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Acetyl Coenzyme A Carboxylase. Purification and Properties of the Bovine Adipose Tissue Enzyme†

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ABSTRACT: Acetyl-CoA carboxylase has been purified over 1000-fold from bovine perirenal adipose tissue. Sedimentation velocity analysis indicates that the enzyme is homogeneous and can exist in polymeric and protomeric states. The polymeric form in the presence of citrate has a filamentous structure, as revealed by electron microscopy, and a high sedimentation coefficient ($s_{20,w}$ = 68 S). Treatment of the polymeric carboxylase with 0.5 M NaCl at pH 9.0 results in its dissociation to a protomeric species having an $s^0_{20,w}$ of 14.7 S and a molecular weight of approximately 560,000. The bovine adipose tissue enzyme is activated by citrate and this activation is associated with an increase in maximal velocity of the carboxylase-catalyzed reaction with no significant effect on K_m values for ATP (Mg^{2+}), bicarbonate, or acetyl-CoA. Following the addition of the polymeric form of the enzyme to assay reaction mixture minus tricarboxylic acid activator, there is a gradual decline in catalytic activity to a level less than 2% of that obtained in the presence of citrate. This decay appears to be associated with depolymerization to the less active protomeric form. Reversibility of the decay in activity is indicated by the nearly instantaneous reactivation produced by citrate. Reactivation is associated with the activator-initiated transition from a 13–15S protomeric species to a 47–50S polymeric form, respectively, as determined by sucrose density gradient centrifugation under assay conditions. Relatively impure carboxylase preparations are markedly activated by (+)-palmitylcarnitine, bovine serum albumin, and dilution, in addition to the citrate activation normally observed. Together these factors produced an activation effect of over 23-fold. Since these activating conditions have no effect on the homogeneous carboxylase, it is evident that this capacity for activation (or deinhibition) is lost during the course of purification. Dilution, (+)-palmitylcarnitine, bovine serum albumin, and purification probably act by a common mechanism, possibly by the removal of an endogenous inhibitory hydrophobic material which is bound to the impure carboxylase.

Acetyl-CoA carboxylases from animal tissues have been shown to be activated by certain tricarboxylic acids, notably citrate and isocitrate (Matsuhashi *et al.*, 1962; Kallen and Lowenstein, 1962; Waite, 1962; Martin and Vagelos, 1962a,b; Waite and Wakil, 1962; Lane and Moss, 1971b; Moss and Lane, 1971), this activation being associated with an increased sedimentation velocity (Vagelos *et al.*, 1962, 1963; Matsuhashi *et al.*, 1964; Numa *et al.*, 1965a,b). In the case of the avian liver carboxylase, the activation and increased sedimentation velocity were shown to be concurrent with the citrate-promoted polymerization of weight homogeneous protomeric subunits of 410,000 (Gregolin *et al.*, 1966b; Gregolin *et al.*, 1968b). The polymerized form of the liver carboxylase has a molecular weight of 4–10 million and was found by electron microscopy to have a unique filamentous structure (Gregolin *et al.*, 1966a). Acetyl-CoA carboxylase, from bovine adipose tissue, recently obtained in our laboratory in homogeneous form, was also found to have a similar filamentous structure (Kleinschmidt *et al.*, 1969).

It has been reported that relatively impure preparations of acetyl-CoA carboxylase from rat liver were markedly activated by (+)-palmitylcarnitine (Fritz and Hsu, 1967; Green-

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span and Lowenstein, 1968) and bovine serum albumin (Marquis *et al.*, 1968; Gibson *et al.*, 1965). Investigations in our laboratory with the crude bovine adipose tissue carboxylase revealed similar effects and, in addition, an apparent activation caused by diluting the enzyme. The availability of the homogeneous adipose tissue enzyme permitted a comparison of these "activating effects" or procedures with pure and impure carboxylase preparations. The present investigation shows that these agents which are effective with crude carboxylase preparations have no activating effect on the pure enzyme. It appears that an inhibitory substance bound by the carboxylase in crude enzyme preparations can be removed either by these agents or extensive purification. The present paper also reports the purification, as well as certain molecular and catalytic properties of the adipose tissue acetyl-CoA carboxylase. Preliminary accounts of this work have already appeared (Kleinschmidt *et al.*, 1969; Moss *et al.*, 1969).

Experimental Procedure

Materials and Miscellaneous Methods. Cellulose phosphate was obtained from Carl Schleicher and Schuell, DL-isocitric acid from Calbiochem, and avidin (10–12 units/mg) from Worthington Biochemical Corp. Carnitine derivatives were generously provided by Dr. Irving Fritz (Banting and Best Institute, Toronto). Coenzyme A thioesters were prepared by the method of Simon and Shemin (1953), and their concentrations determined by the hydroxamate method (Stadtman, 1957). Cellulose phosphate was equilibrated and all other cofactors and reagents were obtained or prepared as previously described (Gregolin *et al.*, 1966a, 1968a).

Preparation of sucrose density gradients followed the method of Martin and Ames (1961). Preparative density gradients (30 ml) contained a 2-ml, 30% (w/v) sucrose cushion upon which a 5–30% (w/v) sucrose gradient was layered; centrifugation was performed in a Spinco SW 25.1 rotor. Analytical gradients (5–20% w/v) were run for 1.5 hr at 38,800 rpm using an SW 39 rotor unless otherwise indicated. Protein was determined by the method of Lowry *et al.* (1951).

