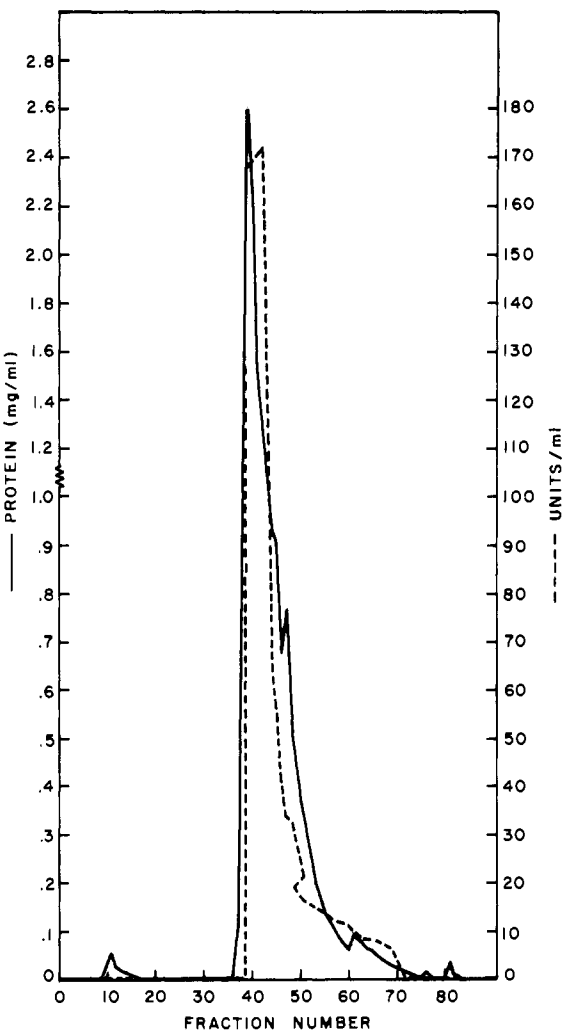

Figure 4. Rechromatography of acetyl-CoA synthetase on DE-52 cellulose: (—) protein, milligrams/milliliter; (- - -) enzyme activity, units/milliliter.

Phillips (1951). Exactly 30 sec after adding the nitroprusside reagent the color was read at 520 nm. One unit of enzyme activity is defined as 1 μmol of substrate reacting per hour.

Isolation of Mitochondria. The mammary gland tissue was taken from Holstein cows at peak lactation, placed in plastic bags, covered with ice, and transported to the cold room. All succeeding operations were carried out at 4°. The gland was freed of excess fat and connective tissue, sliced into thin strips, and ground in an electric meat grinder using a medium grind. One thousand gram aliquots of the ground tissue were homogenized in 2 l. of 0.13 *M* KCl (pH 8.0) in a 1-gal Waring blendor. The blender was operated 20 sec at high speed and then 20 sec at low

