Acetyl Coenzyme A Carboxylase System of *Escherichia coli*

PURIFICATION AND PROPERTIES OF THE BIOTIN CARBOXYLASE, CARBOXYLTRANSFERASE, AND CARBOXYL CARRIER PROTEIN COMPONENTS*

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SUMMARY

The three protein components (biotin carboxylase, carboxyltransferase, and the biotin-containing carboxyl carrier protein of the acetyl coenzyme A carboxylase system have been resolved and purified extensively or to homogeneity from cell-free extracts of *Escherichia coli* B. Carboxylation of acetyl-CoA to form malonyl-CoA requires the presence of all three components. Biotin carboxylase, which catalyzes the first half-reaction,

\[
\text{Me}^{+2} + \text{HCO}^{-} + \text{ATP} \rightleftharpoons \text{CCP-carboxyl carrier protein} - \text{biotin} \rightleftharpoons \text{CCP} + \text{CO}_2 + \text{ADP} + \text{Pi}
\]

has been purified to a homogeneous state and has been crystallized; earlier work in this laboratory showed the enzyme to be composed of two 50,000-dalton subunits. The carboxylation of free d-biotin which can substitute for carboxyl carrier protein-biotin as model substrate is markedly activated by certain organic solvents; an optimal rate enhancement of 10-fold is obtained with 15% (v/v) ethanol. Activation by ethanol affects \( V_{\text{max}} \) and is not accompanied by changes in \( K_m \) values or the state of aggregation of the enzyme. Moreover, none of the carboxyl carrier protein-independent reactions catalyzed by biotin carboxylase, e.g. acetyl-CoA carboxylation, ATP-\[^{14}C\]ADP exchange, and ATP-\[^{32}P\] exchange, are activated by organic solvents.

Carboxyltransferase, the catalyst for the second half-reaction,

\[
\text{CCP-carboxyl carrier protein} - \text{biotin} + \text{acetyl-CoA} \rightleftharpoons \text{CCP-carboxyl carrier protein} - \text{biotin} + \text{malonyl-CoA}
\]

was purified to apparent homogeneity. Biotin carboxylase, although not required for the second half-reaction as measured by carboxyltransferase- and carboxyl carrier protein-independent malonyl-CoA-\[^{14}C\]acetyl-CoA exchange, activates this process indicating the existence of a ternary complex between the three protein components. In addition to the above reaction (Reaction II), carboxyltransferase catalyzes: (a) net transcarboxylation from malonyl-CoA to free d-biotin derivatives in the absence of biotin carboxylase and carboxyl carrier protein, and (b) a slow biotin-independent decarboxylation of malonyl-CoA. The carboxyltransferase component has a molecular weight of 130,000 and is composed of nonidentical polypeptide chains of 30,000 and 35,000 daltons.

Carboxyl carrier protein has been purified extensively by a combination of conventional methods and affinity chromatography with Sepharose-avidin (monomer). Polyacrylamide gel electrophoresis of purified carboxyl carrier protein revealed two major proteins both of which contain biotin and exhibit carboxyl carrier protein activity, i.e. carboxyl carrier protein- and carboxyltransferase-dependent malonyl-CoA-\[^{14}C\]acetyl-CoA exchange.

The two catalytic components of the *E. coli* acetyl-CoA carboxylase system, biotin carboxylase and carboxyltransferase, are devoid of free or covalently bound biotin yet have the ability to carry out their respective model half-reactions utilizing free d-biotin derivatives in place of carboxyl carrier protein. Thus, in addition to possessing binding sites for their respective substrates, each catalytic component must contain a specific binding site for the biotinyl moiety of carboxyl carrier protein. It is evident that during the over-all sequence (Reactions I + II) the carboxylated biotinyl prosthetic group must undergo translocation from the carboxylation site on biotin carboxylase to the transfer site on carboxyl transferase while remaining attached to carboxyl carrier protein through its 14 A side chain.

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tions, the first involving carboxylation of the biotin prosthetic group by an MgATP-dependent process (2 4) and the second, carboxyl transfer from the carboxylated prosthetic group to acetyl-CoA to yield malonyl-CoA (5, 6). Unlike its counterparts in animal tissues (1, 7-12), the carboxylase system from E. coli dissociates readily into three protein components, all of which are essential for acetyl-CoA carboxylation. As will be demonstrated in this and the accompanying papers, the resolved components retain the capacity to carry out their respective partial reactions, thus permitting studies on the carboxylation mechanism and its regulation not possible with unresolved animal carboxylases. Two of these components, i.e. biotin carboxylase and carboxyltransferase, possess catalytic centers for the first (Reaction 1) and second (Reaction 2) half-reactions, respectively, of acetyl-CoA carboxylation.