Assay of Acetyl-CoA Carboxylase. The components of the assay reaction mixture in a final volume of 0.5 ml included (in μmoles, except as indicated): Tris-(Cl^-) buffer, 30; $MgCl_2$, 4; GSH, 1.5; acetyl-CoA, 0.1; ATP, 1; bovine serum albumin, 0.3 mg; potassium citrate, 5; and $KH^{14}CO_3$, 5 (specific activity 250,000 cpm/μmole). The pH of the mixture was adjusted to 7.6 at room temperature. The reaction was initiated by the addition of up to 4×10^{-4} unit of enzyme diluted in 10 mM Tris (Cl^-) buffer (pH 7.5), containing 10 mM potassium citrate–0.1 mM EDTA–5 mM 2-mercaptoethanol. Following a 10-min incubation at 37°, acid-stable radioactivity (as [^{14}C]malonyl-CoA) was determined as described by Gregolin *et al.* (1968a); malonyl-CoA formation follows zero-order kinetics for 15–20 min and is proportional to enzyme concentration. A unit of carboxylase catalyzes the carboxylation of 1 μmole of acetyl-CoA/min under the conditions described. Specific activity is given as units per milligram of protein as determined by the method of Lowry *et al.* (1951), and converted to refractometrically determined protein using a factor of 1.12. This factor was estimated from a refractometric protein concentration determination using a synthetic boundary cell and 1.862×10^{-4} as the specific refractive index increment per milligram per milliliter (Perlman and Longworth, 1948). This factor is in good agreement with that for the avian liver carboxylase (Gregolin *et al.*, 1966b).

Special precautions must be taken when enzyme from the early stages of purification, *i.e.*, prior to cellulose phosphate ion-exchange chromatography, is assayed. The presence of inhibitory substances which are apparently bound to the carboxylase lead to an underestimate of activity. This inhibitory effect can be minimized by the inclusion of a higher concentration (3 mg/ml) of bovine serum albumin in the reaction mixture and assaying the enzyme at a sufficiently high dilution. A series of enzyme dilutions should be run to ascertain the plateau of specific activity when plotted *vs.* enzyme dilution. This is discussed in more detail in a later section.

As has been found to be the case with other acyl-CoA carboxylases, the bovine adipose tissue acetyl-CoA carboxylase is avidin sensitive; pretreatment of avidin with excess *d*-biotin prevents this inhibition.

Electron Microscopy. The carboxylase was dialyzed against and diluted with 50 mM Tris (Cl^-)–10 mM potassium citrate–0.1 mM EDTA–5 mM mercaptoethanol at pH 7.5 to a concentration of 20 μg/ml. A droplet of this solution was applied to a collodion carbon support film (Gordon and Kleinschmidt, 1968) and after 1–2 min blotted with filter paper. Staining was accomplished by transferring a droplet of 4% uranyl acetate to the support film. After 2 min, the film was blotted, washed once with a droplet of water, blotted, and finally heated in an oven at 180° for 10 min to remove the collodion. The specimens were then observed at 40,000–50,000× magnification using a Siemens Elmiskop 1A electron microscope.

Isolation and Purification of Acetyl-CoA Carboxylase. Since the enzyme is unusually stable in its polymeric form, it was possible to carry out most steps in the purification at room temperature. All buffers, except as specifically indicated, used in the purification procedure contained 10 mM potassium citrate in order to maintain the carboxylase in the polymeric state. The results of the purification are summarized in Table I.

Extraction. Perirenal adipose tissue was obtained from dairy cows immediately after slaughter, placed in insulated containers, and rapidly transported to the laboratory. This procedure was followed to delay solidification of the fat which accompanies cooling and renders effective homogenization difficult. After removing connective tissue and blood vessels, 500-g batches of the warm adipose tissue were homogenized in 750 ml of phosphate–citrate buffer[1] for 3 sec at top speed and room temperature in a 4-l. capacity Waring blendor. Preliminary experiments revealed that maximal carboxylase activity in the extract was obtained following a 3- to 5-sec homogenization of the warm tissue. Following homogenization, the extract was poured off and the remaining fat layer rehomogenized in 400 ml of the same buffer. The extracts were pooled and centrifuged at 9000*g* for 12 min at 0–4°. The supernatant solution was filtered through four-ply cheesecloth which removed most of the solidified fat. The solution was held at 0–4° and the ammonium sulfate concentration brought to 30% saturation by the addition of 176 g of the salt per liter of enzyme extract. After storing the suspension overnight at 0–4°, the precipitated protein was collected with a Sharples centrifuge operated at 50,000 rpm with a flow rate of about 170 ml/min. The precipitate was resuspended in 100 ml of a 25% saturated ammonium sulfate solution containing 50 mM Tris (Cl^-) buffer–10 mM potassium citrate (pH 7.3) and was

[1] The phosphate–citrate buffer consisted of 10 mM potassium phosphate–10 mM potassium citrate–1.0 mM EDTA–5 mM mercaptoethanol (pH 7.0).

TABLE I: Purification of Adipose Tissue Acetyl-CoA Carboxylase.

Step	Total Act. (Units)[b]	Protein (mg)[d]	Sp Act. (Units[b]/mg of protein[d])	Yield (%)
1. Initial extract[a]	2300[c]	230,000	0.01	100
2. 0–30%; then 25% saturated $(NH_4)_2SO_4$ fractionation	1450[c]	11,100	0.13	63
3. Cellulose phosphate chromatography	620	310	2.0	27
4. Sucrose density gradient fractionation I	300	25–60	5–12	13[f]
5. Sucrose density gradient fractionation II	140	12–20	7–12	6[f]
6. Sucrose density gradient fractionation III	58	5	12 (10.7)[e]	2.5[f]

[a] From 47.4 kg of bovine perirenal adipose tissue. [b] One unit = 1 μmole of HCO_3^- fixed per min at 37°. [c] Prior to cellulose phosphate chromatography special precautions must be taken to assay the enzyme. See section on Assay of Acetyl-CoA carboxylase. [d] Protein determined by the method of Lowry *et al.* (1951). [e] Based on refractometrically determined protein. [f] The apparent low yield is due to the fact that only those fractions with the highest specific activities were retained for further purification; side fractions with lower specific activities were discarded. Of the total carboxylase activity applied to a gradient, 80–90% could be accounted for as the sum of the activities of the individual gradient fractions.