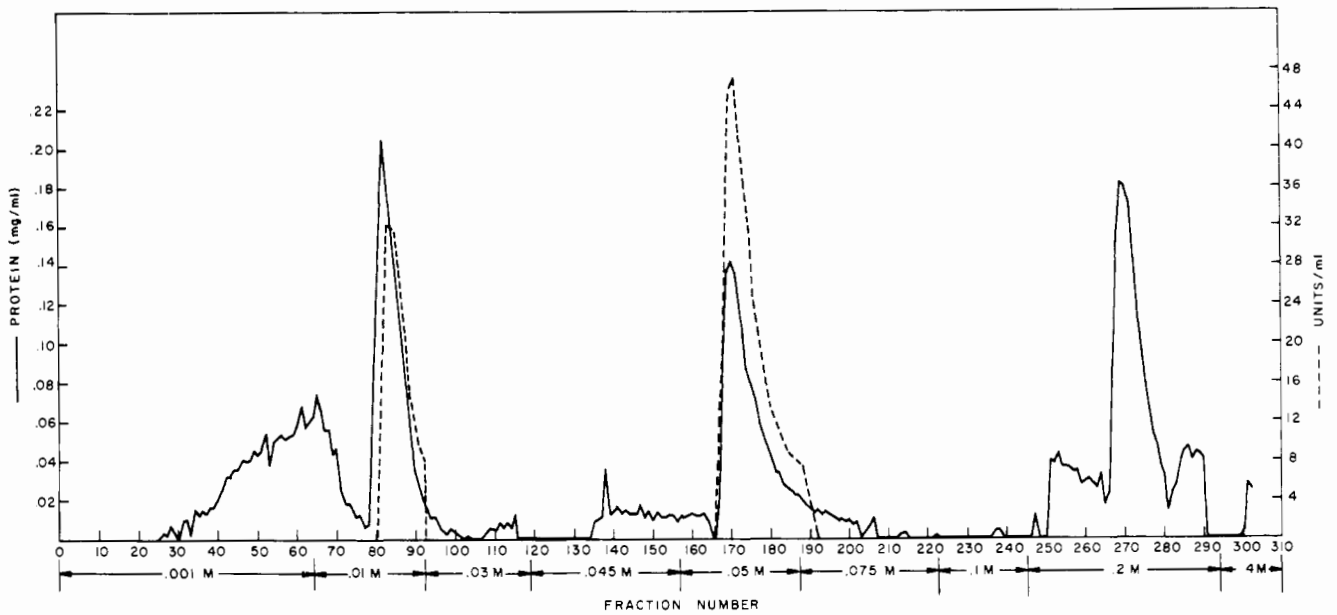

Figure 5. Chromatography of acetyl-CoA synthetase on calcium phosphate gel. The molarities of the different phosphate buffers are shown on the ordinant: (—) protein, milligrams/milliliter; (- - -) enzyme activity, units/milliliter.

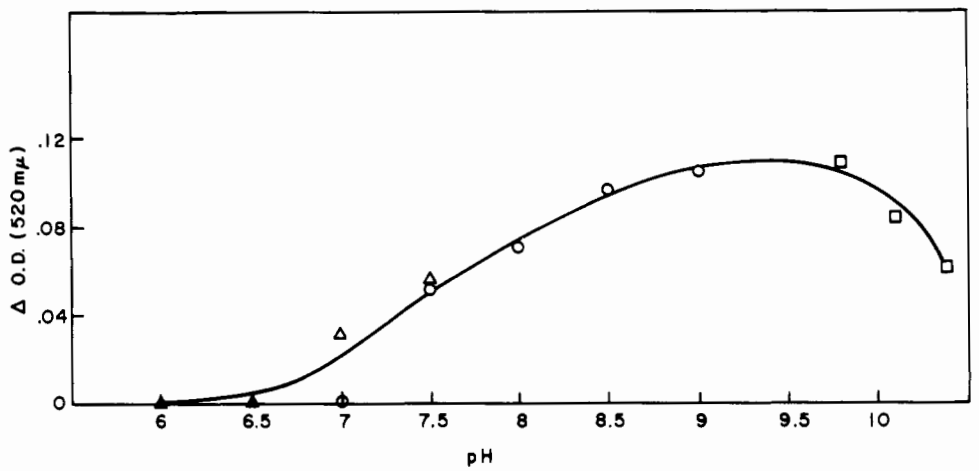

Figure 6. Effect of pH on acetyl-CoA synthetase activity: (O) Tris-HCl buffer; (Δ) potassium phosphate buffer; (□) glycine buffer.

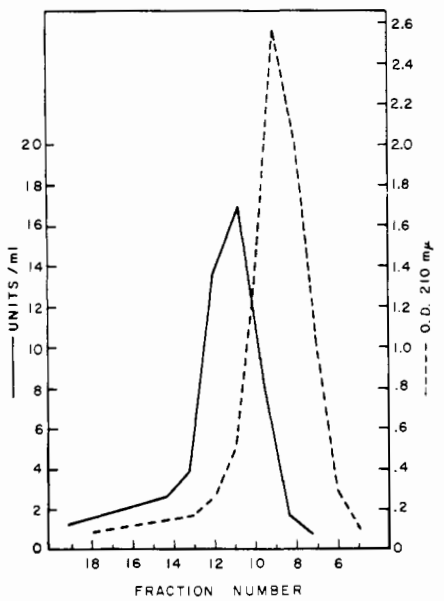

Figure 7. Sucrose density gradient centrifugation of acetyl-CoA synthetase using ovalbumin as a reference. The centrifugation was carried out at 50,000 rpm for 10 hr at 4° using a 5–20% sucrose gradient. Ovalbumin was measured by the absorption at 210 mμ. Acetyl-CoA synthetase was measured by determining enzyme activity (units/milliliter).