\[
\begin{align*}
\text{Me}^+ + \text{HO-CO}_2^- + \text{ATP} &\rightarrow \text{CCP-biotin-CO}_2^- + \text{ADP} + \text{Pi} + \text{OH} \\
\text{CCP-biotin-CO}_2^- + \text{acetyl-CoA} &\rightarrow \text{Me}^+ + \text{malonyl-CoA} \\
\text{Net: HO-CO}_2^- + \text{ATP} + \text{acetyl-CoA} &\rightarrow \text{Me}^+ + \text{malonyl-CoA} + \text{ADP} + \text{Pi} \\
\end{align*}
\]

as described by Callanan et al. (33) to reduce the pore size and prevent the escape of carboxyl carrier protein.

**Enzyme Assays**

**Biotin Carboxylase:** [Ucarboxylase Fixation Assay—To obviate high blanks due to 14C contaminant(s) in commercial NaHCO3 not removed by gassing with CO2. H14CO3- was purified as follows. The reaction mixture contains 100 mM triethanolamine (Cl-) buffer, pH 8.0; 1 mM ATP; 8 mM MgCl2; 8 mM KHCO3 (300 cpm per mole); 50 mM potassium d-biotin; 3 mM glutathione; 0.3 mg of bovine serum albumin; 0.05 mM of ethyl alcohol; and 0.1 to 1.0 millimolar of biotin carboxylase in a total volume of 0.5 ml. The reaction is initiated with enzyme, incubated for 10 min at 30°, and carboxylation is terminated by rapid transferral of a 0.4-ml aliquot to 1 ml of ice-cold water containing 2 drops of 1-octanol. CO2 is bubbled through the solution until neutral, 0.1 N HCl, water (until neutral), 0.1 N NaOH, water (until neutral), 0.05 M potassium phosphate, pH 7.0, and finally, 20 mM potassium phosphate, pH 7.0, containing 0.1 mM EDTA and 5.0 mM 2-mercaptoethanol.

**Experimental Procedure**

**Materials**

Full or 1/2 log phase E. coli B cells grown on enriched medium were purchased from Grain Processing Corp., Muscatine, Iowa. Calcium phosphate gel was prepared according to the method of Keilin and Hartree (27) except that the gel was washed more extensively with distilled water until the washes were chloride-free. Standard and type 20 DEAE-cellulose and standard phosphocellulose ion exchangers were obtained from the Schleicher and Schuell Company. DEAE-cellulose was equilibrated by washing with 0.5 M potassium phosphate, pH 7.0, followed by 0.02 M potassium phosphate, pH 7.0, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol. Phosphocellulose was equilibrated by successive washing with 0.1 N NaOH, water (until neutral), 0.1 N HCl, water (until neutral), 0.05 M potassium phosphate, pH 7.0,
0.5 ml containing 50 mM Tris (Cl-) buffer, pH 8.0; 85 PM [3-14C]-malonyl-CoA (4 to 100 cpm per nmole); 10 mM d-biotin methyl ester; 0.5 mg of bovine serum albumin; and up to 1 milliunit of carboxyltransferase. After 5, 10, 15, and 20 min of incubation at 30°, 0.1-ml aliquots are transferred to scintillation vials containing 0.1 ml of 5 N HCl. The mixture is taken to dryness at 95° in a forced-draft oven after which water and scintillator are added and residual 14C acid-stable radioactivity determined. The 14C-carboxyl group of N-carboxybiotin methyl ester generated during the reaction are volatile under these conditions, whereas [14C]-malonyl-CoA is not. The amount of carboxyl transfer or decarboxylation at zero time and after incubation. Malonyl-CoA decarboxylase activity is estimated as described above but with the d-biotin methyl ester omitted; carboxyl transfer is equal to acid-stable radioactivity lost during incubation with d-biotin methyl ester minus that lost in the absence of the d-biotin derivative.

It has been established (5, 34) with the use of [3-14C]malonyl-CoA as carboxyl donor and a d-biotin derivative as acceptor that the acid-labile product stable to gassing with CO2 is the II-N-carboxybiotin derivative. Under the conditions described, the rate of carboxyl transfer is approximately 30 times faster than malonyl-CoA decarboxylation. The assay follows zero-order kinetics and activity is proportional to enzyme concentration.