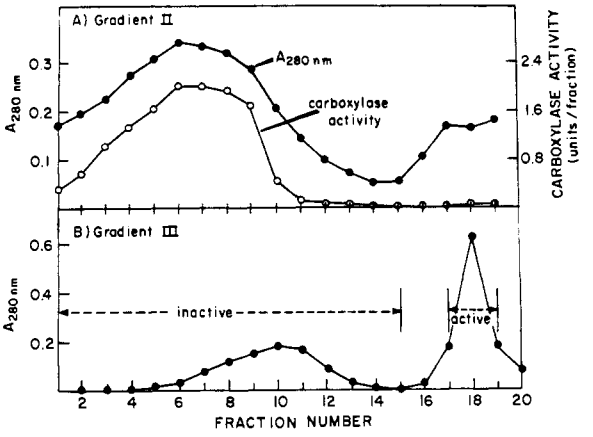

FIGURE 1: Purification of adipose tissue acetyl-CoA carboxylase in the polymeric (A) and protomeric (B) states by sucrose density gradient centrifugation. (A) Carboxylase (5 mg, 28 units) from sucrose density gradient fractionation I (step 4, Table I) in 2 ml of 10 mM Tris (Cl^-) buffer (pH 7.5), containing 10 mM potassium citrate–0.1 mM EDTA–5 mM mercaptoethanol, was applied to a 30-ml linear 5–30% sucrose density gradient (see Materials and Miscellaneous Methods) containing the same buffer mixture. (B) Carboxylase (3 mg, 24 units) from sucrose density fractionation II (step 5, Table I) in 2 ml of buffer containing 0.5 M NaCl–50 mM Tris (Cl^-) (pH 8.0)–0.1 mM EDTA–5 mM mercaptoethanol was applied to a 30-ml linear sucrose density gradient containing the same buffer mixture. Centrifugation and analysis of the gradients were as described in Experimental Procedure. The bottom of the gradient corresponds to fraction 1 in A and B.

then allowed to extract for 10 min at 0°. This and all solutions used subsequently in the purification procedure contained 0.1 mM EDTA and 5 mM 2-mercaptoethanol. After centrifugation at 34,000*g* for 12 min, the supernatant solution was discarded, and the precipitate was extracted three additional times in a similar manner. All subsequent purification steps, with the exception of the final sucrose density gradient centrifugation, were performed at room temperature.

Cellulose Phosphate Chromatography. The precipitated protein from the preceding step was dissolved in 400 ml of 10 mM Tris (Cl^-) buffer containing 10 mM potassium citrate at pH 7.0 and dialyzed against repeated changes of buffer over a 9-hr period. The dialyzed enzyme solution (approximately 11 g of protein) was applied to an 8 × 30 cm column of cellulose phosphate (see Materials and Miscellaneous Methods) which had been previously equilibrated with 5 l. of the phosphate–citrate buffer[1]. An 8-l. linear citrate gradient (10–200 mM potassium citrate gradient containing 10 mM potassium phosphate buffer at pH 7.0) was used to elute the enzyme. The enzyme appeared in the eluate after approximately 4 l. had passed through the column. The fractions containing carboxylase activity were pooled and precipitated by overnight dialysis against an equal volume of saturated ammonium sulfate. The precipitate can be stored under 50% saturated ammonium sulfate[2] at 0–4° for at least 1 week without significant loss of activity.

Sucrose Density Gradient Fractionation I and II. An aliquot of the ammonium sulfate suspension was centrifuged at 35,000*g* for 12 min. The precipitated protein (about 45 mg of protein) was dissolved in 6 ml of 10 mM Tris (Cl^-) buffer containing 10 mM potassium citrate at pH 7.6 to give a final carboxylase concentration of approximately 15 units/ml and dialyzed overnight against the same buffer. The relative insolubility of the polymeric form of the carboxylase limited the amount of enzyme that could be placed on a gradient. The carboxylase solution was applied (2 ml/gradient) to three 5–30% sucrose gradients (w/v), containing the buffer indicated above (see Materials and Miscellaneous Methods). After centrifugation for 4.5 hr at 24,800 rpm and 25°, 20 1.5-ml fractions were collected. Protein, estimated from absorbance measurements at 280 mμ, was localized in a rapidly sedimenting (about 50 S) and a slowly sedimenting (<20 S) peak. Carboxylase activity was associated with the faster moving peak; these fractions were pooled and precipitated by dialysis against a tenfold excess of 50% saturated ammonium sulfate,[2] containing 10 mM potassium citrate. The precipitated protein (sucrose density gradient preparation I) was dialyzed, and the sucrose density gradient centrifugation procedure described above was repeated giving rise to sucrose density gradient preparation II (Figure 1A).

Sucrose Density Gradient Fractionation III. In order to eliminate high molecular weight contaminants which may cosediment with the polymeric form of the carboxylase, the enzyme was dissociated to its protomeric form with 0.5 M NaCl at pH 8.0 and subjected again to sucrose density gradi-

[2] Saturated ammonium sulfate was neutralized with NH_4OH, so that when diluted fivefold with water, the pH was 7.5.

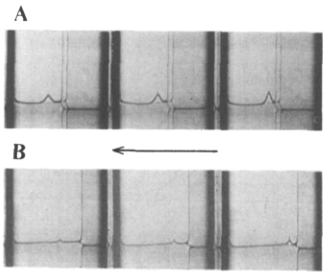

FIGURE 2: Sedimentation patterns of the protomeric (A) and polymeric (B) forms of adipose tissue acetyl-CoA carboxylase. (A) Enzyme in 0.5 M NaCl–50 mM Tris (Cl^-) buffer (pH 9.0 at 4°)–0.1 mM EDTA–5 mM mercaptoethanol; enzyme concentration, 2 mg/ml; centrifugation at 30,000 rpm and 4°; photographs taken at 32, 48, and 64 min after reaching speed. (B) Enzyme in 10 mM Tris (Cl^-) buffer (pH 7.5)–10 mM potassium citrate–0.1 mM EDTA–5 mM mercaptoethanol; enzyme concentration, 0.8 mg/ml; centrifugation at 22,000 rpm and 20°, photographs taken at 8, 16, and 24 min. Carboxylase from step 6 Table I was used in these experiments.