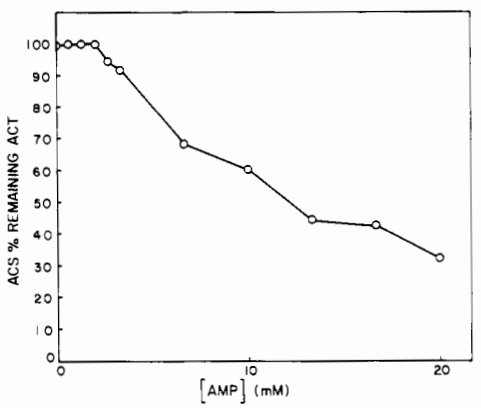

Figure 8. Effect of AMP concentration on acetyl-CoA synthetase (ACS) activity.

speed. The homogenate was transferred to 1-l. plastic centrifuge bottles and centrifuged for 15 min at 1000*g* in a 6-l. MSE refrigerated centrifuge. The supernatant was filtered through four layers of cheesecloth. The 1000*g* supernatant was centrifuged 20 min at 20,000*g* in a Sorvall RC 2-B centrifuge using a GSA rotor. The 20,000*g* pellet was suspended in 10 vol of 0.13 *M* KCl (pH 8.0) by brief blending using a 1 qt Waring Blendor. These suspensions were transferred to 1-l. plastic bottles and frozen at −20°.

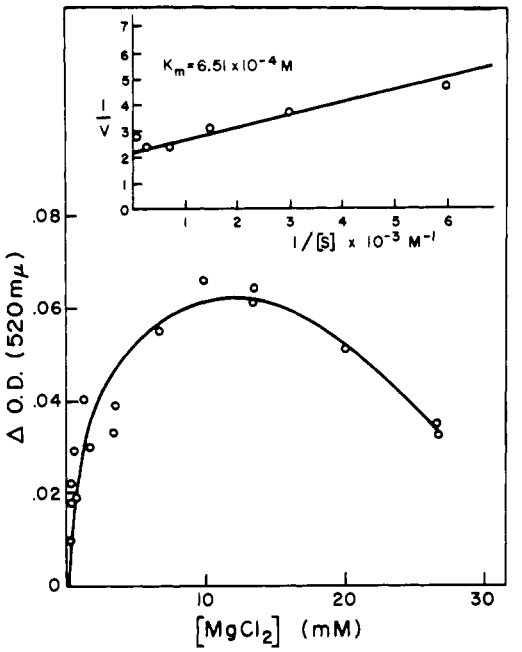

Figure 9. Effect of Mg concentration on acetyl-CoA synthetase activity. The inset is the Lineweaver–Burk plot of the same data.

In order to solubilize acetyl-CoA synthetase the suspensions were frozen and thawed three times, usually over a period of at least 3 weeks. Some suspensions were stored several months before the first thaw. After the first thaw, 5 or 10 ml was taken for subsequent freezing and thawing and finally for enzyme assay. The remaining suspension was adjusted to pH 8.0 with 1 *M* KOH and made 0.1 *M* in mercaptoethanol prior to the last two freeze–thaw treatments. After the final thaw, the material was centrifuged at 20,000*g* for 30 min and the supernatant was taken for further fractionation using ammonium sulfate.

Ammonium Sulfate Fractionation. The precipitate obtained after 40% saturation with solid ammonium sulfate was discarded. The precipitate obtained after 70% saturation contained the enzyme and is referred to as the ammonium sulfate fraction. This fraction was suspended in a minimum volume of Tris buffer (pH 8.5) and frozen.

Table II. Substrate Specificity of Acetyl-CoA Synthetase (Assay Conditions Are Those Described in Experimental Section)

Substrates tested	Rel act.	Substrates tested	Rel act.
Acetate	100	Heptanoate	0
Propionate	65	Octanoate	0
Butyrate	0	Acrylate	151
Valerate	0	Maleate	25
Hexanoate	0	Crotonate	0

Chromatography Using DE-23 Cellulose. DEAE-cellulose was washed with KOH and then HCl, degassed, and suspended as a thick slurry in 0.005 *M* Tris-HCl–3 m*M* 2-mercaptoethanol buffer (pH 7.5). The column dimensions used were 1.7 × 42 cm. The column was equilibrated overnight at 4° using the above buffer at a flow rate of 15–20 ml/hr. Usually 1 g of protein (ammonium sulfate precipitate) was dialyzed 30 min against 100 vol of the above buffer, diluted to 10 mg of protein/ml, and added to the top of the column. The column was first eluted with 140 ml of the above buffer and then eluted with 160 ml of 0.01 *M* Tris-HCl–3 m*M* 2-mercaptoethanol buffer (pH 7.5). Finally, the column was eluted with 600 ml of a linear KCl gradient of 0–0.6 *M* KCl in 0.01 *M* Tris-HCl–3 m*M* 2-mercaptoethanol buffer (pH 7.5). Six-milliliter fractions of eluate were collected. The tubes with highest enzyme activity were pooled and concentrated by ultrafiltration. The concentrated protein solution was stored at −60°.