**Carboxyltransferase Spectrophotometric Assay** This assay can be employed only with enzyme carried beyond Step 3 of Table I; it involves coupling the acetyl-CoA generated in the reaction to the combined citrate synthase-malate dehydrogenase reactions and following NADH reduction spectrophotometrically. The reaction mixture contains 100 mM Tris (Cl-) buffer, pH 8.0, 0.1 mM malonyl-CoA, 5 mM d-biotin methyl ester, 10 mM L-malate, 0.5 mM NADH, 0.06 mg of bovine serum albumin, 18 units of malate dehydrogenase, 3.5 units of citrate synthase, and 1 to 10 milliunits of carboxyltransferase. The reaction is conducted at 30° and is initiated by the addition of d-biotin methyl ester; NADH formation is followed at 340 nm for 3 min.

One unit of carboxyltransferase catalyzes the formation of 1 μmole of free carboxybiotin methyl ester per min.

**Carboxyl Carrier Protein. Malonyl-CoA-[14C]Acetyl-CoA Exchange Assay**—Carboxyltransferase catalyzes an isotopic exchange between [14C]acetyl-CoA and malonyl-CoA which is dependent upon carboxyl carrier protein (see Reaction 2 and Refs. 5, 6, and 13). The reaction mixture contains 50 mM imidazole (Cl-) buffer, pH 6.5, 0.1 mM malonyl-CoA, 0.2 mM [1- or 2-14C]-acetyl-CoA (600 cpm per nmole), 1 mM diethanolamine, 10 milliunits of carboxyltransferase, and carboxyl carrier protein in a total volume of 0.25 ml. After initiating the reaction (30°) with carboxyltransferase, 50 μl aliquots are transferred at 2, 4, 6, and 8 min to scintillation vials containing 0.1 ml of 6 N HCl, the mixture taken to dryness at 95°, and the acid-stable 14C activity incorporated into malonyl-CoA determined as indicated for the carboxyltransferase assay (see preceding section). The rate of exchange is proportional to carboxyl carrier protein concentration up to a level of 0.20 nmole of covalently bound biotinyl prosthetic group at the specified level of carboxyltransferase. Since the exchange rate also depends upon the concentration of carboxyltransferase, the amount of purified transferase added must be adjusted to compensate for that present in the carboxyl carrier protein preparation. Following the first step in the purification of carboxyl carrier protein involving exposure to 6 M guanidine hydrogen chloride, the preparation is free of carboxyltransferase activity. Care must also be taken to avoid high salt concentrations since the exchange reaction is markedly inhibited by most salts at >0.1 M.

**Carboxyl Carrier Protein. Stoichiometric Carboxylation Assay**—Carboxyl carrier protein may also be assayed by determining the extent of its carboxylation with H14CO3 of known specific activity in the presence of Mg2+ ATP and excess biotin carboxylase (see Reaction 1). The extent of carboxylation of dialyzed preparations provides a stoichiometric measure of the amount of biotinyl prosthetic group present as carboxyl carrier protein. The assay mixture contains (final volume, 0.05 ml): 100 mM triethanolamine (Cl-) buffer, pH 8.0, 20 μg of bovine serum albumin, 1 mM ATP, 8 mM MgCl2, 8 mM KH2CO3 (20 × 100 cpm per nmole), 10 to 20 milliunits of biotin carboxylase, and carboxyl carrier protein. The mixture is incubated for 10 min at 30°; 1.0 ml of cold water is added, followed by 3 drops of 1-octanol after which CO2 is bubbled through the mixture at 0° for 30 min. The solution is transferred to a counting vial containing 0.1 ml of 0.1 N NaOH and the amount of 14C activity stable to gassing with CO2 (i.e., carboxyl biotinyl carrier protein) determined.
Treatment of the carboxyl carrier protein with avidin abolishes its ability to support the malonyl-CoA-to-acetyl-CoA exchange reaction and its capacity to undergo carboxylation by biotin carboxylase; prior loading of avidin with free biotin renders it ineffective as inhibiton of both processes.

RESULTS

Isolation and Purification of Biotin Carboxylase from E. coli B

The first three steps of the purification procedure for biotin carboxylase are identical with those for the purification of carboxyltransferase to be described later. All manipulations are carried out at 2-4°C and all solutions employed contain 1 mM EDTA and 5 mM 2-mercaptoethanol. The results of a typical biotin carboxylase purification are summarized in Table I. A kilogram of E. coli B cells, 3/4 log phase, grown on enriched medium are suspended in 4 liters of 0.1 M potassium phosphate buffer, pH 7.0. The suspension is passed twice through a precooled Manton-Gaulin submicron dispersor at 9000 p.s.i. The cell breaker is washed two or three times with 500 ml of buffer; the washes are combined with the extract, the pooled extract centrifuged at 20,000 × g for 30 min, and the supernatant solution retained.