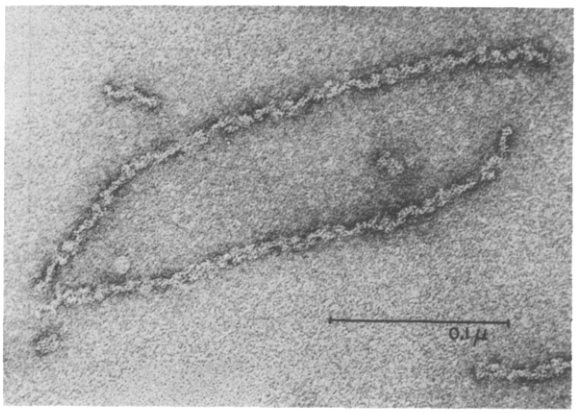

FIGURE 3: Electron micrograph of the filamentous form of bovine perirenal adipose tissue acetyl-CoA carboxylase in the presence of tricarboxylic acid activator (citrate). The methods employed are described in Experimental Procedure. Carboxylase from step 6 Table I was used for electron microscopy.

ent centrifugation. The precipitated enzyme from the preceding step was recovered by centrifugation and dissolved in 0.5 M NaCl in 50 mM Tris (Cl^-) buffer at pH 8.0 to give a final carboxylase concentration of about 15 units/ml, and this solution was dialyzed at 2° for 8 hr against the same buffer. Two milliliters of the enzyme solution was applied to a 5–20% sucrose gradient containing 0.5 M NaCl and the buffer indicated above. As shown in Figure 1B two well-separated protein peaks were obtained, a slow-moving symmetrical peak which contained most of the protein applied to the gradient and a smaller fast-moving peak. The slowly sedimenting, active enzyme was pooled and precipitated by dialysis against 60% saturated ammonium sulfate[2] containing 10 mM potassium citrate. The precipitated carboxylase can be stored under 60% saturated ammonium sulfate containing 10 mM potassium citrate at 0–4° for at least 3 weeks with little loss of activity. Preparations carried to this stage of purification were used for the determination of the physical properties of the enzyme.

Results

Hydrodynamic Properties and Electron Microscopy. The results summarized in Table I show that the carboxylase is purified over 1000-fold from the initial adipose tissue extract using the procedure outlined. The maximal specific activity achieved, 10.7 μmoles of acetyl-CoA carboxylated per min per mg of refractometrically determined protein, closely approximates that of the avian liver acetyl-CoA carboxylase (Gregolin *et al.*, 1968a). Several criteria of purity suggest that the adipose tissue carboxylase is homogeneous. Figure 2A shows a sedimentation velocity pattern of the protomeric species of the enzyme which is obtained by dissociation of the polymeric form in 0.5 M NaCl at pH 9.0. It is evident that a single symmetrical sedimenting boundary is obtained with the purified carboxylase preparation (step 6, Table I). The sedimentation coefficient of the protomeric form exhibits a marked dependence on protein concentration which is suggestive of molecular asymmetry; a plot of $1/s_{20,w}$ *vs.* enzyme concentration (not shown) is linear and extrapolates to an $s^0_{20,w}$ of 14.7 S. Viscosity measurements obtained with the avian liver acetyl-CoA carboxylase protomer indicate that it is rather asymmetric (Moss and Lane, 1972). In the presence of citrate, the bovine adipose tissue carboxylase sediments as a high molecular weight species having an $s_{20,w}$ of 68 S (Figure 2B). The schlieren patterns of the polymeric form reveal a single sedimenting boundary which has hypersharp character consistent with its filamentous structure as visualized by electron microscopy (Figure 3). Due to the low solubility of the polymeric form of the enzyme in dilute buffer at neutral pH values, the protein concentration that could be used in sedimentation velocity experiments was limited.

Electron micrographs of the carboxylase in the presence of citrate, illustrated in Figure 3, show that the enzyme exists as long, filamentous structures, with widths of from 70 to 100 Å and lengths of about 0.3 μ. Helical and irregularly twisted regions were evident along the filament axis. The lengths of the filaments varied with buffer and activator concentration with lengths of up to 1 μ having been observed. Alternatively, the presence of the reactants, such as malonyl-CoA which lead to enzyme–CO_2^- formation, resulted in the disruption of the filamentous structures. This observation is supported by sucrose density gradient centrifugation experiments to be discussed later.

Sedimentation equilibrium experiments by the method of Yphantis (1964) on the protomeric form of the carboxylase in 0.5 M NaCl at pH 9 gave a molecular weight of 560,000 when extrapolated to zero enzyme concentration. Plots of log concentration *vs.* (radius)^2 were linear, which provides further evidence for homogenity of the enzyme preparation.

Kinetic Studies. Acetyl-CoA carboxylases from a number of animal tissues have been shown (Matsuhashi *et al.*, 1962; Kallen and Lowenstein, 1962; Waite, 1962; Martin and Vagelos, 1962a,b; Waite and Wakil, 1962; Lane and Moss, 1971b; Moss and Lane, 1971) to be activated by certain tricarboxylic acids, most notably citrate. When the adipose tissue carboxylase, dialyzed against Tris (Cl^-) buffer (pH 7.6) in the absence of citrate, is added to assay reaction mixture without activator or bovine serum albumin, it is initially active, but decays slowly to a less active state (Figure 4; curve A). Other experiments show that the presence of bovine serum albumin (0.6 mg/ml) in the assay reaction mix retards the rate of activity decay. As shown in Figure 4 (curves C, D, and E) the carboxylase is instantaneously reactivated upon addition of citrate. The citrate effect increased with increasing length of preliminary incubation in assay reaction mixture minus activator. Addition of citrate at 5.5 min resulted in a 75-fold activation