Chromatography Using DE-52 Cellulose. The DEAE-cellulose was washed and degassed as described above and suspended in 0.005 *M* Tris-HCl–3 m*M* 2-mercaptoethanol buffer (pH 7.5) and a column 1 × 40 cm in dimensions was prepared. The column was equilibrated overnight with the above buffer at a flow rate of 20 ml/hr. From 60 to 80 mg of enzyme protein, isolated from the DE-23 cellulose column, was added to the DE-52 cellulose column. The column was eluted first with 75 ml of 0.005 *M* Tris-HCl–3 m*M* 2-mercaptoethanol buffer (pH 7.5). Then the column was eluted with 400 ml of a linear KCl gradient of 0.15–0.4 *M* KCl in 0.01 *M* Tris-HCl–3 m*M* 2-mer-

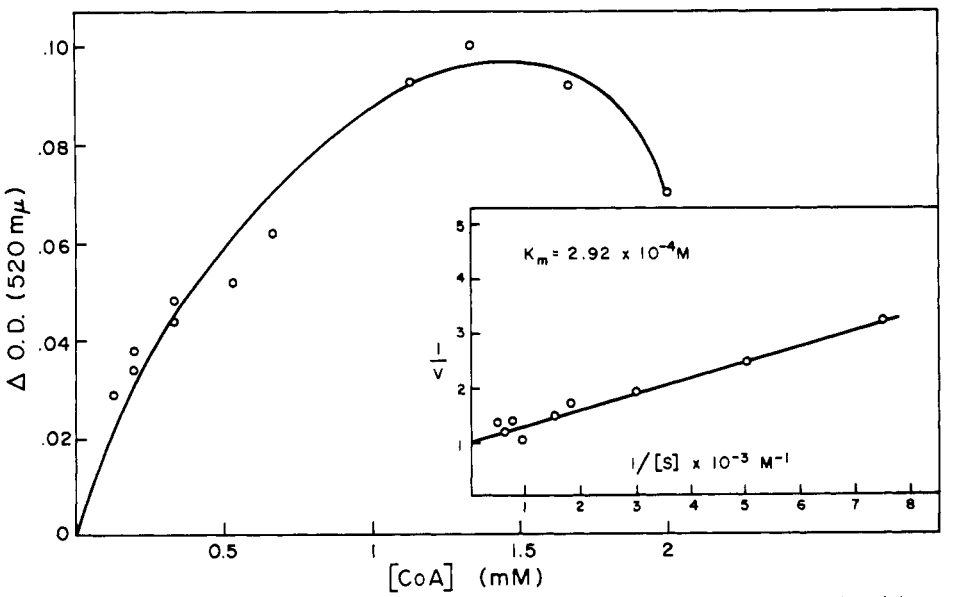

Figure 10. Effect of CoA concentration on acetyl-CoA synthetase activity. The inset is the Lineweaver–Burk plot of the same data.