Ammonium Sulfate Fractionation (85 to 42% Saturation)—The supernatant solution is brought to 25% saturation by addition of solid ammonium sulfate (144 g per liter); the pH was maintained at 7.0 with dilute ammonium hydroxide. After 15 min, the mixture is centrifuged and the supernatant recovered and brought to 45% saturation with solid ammonium sulfate (125 g per liter). The precipitate is recovered by centrifugation and extracted with 4 liters of a 42% saturated ammonium sulfate solution containing 50 mM potassium phosphate buffer, pH 7.0. After removing the supernatant, the precipitate is either stored at −90°C or fractionated immediately with calcium phosphate gel for the purification of biotin carboxylase (below) or carboxyltransferase.

Calcium Phosphate Gel Fractionation—The precipitated protein from the preceding step (approximately 27 g of protein) is dissolved in 200 ml of 5 mM K2HPO4, containing 20% glycerol and dialyzed overnight against 10 liters of the same solution. The solution is then dialyzed against the same buffer and crystallization usually begins within 24 hours. As shown in Fig. 1 (inset), the crystals appear as prisms ranging from 0.01 to 0.025 mm. Crystallization can be accelerated by use of seed crystals or by 10% ethane in the medium (35); crystals prepared in the presence of ethanol are larger and appear as plates which crack spontaneously.

Isolation and Purification of Carboxyltransferase from E. coli B

The preparation of a cell-free extract of E. coli B cells and its fractionation with ammonium sulfate (25 to 42% saturated ammonium sulfate fraction) are carried out as described in the preceding section except that 2 kg of packed cells are used. All subsequent steps are performed at 2-4°C with solutions containing 1 mM EDTA and 5 mM 2-mercaptoethanol. The results of a typical purification are summarized in Table II.

Calcium Phosphate Gel Fractionation—The precipitate obtained by ammonium sulfate fractionation (25 to 42% saturation) as described in the preceding section is dissolved in 250 ml of 2 mM K2HPO4 and dialyzed against 25 liters of the same buffer overnight. The dialyzed enzyme is diluted with buffer to a protein concentration of 20 mg per ml and sufficient calcium phosphate gel (30 mg dry weight per ml) is added to adsorb 65% of the protein; this usually requires a gel-protein ratio of approximately 2.5:1.0. After stirring for 30 min, the mixture is centrifuged and the supernatant solution containing less than 10% of the original biotin carboxylase activity is discarded. The gel is washed once with 2 liters of 5 mM potassium phosphate buffer, pH 7.0. The precipitated enzyme is eluted by resuspending the gel precipitate in 2 liters of 0.12 M potassium phosphate buffer, pH 7.0, stirring for 30 min, and centrifuging; this process is repeated two additional times and the eluates combined and brought to 60% saturation with solid ammonium sulfate (213 g per liter). While all of the biotin carboxylase activity is recovered in this eluate, carboxyltransferase activity remains adsorbed to the gel. Approximately 6 g of protein are recovered in the 0.12 M buffer eluate and this can be stored frozen at −90°C as a pellet after centrifugation or fractionated immediately by DEAE-cellulose chromatography.

DEAE-cellulose Chromatography—The precipitated enzyme is dissolved in 75 ml of 10 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol. After dialysis against the same buffer and centrifugation, the supernatant (about 6 g of protein) is applied to a 3-liter DEAE-cellulose column (9 × 50 cm) previously equilibrated with dialysis buffer. Elution is accomplished with 8 liters of the same 10 mM potassium phosphate buffer and the eluate is collected fractionally. Biotin carboxylase activity appears with the first protein eluted from the column and precedes the major “breakthrough” protein peak. This step achieves a purification of at least 100-fold, giving rise to approximately 30 to 40 mg of protein in the biotin carboxylase peak; the active fractions are pooled and subjected immediately to chromatography on cellulose phosphate.

Cellulose Phosphate Chromatography—The pooled fractions from DEAE-cellulose chromatography (about 40 mg of protein) are applied directly to a 50-ml cellulose phosphate column (2 × 30 cm) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol. Elution is carried out with a 1-liter linear potassium phosphate gradient (10 to 500 mM, pH 7.0, containing 20% glycerol). The effluent is collected fractionally and is monitored for biotin carboxylase activity and protein; carboxylase activity is eluted at a phosphate concentration of approximately 0.1 M. The fractions containing maximal activity are placed in dialysis bags, and dialyzed against a solution containing 50 mM potassium phosphate buffer, pH 7.0, 20% glycerol, and sufficient ammonium sulfate to bring the solution to 60% saturation at equilibrium. After 1 to 2 days the flocculated protein is recovered by centrifugation and stored as a pellet at −90°C (no loss of activity for several months) or is subjected immediately to the crystallization procedure.