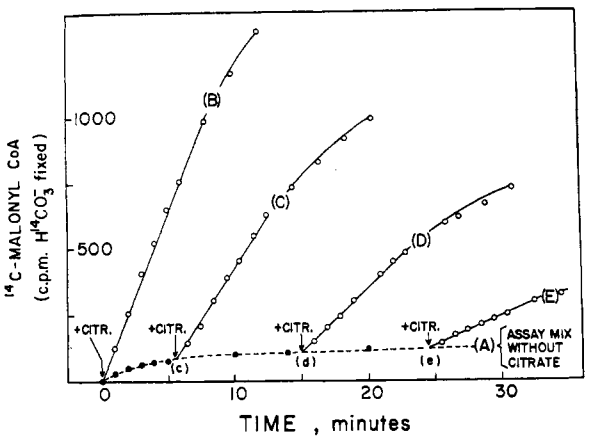

FIGURE 4: Kinetics of reversible inactivation of the polymeric carboxylase in assay reaction mixture minus tricarboxylic acid activator. 27 munits of the homogeneous carboxylase (step 5, Table I) in its polymeric state in 10 mM Tris (Cl^-) buffer (pH 7.6) containing 0.1 mM EDTA–5 mM mercaptoethanol was used to initiate the carboxylation reaction in 5.6 ml of standard assay reaction mixture minus citrate and bovine serum albumin. After 5.5, 15, and 24.5 at 37°, citrate (10 mM, final concentration) was added to a 1.5-ml aliquot; 0.1-ml aliquots were withdrawn as indicated and acid-stable ^{14}C activity (as malonyl-CoA) was determined as described in Experimental Procedure. To obtain the data for curve B, the same procedure was followed except that 10 mM citrate was present in the reaction mixture from zero time.

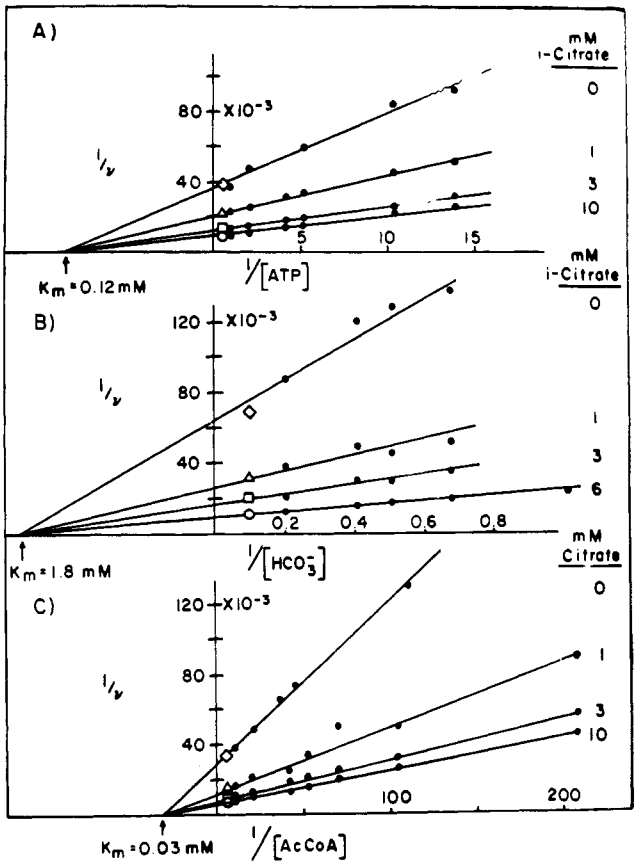

FIGURE 5: Effect of tricarboxylic acid activator on the K_m and V_{max} values for ATP, HCO_3^-, and acetyl-CoA. Acetyl-CoA carboxylation rates were determined as described in Experimental Procedure except for the omission of bovine serum albumin from assay reaction mixtures and for the variable additions specified. A 5- to 8-min preliminary incubation of the enzyme in an incomplete reaction mix was permitted to allow the enzyme to decay to the "catalytically inactive" state before initiating the reaction. When ATP was the variable substrate, an 8-min preliminary incubation was used and the reaction was initiated with ATP (± isocitrate), whereas when bicarbonate or acetyl-CoA were variable substrates, a 5-min preliminary incubation was employed and the reactions were started with acetyl-CoA (± tricarboxylic acid activator). Since some enzyme activity was lost during preliminary incubation without activator, somewhat higher than usual enzyme levels were used (6–10 μg of carboxylase/ml of reaction mixture). Zero-order kinetics were obtained during the 3-min course of the assays. Initial velocity, v, is expressed as cpm of [^{14}C]bicarbonate fixed per min per 0.1-ml aliquot; the specific activity of the [^{14}C]bicarbonate used was 250 cpm/nmole.

(Figure 4; point c); in contrast, shorter preliminary incubations resulted in much lower apparent citrate effects. The reversible decay of catalytic activity is correlated with depolymerization of the polymeric filamentous form of the carboxylase to its protomeric form (see next section, Effect of Activator and Reaction Mix on Polymeric State). A fraction of the enzyme is irreversibly inactivated under assay conditions in the absence of activator as indicated by the decreasing slopes of the rate lines (unbroken lines C, D, and E in Figure 4) with increasing time of exposure to assay mix minus citrate at 37°. The half-life for this irreversible decay of activity was 10 min.

Earlier investigations in this laboratory (Gregolin *et al.*, 1968a) with the avian liver carboxylase indicated that the primary kinetic effect of tricarboxylic acid activator was on V_{max} rather than on the K_m values for substrates. In contrast to this, recent reports from Numa's laboratory (Numa *et al.*, 1970; Hashimoto *et al.*, 1970) on the rat liver enzyme suggest that citrate reduces the apparent K_m value for acetyl-CoA. However, in this case the reaction was initiated with enzyme that had undergone preliminary activation with high levels of citrate (10 mM) prior to initiating the reaction. In view of the facts presented in this and an earlier paper (Moss and Lane, 1972) which indicate the slow attainment of polymer–protomer equilibrium when the reaction is initiated with polymer, it was imperative to reexamine the activator effects on K_m and V_{max} following equilibration of the enzyme at each of the variable reaction conditions. In the present experiments care was taken to allow the carboxylase to equilibrate prior to initiating the reaction. Failure to take this into consideration resulted in hybrid reaction rates primarily characteristic of the polymer, even in the absence of citrate. When these precautions were taken, linear reaction rates were obtained.