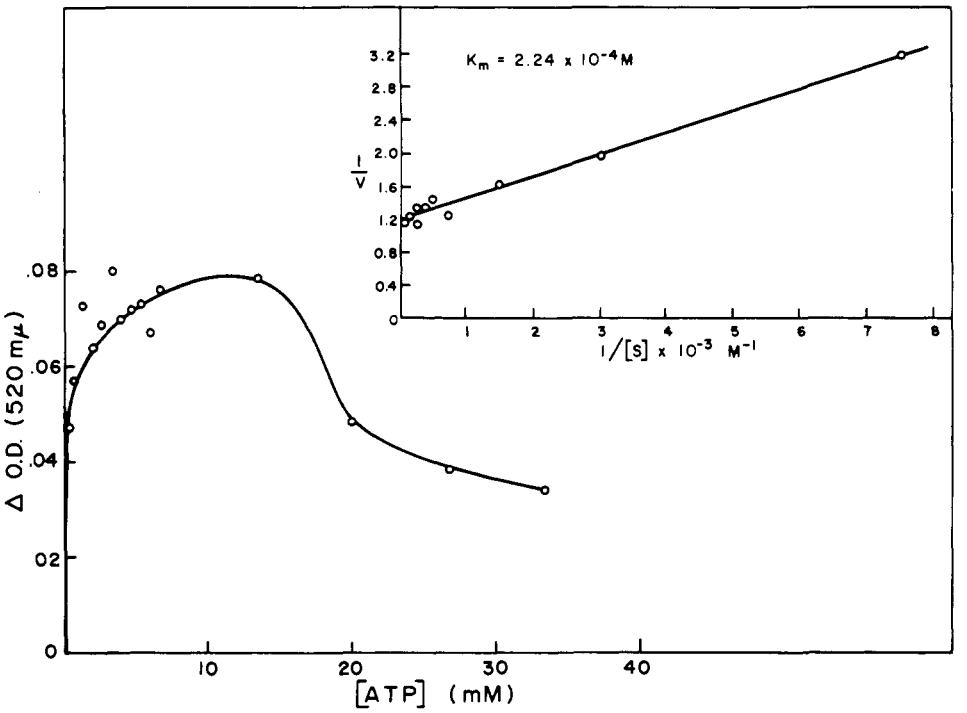

Figure 11. Effect of ATP concentration on acetyl-CoA synthetase activity. The inset is the Lineweaver-Burk plot of the same data.

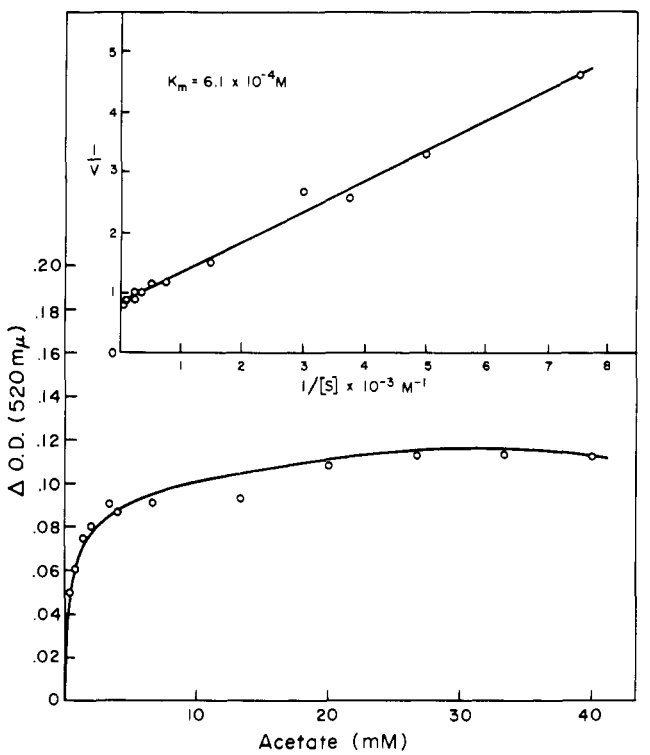

Figure 12. Effect of acetate concentration on acetyl-CoA synthetase activity. The inset is the Lineweaver-Burk plot of the same data.

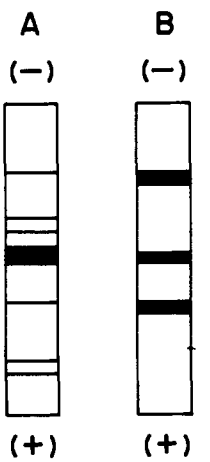

Figure 13. Schematic presentation of polyacrylamide gel electrophoresis of purified acetyl-CoA synthetase. A pH 8.3 buffer system (Davis) was used. Twenty micrograms of protein was layered on top of the gel. The electrophoresis was carried out for 30 min using 6 mA/gel at 4°; (A) represents protein bands and (B) represents enzymatic activity.

captoethanol buffer (pH 7.5). Six-milliliter fractions were collected.

Calcium Phosphate Gel Chromatography. Calcium phosphate gel was suspended in 0.001 *M* potassium phosphate buffer (pH 7.0) and packed in a column 2.0 × 12 cm. From 20 to 50 mg of protein was diluted to 150 ml with 0.001 *M* $KHPO_4$ (pH 7.0) and added to the column at a flow rate of 60 ml/hr. The column was then eluted with a stepwise gradient of increasing concentrations of potassium phosphate buffer, 0.001–0.4 *M*, pH 7.0. Six-milliliter fractions were collected.