Crystallization—The precipitated enzyme from the preceding step is dissolved in a minimal volume of 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 2 mM dithiothreitol to produce a protein concentration of 4 to 6 mg per ml. The solution is then dialyzed against the same buffer and crystallization usually begins within 24 hours. As shown in Fig. 1 (inset), the crystals appear as plates which crack spontaneously.
three times with 3 liters of 0.5 M potassium phosphate buffer, pH 7.0. The pooled 0.5 M eluates which contain 80 to 90% of the original carboxyltransferase activity are brought to 60% saturation with solid ammonium sulfate (390 g per liter) and allowed to stir for 20 min. After centrifugation, the precipitate is dissolved in 200 ml of 25 mM potassium phosphate buffer, pH 7.0, and then dialyzed against 30 liters of the same buffer overnight.

First DEAE-cellulose Chromatography—The dialyzed enzyme (about 5 g of protein) is applied to a 3.8-liter DEAE-cellulose column (9 X 60 cm) previously equilibrated with 25 mM dialysis buffer. The column is washed with 4 liters of 25 mM potassium phosphate buffer, pH 7.0, after which the enzyme is eluted with an 8-liter linear phosphate gradient (25 to 500 mM, pH 7.0). Carboxyltransferase is eluted at a phosphate concentration of approximately 0.2 M. The most active fractions are pooled and the enzyme precipitated by bringing the ammonium sulfate concentration to 60% saturation with solid ammonium sulfate. After collecting the precipitated protein by centrifugation, the pellet is dissolved in 40 ml of 25 mM phosphate buffer, pH 7.0, and then dialyzed overnight against 6 liters of the same buffer.

Phosphocellulose Chromatography—The dialyzed enzyme (about 360 mg of protein) is applied to a 410-ml phosphocellulose column (2.5 X 90 cm) previously equilibrated with 25 mM dialysis buffer, pH 7.0. After passing 200 ml of the same buffer through the column, the enzyme is eluted with a 3-liter linear phosphate gradient (25 to 300 mM potassium phosphate, pH 7.0). The enzyme appears in the column eluate at a phosphate concentration of approximately 0.15 M. As shown in Fig. 2, two distinct peaks of carboxyltransferase activity are observed. The major activity peak (Peak I, at 1650 ml eluate volume) which comprised approximately 80% of the total activity is retained and further purified; although Peak II has not been further purified, its kinetic properties appear indistinguishable from those of

Peak I. The pooled fractions from Peak I (Fig. 2) are placed in dialysis bags and the enzyme is precipitated by dialysis against sufficient 65% saturated ammonium sulfate containing 50 mM phosphate buffer, pH 7.0, such that at equilibrium the percentage of saturation reaches 60. The precipitate is recovered by centrifugation, dissolved in 2 ml of 25 mM phosphate buffer, pH 7.0, and is then dialyzed overnight against 1 liter of the same buffer.

Second DEAE-cellulose (Type 20) Chromatography—The dialyzed enzyme (about 20 mg of protein) from the preceding step is applied to a 120-ml DEAE-cellulose (type 20) column (1.5 X 90 cm) and the carboxyltransferase eluted with a 500-ml linear potassium phosphate, pH 7.0, gradient (50 to 250 mM). Car-
boxytransferase activity, which appears in the eluate when the phosphate concentration reaches about 150 mM, is exactly coincident with the protein peak. Fractions having maximal enzyme activity are pooled and precipitated by dialysis against 65% saturated ammonium sulfate as described previously. After centrifugation the pellet is stored at -90°C.

**Isolation and Purification of Biotin-containing Carboxyl Carrier Protein(s) from E. coli B**

Previous investigations in this laboratory (4, 5) with a biotin auxotroph (strain SA 283) of E. coli grown on d-[14C]biotin revealed that >90% of the biotin present in the cell-free extract was recovered in the 25 to 42% saturated ammonium sulfate fraction routinely used for the preparation of biotin carboxylase (Table I) and carboxytransferase (Table II). Moreover, >80% of this protein-bound biotin appears in the gel supernatant from the subsequent steps of the fractionation of biotin carboxylase and carboxytransferase on calcium phosphate gel. Although any of these fractions, i.e. the 25 to 42% ammonium sulfate fraction or the gel supernatants remaining after the adsorption of biotin carboxylase (Table I) or carboxytransferase (Table II), can be used as starting material for the purification of the carboxyl carrier protein, the gel supernatant is generally employed. The carrier protein in this fraction is precipitated at a concentration of 80% saturated ammonium sulfate and the protein pellet, recovered by centrifugation, is redissolved in 50 mM potassium phosphate buffer, pH 7, containing 1 mM EDTA. This solution is brought to 6 M guanidine HCl with the crystalline salt, is stirred for 1 hour at 0-2°C, and then is dialyzed overnight against two changes of the 50 mM phosphate buffer. The voluminous precipitate of denatured protein is removed by centrifugation and washed twice with the 50 mM phosphate buffer. The original supernatant solution and washings containing the carboxyl carrier protein are combined; protein is precipitated by the addition of sufficient solid ammonium sulfate to bring the concentration to 80% saturation. The suspension is centrifuged and the protein pellet dissolved in 0.5 M KCl containing 50 mM potassium phosphate, pH 7.0, and 0.1 mM EDTA. After centrifugation to clarify the solution, the supernatant is retained for purification of carboxyl carrier protein by affinity chromatography on Sepharose-avidin.