As is evident from Figure 5, the sole effect of tricarboxylic acid activator appears to be on V_{max}, which is increased in all cases; there is no detectable effect on K_m's. Isocitrate, rather than citrate, was utilized as activator, particularly when ATP was the variable substrate because of its lower affinity for Mg^{2+}. Under the conditions employed ATP was almost totally (>99%) in the form of ATP–Mg complex at all concentrations of isocitrate (Gregolin *et al.*, 1968a).

In addition to acetyl-CoA, the purified enzyme also carboxylates propionyl-CoA and butyryl-CoA at 65 and 10%, respectively, the rate with acetyl-CoA under standard assay conditions (citrate present). In the presence of activator, the K_m values for acetyl-CoA and propionyl-CoA are identical, *i.e.*, K_m = 20–30 μM. The pH optima for the purified enzyme are 7.9–8.0 with acetyl-CoA, 7.6–7.7 with propionyl-CoA, and 7.3–7.4 with *n*-butyryl-CoA as acceptor substrates. The apparent K_a values for citrate and DL-isocitrate were dependent upon the time of preliminary incubation of the enzyme in assay reaction mixture prior to the addition of activator, *i.e.*, longer times of preliminary incubation led to increased K_a

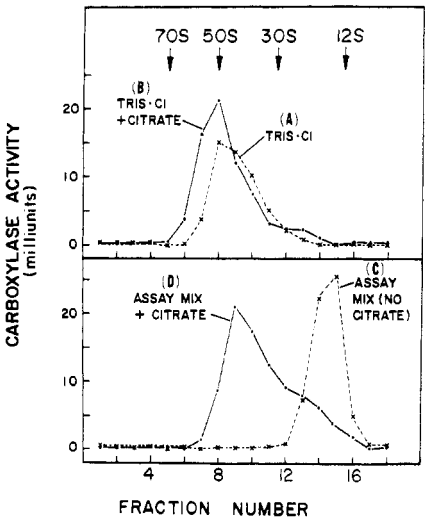

FIGURE 6: Sedimentation velocity of adipose tissue acetyl-CoA carboxylase in Tris (Cl^-) buffer and assay reaction mixture in the presence and absence of citrate. Analytical sucrose density gradient centrifugation was carried out as described in Experimental Procedure. Sucrose density gradients contained: (A) Tris·Cl = 10 mM Tris (Cl^-) buffer (pH 7.6)–0.1 mM EDTA–5 mM mercaptoethanol; (B) Tris·Cl + citrate = as in A plus 10 mM potassium citrate; (C) assay mix (no citrate) = standard carboxylase assay reaction mixture (see Experimental Procedure) minus citrate and bovine serum albumin; and (D) assay mix + citrate = as in C plus 10 mM potassium citrate. Twelve micrograms (90 munits) of purified carboxylase (step 5, Table I) was diluted in the respective buffers or reaction mixtures (A–D) and layered onto their respective gradients. Gradients were centrifuged at 25° after which the tubes were punctured, and the contents were collected fractionally and assayed for carboxylase activity. The recovery of enzyme activity applied to the gradients ranged from 65 to 90%. External gradient markers were 70, 50, and 30 S. *Escherichia coli* ribosomes and 12S avian liver fatty acid synthetase (Yang *et al.*, 1965).

values. Following a 10-min preliminary incubation, maximal K_a values of 3–4 and 7–8 mM were obtained for citrate and DL-isocitrate, respectively. With the avian liver enzyme, the apparent K_a for tricarboxylic acid activator appears to depend (Gregolin *et al.*, 1966b; Gregolin *et al.*, 1968b; Moss and Lane, 1971) upon the position of the polymer $\rightleftarrows$ protomer equilibrium and factors which tend to displace it. Moreover, it has also been observed (Moss and Lane, 1971) that a lower activator concentration is needed to retard depolymerization than is required to effect polymerization of carboxylase initially in the protomeric state. Thus, it was not surprising to find with the adipose tissue enzyme that prolonged preliminary incubation without activator, which lowers the polymer/protomer ratio, causes an increase in the apparent K_a for citrate or isocitrate.

Effect of Activator and Assay Reaction Mix on Polymeric State. Insight into the effect of activator and assay reaction mixture on the state of aggregation of the carboxylase was obtained by sucrose density gradient centrifugation. As shown in Figure 6, the carboxylase exists as a rapidly sedimenting species in Tris (Cl^-) buffer (pH 7.6) in the presence (B) or absence of citrate (A); the filamentous nature of the 45–50S species obtained under these conditions was confirmed by electron microscopy. Like the experiments (Figure 2A) conducted with the analytical ultracentrifuge at higher carboxylase concentrations, the polymeric form is dissociated to a slowly sedimenting 13–15S protomeric form by the addition of 0.5 M NaCl to sucrose density gradients containing Tris (Cl^-) buffer without citrate (Moss and Lane, unpublished experiments). The polymeric state of the bovine adipose tissue enzyme in Tris (Cl^-) buffer without citrate stands in contradistinction to the avian and rat liver carboxylases, which exist as low S species under these conditions, and further illustrates the greater stability of the bovine adipose tissue carboxylase filaments. However, when the enzyme was subjected to sucrose density gradient centrifugation in assay reaction mix minus citrate (Figure 6C), the 13–15S species, previously shown to be protomeric (Figure 2A), was formed. In contrast, centrifugation in assay mix plus citrate (Figure 6D) yields the rapidly sedimenting polymeric form. It is evident, therefore, that the presence of citrate is not necessary to retain the enzyme in the polymeric state in dilute Tris (Cl^-) buffer. However, upon addition of the components of the assay mix (MgATP, HCO_3^-, and acetyl-CoA), citrate is required for maintenance of the polymeric state (Figure 6C,D). It has been established with the avian liver enzyme (see Discussion and Gregolin *et al.*, 1966b, 1968b) that exposure to malonyl-CoA or certain components of the assay mix, notably MgATP and HCO_3^-, gives rise to the carboxylated form of the enzyme (enzyme–CO_2^-) and thereby promotes depolymerization.