Electrophoresis. Polyacrylamide disc gel (5.5% acrylamide) electrophoresis was performed by the method of Davis (1964). After electrophoresis the gels were fixed and stained by the procedure of Chrambach et al. (1965).

Molecular Weight Measurement. The molecular weight of acetyl-CoA synthetase was estimated by sucrose density gradient centrifugation according to the method of Martin and Ames (1961). A Beckman Model L-2 65 B preparative ultracentrifuge with a SW 57 swinging bucket rotor was used.

RESULTS

Purification. A summary of the purification of acetyl-

CoA synthetase is shown in Table I. It was found that very little purification of the $(NH_4)_2SO_4$ fraction could be achieved using gel filtration employing Sephadex G-100 or G-200 and Bio-Gels P-100 or P-200. Also the enzyme could not be purified using carboxymethylcellulose as a cation exchange support. The enzyme could be purified extensively using TEAE- or DEAE-cellulose and Tris buffers but not $KHCO_3$ buffers.

The enzyme assay was linear with time and increasing protein concentrations (Figures 1 and 2).

Chromatography using DE-23 cellulose and a linear KCl gradient gave a major purification (Figure 3). The most active enzyme fractions from this step were concentrated by ultrafiltration and rechromatographed using DE-52 and a linear KCl gradient. The enzyme and protein eluted in one symmetrical peak (Figure 4). However, this enzyme preparation obtained from the DE-52 cellulose column resolved into many protein peaks when rechromatographed using calcium phosphate gel and potassium phosphate buffers (Figure 5). Extensive purification was thus achieved using calcum phosphate gel. The enzyme activity eluted in two peaks (Figure 5). The first enzyme peak became inactive within a few hours after emerging from the calcium phosphate gel column. The most active fractions were from the second enzyme peak. These fractions were combined, concentrated by ultrafiltration, diluted with 0.005 *M* Tris-HCl (pH 8.5), reconcentrated, and finally stored at −80°. The purified enzyme was stable when stored under these conditions.

Properties of Bovine Mammary Acetyl-CoA Synthetase. The pH optimum was between 8.5 and 9 (Figure 6). The molecular weight of the enzyme was determined on the purified enzyme (Table I), using sucrose density gradient centrifugation and ovalbumin as a reference. The molecular weight was estimated to be 63,000 and the sedimentation coefficient was estimated to be 4.4 S (Figure 7). A similar experiment was conducted using enzyme isolated from a cellulose ion exchange column and using tRNA as a reference. The molecular weight was estimated to be 62,000 and the sedimentation coefficient 4.5 S.

AMP is not a strong inhibitor of mammary acetyl-CoA synthetase activity. There is 50% inhibition at an AMP concentration of about 50 m*M* (Figure 8).

The K_m values for Mg^{2+} coenzyme A, ATP, and acetate were determined from Lineweaver-Burk plots to be 6.5×10^{-4}, 2.92×10^{-4}, 2.24×10^{-4}, and 6.10×10^{-4} *M*, respectively (Figures 9–12). These values are of the same order of magnitude as those reported by others for the heart enzyme (Webster, 1965; Farrar, 1970).

The enzyme is most active on acrylate followed by acetate, propionate, and maleate. Longer chain fatty acids were not substrates for the enzyme (Table II).

Using polyacrylamide gel electrophoresis the purified enzyme could be separated into three active proteins. When stained with Amido Black only one protein band was observed. However, when the gels were stained with Coomassie Blue seven protein bands were evident (Figure 13). Three of these protein bands corresponded to the active enzyme. More than 90% of the protein was in one band which was active catalytically.

DISCUSSION

We believe the enzyme preparation to be at least more than 90% pure and to our knowledge probably is the purest preparation of acetyl-CoA synthetase obtained thus far from any ruminant tissue. When the enzyme was examined on polyacrylamide gel electrophoresis it was apparent that mammary acetyl-CoA synthetase exists as more than one molecular species.

The enzyme has been characterized further and found to be a glycoprotein (Stamoudis, 1973). The details of these experiments are reported in a separate paper. The apparent multiple forms of acetyl-CoA synthetase may be due to a difference in carbohydrate composition. The carbohydrates may be important in determining enzyme activity and substrate specificity. The fact that the enzyme has been highly purified provides an opportunity for extensive characterization of the protein. The discovery that the enzyme is a glycoprotein further supports our proposal that the acetate activation reaction catalyzed by acetyl-CoA synthetase is an important regulatory step in acetate utilization by ruminant mammary tissue (Cook et al., 1969; Quraishi and Cook, 1972).

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