**Preparation and Calibration of Sepharose-Avidin**—Since the dissociation constant for the avidin tetramer-biotin, complex is unusually low (Kd ≈ 10^-15 M) for affinity chromatography (1), whereas the affinity of the avidin monomer for biotin is much weaker (1), a procedure designed to produce Sepharose-avidin monomer was developed. Cyanogen bromide-activated Sepharose 4B is prepared (37) using 100 mg per ml of packed Sepharose. The coupling of avidin is performed at low pH (pH 5.5 in 0.1 M sodium acetate) to minimize the number of cross-links per avidin molecule in order that the subunits not covalently linked can be dissociated subsequently. One milliliter of avidin solution (1.5 mg per ml) in coupling buffer is used per ml of packed CNBr-activated Sepharose and coupling is allowed to proceed for 16 hours at 4°C. The Sepharose-avidin tetramer is then washed with distilled water and treated with 1 M Tris base overnight; the tetramer form is washed with distilled water and stored in 0.2% NaN3 at 4°C. It is recommended that Sepharose-avidin be stored in the tetramer form because it is considerably more stable than is the monomer form. Although the stability of the tetramer has not been studied rigorously, our observations suggest a 10 to 15% loss of activity per month.

The Sepharose-avidin monomer is generated as needed by dissociating the subunits of the tetramer form covalently linked to the Sepharose matrix with 6 M guanidine HCl. The appropriate amount of the tetramer is washed on a Millipore filter with distilled water and resuspended in 5 volumes of 0 M guanidine HCl in 50 mM potassium phosphate, pH 7.0, containing 5 mM EDTA. The resuspended pellet is left for 30 min at 0°C and the solution then removed by filtration; this step is repeated three additional times. After the fourth washing, the gel is washed repeatedly with distilled water and finally is resuspended in 0.1 M phosphate buffer, pH 7.0, containing 1 mM EDTA.

The biotin-binding capacity of Sepharose-avidin is determined by allowing an aliquot of the tetramer or monomer suspension to interact with an excess of [14C]biotin (approximately 70,000 cpm per nmole) for 20 min at 25°C with occasional stirring. The Sepharose-avidin gel is collected on a Millipore filter, washed, and counted. Normally, 40 to 50% of the avidin added in the initial coupling reaction becomes covalently bound yielding the tetramer which contains about 0.7 mg of avidin per ml of gravity-packed gel (binding capacity, 7 μg of free d-biotin per ml). Treatment of the tetramer with 6 M guanidine HCl results in the dissociation of the bulk, 65 to 70%, of avidin subunits, the theoretical value being 75%. Thus, the residual covalently linked avidin subunits (monomer) have a biotin-binding capacity of 2 to 2.5 μg per ml of gravity-packed gel.

It became apparent that Sepharose-avidin monomer possessed multiple classes of biotin-binding sites which differ in their affinities for free biotin and the biotinyl prosthetic group. Thirty-six per cent of the bound [14C]biotin exchanges readily with unlabeled d-biotin (10 mM), whereas an additional 26% is eluted only with strong eluting agents, i.e. neutral (pH 7.0) or acidic (pH 1.5) 6 M guanidine HCl; moreover, another fraction appears to be bound almost irreversibly.