Thus, the decay of catalytic activity produced by incubating the carboxylase in assay reaction mixture without citrate (Figure 4; curve A) apparently occurs concurrent with the transition of the polymeric 45–50S species to the protomeric 13–15S form. This dissociative process can be reversed by the addition of citrate to carboxylase previously incubated in citrate-free assay reaction mix; the carboxylase now sediments as the high S species on sucrose density gradients containing assay reaction mixture and citrate (Figure 6D) and appears filamentous by electron microscopy. This citrate-dependent reaggregation of the carboxylase is consistent with the citrate reactivation observed in the previous kinetic experiments (Figure 4; curves C, D, and E), and indicates that the reactivation is associated with polymerization. However, the time required for the gradient experiment permits us to view only the extremes of the decay curves, *i.e.*, the polymeric form in Tris (Cl^-) buffer and in assay reaction mix plus citrate, and the protomeric species in reaction mix minus citrate. The tight coupling of changes in catalytic activity to changes in polymeric state has been confirmed with the avian liver enzyme (Moss and Lane, 1972).

Activation Phenomena Associated with Crude Carboxylase Preparations. Our initial experiments with crude carboxylase preparations (*i.e.*, steps 1 and 2 in Table I) suggested that an inhibitory compound(s) may mask the full expression of enzyme activity. Introduction of (+)-palmitylcarnitine into the assay mix or extensive dilution of the enzyme prior to assay resulted in a substantial enhancement of activity, as shown in Figure 7 (lines A and C, respectively). Dilution and assay in the presence of (+)-palmitylcarnitine resulted in an almost additive eightfold activation (Figure 7; line B). With the undiluted carboxylase preparation increasing the (+)-palmitylcarnitine concentration produces a hyperbolic activation response (Figure 7; curves I, II), whereas, with the diluted preparations, (+)-palmitylcarnitine becomes inhibitory at high concentrations (Figure 7; curves III, IV, V). When the experiments were performed with the homogeneous carboxylase, it was observed that dilution had no effect whatsoever (Figure 7; line D), and palmitylcarnitine was slightly inhibitory (Figure 7; line E) at a concentration which produced maximal stimulation with the crude preparation. Experiments (not shown) conducted with the naturally occurring (−) isomer of palmitylcarnitine show that it too activates the

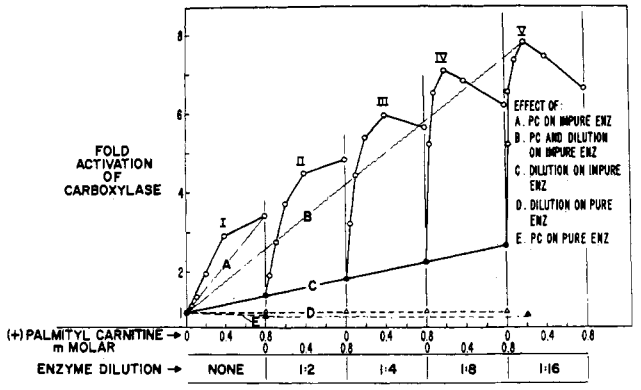

FIGURE 7: Activation of crude acetyl-CoA carboxylase by (+)-palmitylcarnitine and dilution. The carboxylation reaction was initiated by addition of ammonium sulfate-fractionated (130 μg, when no dilution was effected; step 2, Table I; specific activity, 0.1 unit/mg) or homogeneous (0.05–1.0 μg; step 6, Table I) carboxylase to the standard carboxylase assay reaction mixture without bovine serum albumin, but containing 10 mM potassium citrate; (+)-palmitylcarnitine was added as indicated. Enzyme dilutions were made immediately prior to assay with 10 mM Tris (Cl^-) buffer (pH 7.0), containing 10 mM potassium citrate–0.1 mM EDTA–5 mM mercaptoethanol. Acetyl-CoA carboxylation rates were determined as described in Experimental Procedure.

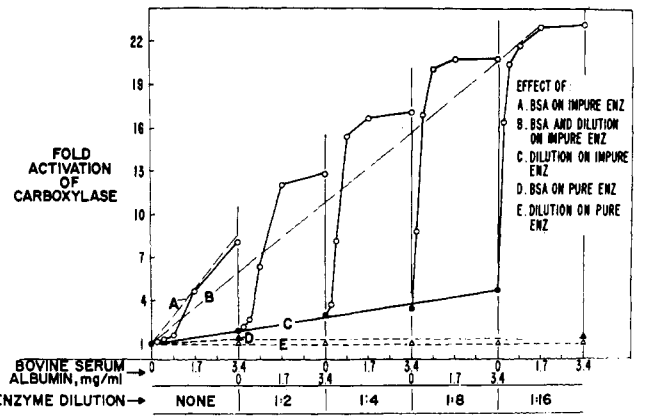

FIGURE 8: Activation of crude acetyl-CoA carboxylase by bovine serum albumin and dilution. Conditions and procedure were the same as in Figure 7 except that bovine serum alumin was added as indicated.

crude enzyme preparation, although to a much lesser degree (+) isomer.