**Affinity Chromatography of Carboxyl Carrier Protein on Sepharose-Avidin Monomer**—Carboxyl carrier protein (1.22 g of protein in 100 ml of 0.5 M KCl-50 mM phosphate buffer, pH 7.0) partially purified as described above by fractionation with ammonium sulfate, calcium phosphate gel, and guanidine HCl, is stirred with 70 ml of gravity-packed monomer gel (biotin binding capacity, about 2.5 μg per ml of gel). Essentially complete adsorption occurs within 1 to 2 hours. The suspension of Sepharose-avidin monomer-carboxyl carrier protein complex is then poured into a 4.2-em diameter column (5.5 cm in height); the height to diameter ratio of the packed gel in the column should not exceed 3:1 in order to maintain a adequate flow rate. The column is eluted sequentially with 650 ml of 0.5 M KCl containing 50 mM potassium phosphate, pH 7, and 1 mM EDTA; 300 ml of 0.5 M KCl containing 0.2 M sodium glycinate buffer, pH 9, and 1 mM EDTA; 200 ml of the preceding buffer containing 10 mM d-biotin; 200 ml of 6 M guanidine HCl containing 50 mM potassium phosphate buffer, pH 7, and 1 mM EDTA; and finally with 6 M guanidine HCl containing 50 mM NaPO4, pH 2, and 1 mM EDTA. As shown in Table III, the bulk (about 60%) of the carboxyl carrier protein activity, but only a small fraction of the protein applied to the column, is eluted by the latter three eluants (Fractions II, B, C, and D, Table III); the extent of purification by affinity chromatography ranges from 20 to 70 fold in the three fractions.

**Properties of Acetyl-CoA Carboxylase System**

**Reconstitution of Acetyl-CoA Carboxylase Activity with Purified Biotin Carboxylase, Carboxyltransferase, and Carboxyl Carrier Protein**—The common initial steps for the purification of biotin
The calcium phosphate gel supernatant fraction (18,800 mg of protein) after adsorption of biotin carboxylase (Step 3 of Table I, but beginning with 1.2 kg of packed Escherichia coli B cells) was treated with 6 M guanidine HCl and prepared for affinity chromatography as described in the text. The carboxyl carrier protein preparation (1220 mg of protein in 100 ml of 0.5 M KCl containing 50 mM phosphate, pH 7, and 1 mM EDTA) was treated with 70 ml of gravity-packed Sepharose-avidin monomer (binding capacity, 7.4 nmoles or 180 µg of d-biotin). After allowing 2 hours with occasional stirring, the suspension was poured into a column and sequential elution accomplished as described in the text. Fractions II, A and B, were brought to 80% saturation with solid ammonium sulfate and the precipitated protein dissolved in and dialyzed against 50 mM potassium phosphate, pH 7, containing 2 mM diethiothreitol and 1 mM EDTA. Fractions II, C and D, were treated similarly except that guanidine HCl was removed by dialysis prior to precipitation of protein with ammonium sulfate. Malonyl-CoA-[^14C]acetate exchange activity was determined in the presence of carboxyltransferase as described under "Enzyme Assays." Biotin content was determined either by the stoichiometric carboxylation assay (see "Enzyme Assays") or by the avidin-[^125I]biotin binding assay described in Table II of the accompanying paper (13). Protein was determined by the method of Lowry et al. (30).

### Table III

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity[^a]</th>
<th>Biotin content[^a]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg nmoles/min/mg protein</td>
<td>µg/µg protein</td>
</tr>
<tr>
<td>I. Before chromatography...</td>
<td>1220</td>
<td>1.0</td>
</tr>
<tr>
<td>II. After chromatography (Sepharose-avidin monomer column eluates):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. 0.5 M KCl, pH 7-9...</td>
<td>1200</td>
<td>0.42</td>
</tr>
<tr>
<td>B. 10 mM d-biotin-0.5 M KCl, pH 9...</td>
<td>9.5</td>
<td>33</td>
</tr>
<tr>
<td>C. 6 M guanidine HCl, pH 7...</td>
<td>9.4</td>
<td>20</td>
</tr>
<tr>
<td>D. 6 M guanidine HCl, pH 2...</td>
<td>2.8</td>
<td>68</td>
</tr>
</tbody>
</table>

[^a]: Determined by the stoichiometric carboxylation assay.
[^b]: Determined by the avidin-[^125I]biotin binding assay.

Carboxylase, carboxyltransferase, and carboxyl carrier protein (through Step 2 of Tables I and II, 25 to 42% fractionation) yield a preparation which contains all three components and actively catalyzes the carboxylation of acetyl-CoA, i.e., 1 to 2 nmoles per min per mg of protein under the conditions described in Table IV. Nonetheless, the three components are largely resolved by the next step (calium phosphate gel fractionation) and are completely separated by subsequent steps in the purification procedures. Thus it was found (4, 5) that chromatographically purified biotin carboxylase and carboxyltransferase isolated from an E. coli biotin auxotroph (strain SA 283) grown on [2-[^14C]biotin (7 × 10^5 cm per µmole) were free of the [[^14C]]biotin-containing carboxyl carrier protein. Both enzymes, prepared from [[^14C]]biotin-labeled cells as described above and carried through their respective purification procedures outlined in Tables I and II, contained <5 cm per µg of protein. Moreover, carboxyl carrier protein purified by affinity chromatography (Table III) contains no detectable biotin carboxylase or carboxyltransferase activity.