Enhancement of activity was also noted when bovine serum albumin was added to the assay mix (Figures 8). Assay of the undiluted enzyme in the presence of bovine serum albumin resulted in an eightfold activation (Figure 8; line A). Dilution of this carboxylase preparation prior to assay resulted in a fivefold increase in activity (Figure 8; line C) while dilution and assay in the presence of bovine serum albumin gave an almost additive stimulation (Figure 8; line B). In contrast, with the homogeneous enzyme, dilution and assay in the presence of bovine serum albumin produce only a marginal increase in activity (Figure 8; line D). The combined addition of bovine serum albumin and (+)-palmitylcarnitine to the assay reaction mixture results in an additive activation when undiluted enzyme is used (Moss and Lane, unpublished experiments). Since serum albumin binds apolar compounds, such as fatty acids and their CoA derivatives (Spector, 1969; Goodman, 1957; Dole and Meinertz, 1960; Foster, 1960; Spector *et al.*, 1969), and palmitylcarnitine is thought to act by competitive displacement of these substances (Fritz and Hsu, 1967), the activation phenomena observed in the present studies are best explained by removal of endogenous hydrophobic inhibitor(s) from the crude carboxylase. The fact that palmitylcarnitine and bovine serum albumin have no significant activating effect on the homogeneous carboxylase strongly supports this interpretation.

Discussion

Acetyl-CoA carboxylase from animal tissues appears to be capable of oscillating between catalytically active polymeric and catalytically inactive protomeric states, the level of carboxylase activity presumably determined by the position of the protomer–polymer equilibrium (Lane and Moss, 1971a; Moss and Lane, 1971, 1972; Lane *et al.*, 1970; Lane and Moss, 1971b). Tricarboxylic acid activators, such as citrate and isocitrate, are capable of shifting this equilibrium toward the catalytically active state, apparently by inducing a productive conformational change in the carboxylase protomer which favors polymerization. As shown in the present investigations and those of Gregolin *et al.* (1968b, 1966a), in this laboratory, under conditions approximating those of the enzymatic assay, citrate or isocitrate induces a change in $s_{20,w}$ of the carboxylase from 13–15 S to 45–50 S, a rapid transition from protomeric to filamentous form as judged by electron microscopy and a large increase in intrinsic viscosity. As shown in other investigations (Moss and Lane, 1972), the close correlation of the kinetics of depolymerization of the filamentous form with the rate of loss of enzymatic activity indicates that catalytic activity is determined by the polymeric state of the enzyme.

The position of the protomer–polymer equilibrium can be shifted toward the protomer by converting the enzyme to its carboxylated form, enzyme-biotin-CO^{-2} (Gregolin *et al.* 1966b, 1968b), or by the binding of certain hydrophobic compounds, *i.e.*, long-chain fatty acyl-CoA derivatives (Bortz and Lynen, 1963; Numa *et al.*, 1965b) or fatty acids (Yugari and Matsuda, 1967; Levy, 1963). It appears (Moss and Lane, 1971) that introducing a carboxyl group at the 1′-N position of the biotinyl prosthetic group causes sufficient conformational strain, such that activators, *i.e.*, citrate and isocitrate, are able to constrain the enzyme and prevent depolymerization. Malonyl-CoA, which can carboxylate the enzyme, is a competitive inhibitor of the overall forward reaction with respect to citrate (Gregolin *et al.*, 1966b). This competitive kinetic relationship apparently results from the opposing effects of citrate and malonyl-CoA on the protomer–polymer equilibrium. Like inhibition by malonyl-CoA, that by long-chain acyl-CoA derivatives (K_i = (3–8) $\times 10^{-7}$ M) (Numa *et al.*, 1965a; Bortz and Lynen, 1963; Numa *et al.*, 1965b) and fatty acids can be explained by their effects on this equilibrium. Fatty acyl-CoA derivatives, such as palmityl-CoA, stearyl-CoA, and oleyl-CoA, are also competitive inhibitors with respect to citrate (Numa *et al.*, 1965a). Furthermore, these derivatives promote depolymerization of the carboxylase which can be prevented by citrate (Numa *et al.*, 1965a,b). Reversal of the inhibition of acetyl-CoA carboxylase by fatty acyl-CoA derivatives with (+)-palmitylcarnitine or cetyltrimethylammonium ion is probably attributable to their displacement from the enzyme (Greenspan and Lowenstein, 1968). Evidence presented in this paper and elsewhere (Fritz and Hsu, 1966, 1967; Greenspan and Lowenstein, 1968;

Marquis *et al.*, 1968; Moss *et al.*, 1969) indicates that an endogenous hydrophobic inhibitor(s) of acetyl-CoA carboxylase is present in crude enzyme extracts of adipose tissue and liver. The activating effect of (+)-palmitylcarnitine or serum albumin on impure acetyl-CoA carboxylase preparations may also be due to the displacement of hydrophobic inhibitors, since homogeneous carboxylase preparations are unaffected by these substances. Impure preparations of acetyl-CoA carboxylase from bovine adipose tissue are markedly activated by palmitylcarnitine (Figure 7), bovine serum albumin (Figure 8), or extensive dilution of the enzyme preparation prior to assay (Figures 7 and 8). On the other hand, none of these agents (palmitylcarnitine, serum albumin, or dilution), which activate the impure carboxylase, have an activating effect on the homogeneous enzyme. It is evident that an inhibitory substance, apparently hydrophobic in nature, is removed either by purification of the enzyme or by the agents or treatments mentioned above.

Cytoplasmic filaments with dimensions similar to those of the citrate-activated bovine adipose tissue carboxylase (Figure 3) have been observed *in situ* (Bloom and Fawcett, 1968; Schotz *et al.*, 1969; Sheldon and Ferguson, 1970). Sheldon and Ferguson (1970) recently reported the occurrence of filamentous structures surrounding the fat droplets in thin sections of developing adipose tissue of yellow obese mice. This and the fact that purified acetyl-CoA carboxylases isolated from several sources (bovine adipose tissue, avian (Gregolin *et al.*, 1966a) and human (Kleinschmidt *et al.*, 1969) liver) are filamentous suggests that these structures may have physiological relevance.

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