As shown in Table IV reconstitution of acetyl-CoA carboxylase activity is accomplished when all three purified protein components are present; any combination of two components is incapable of catalyzing acetyl-CoA carboxylation or the reverse reaction. The rate of acetyl-CoA carboxylation is dependent upon carboxyl carrier protein concentration (Table IV), as well as the concentrations of the biotin carboxylase and carboxyltransferase components (results not shown). Although both biotin carboxylase and carboxyltransferase catalyze model partial reactions (5-7, 13, 24) in which free biotin derivatives, e.g., 10 to 50 mM d-biotin, biocytin, or d-biotin methyl ester, can substitute for carboxyl carrier protein, these compounds were unable to replace the carrier protein for acetyl-CoA carboxylation under the conditions described in Table IV.

The ease with which the three proteins of the acetyl-CoA carboxylase system can be resolved during purification suggests that if a ternary multienzyme complex functions in the overall reaction, the complex must dissociate readily. Several findings suggest the participation of such a complex. As shown in Table
TABLE V
Effect of dilution and purified biotin carboxylase on carboxyltransferase- and carboxyl carrier protein-dependent malonyl-CoA-[14C]acetyl-CoA exchange

A, dialyzed ammonium sulfate fraction (Step 2, Tables I and II), 146 mg of protein per ml, was diluted with 0.05 m potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 5 mM β-mercaptoethanol to the final protein concentrations indicated. The undiluted control and the diluted solutions were preincubated for 1 hour at 30° before the exchange activity of 0.5 mg of protein was tested at 30° for 10 min as described under "Enzyme Assays." Where indicated, the enzyme was concentrated by precipitation with solid ammonium sulfate at 80% saturation; the precipitated protein was dialyzed overnight at a protein concentration of 50 mg per ml and then assayed. B, malonyl-CoA-[14C]acetyl-CoA exchange activity was determined in a volume of 0.250 ml as described under "Enzyme Assays." The biotin content of the carboxyl carrier protein (CCP) was 4.5 nmoles per mg and the specific activity of the carboxyltransferase (CT) was 1.1 pmoles per min per mg as measured with d-biotin at pH 4.5 (5). Biotin carboxylase (BC) equivalent to 23.7 milliunits (1.5 units per mg, without ethanal) was added as indicated.

<table>
<thead>
<tr>
<th>Conditions of preliminary incubation</th>
<th>Malonyl-CoA-[14C]acetyl-CoA exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/min</td>
</tr>
<tr>
<td>A. 146 mg per ml, not preincubated</td>
<td>0.75</td>
</tr>
<tr>
<td>146 mg per ml, 1 hr at 30°</td>
<td>0.85</td>
</tr>
<tr>
<td>40 mg per ml, 1 hr at 30°</td>
<td>0.72</td>
</tr>
<tr>
<td>10 mg per ml, 1 hr at 30°</td>
<td>0.50</td>
</tr>
<tr>
<td>3 mg per ml, 1 hr at 30°</td>
<td>0.21</td>
</tr>
<tr>
<td>5 mg per ml, 64 hrs at 4°</td>
<td>0.12</td>
</tr>
<tr>
<td>5 mg per ml, 64 hrs at 4°, then concentrated to 50 mg per ml and assayed</td>
<td>0.60</td>
</tr>
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</table>

B. CT, 44 μg

<table>
<thead>
<tr>
<th>Additions</th>
<th>Without BC</th>
<th>With BC</th>
</tr>
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<tbody>
<tr>
<td>CCP, 110 μg</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CT, 44 μg + CCP, 110 μg</td>
<td>0.38</td>
<td>1.1</td>
</tr>
<tr>
<td>CCP, 220 μg</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CT, 44 μg + CCP, 220 μg</td>
<td>1.1</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* The small amount of carboxylase activity observed in the absence of added bicarbonate is due presumably to the unavoidable contamination of certain reaction mixture components, e.g., buffer, with traces of bicarbonate.

V. A reversibly lost by the unresolved carboxylase system of malonyl-CoA-[14C]acetyl-CoA exchange activity during a 1-hour preliminary incubation is strongly dependent upon enzyme concentration. Moreover, this activity loss during prolonged incubation of a dilute enzyme preparation can be largely regained by concentrating the inactivated preparation. Another indication that a productive ternary complex is formed derives from the observation (Table V A) that the carboxylcarrier protein-dependent, carboxyltransferase-catalyzed malonyl-CoA-[14C]-acetyl-CoA exchange is activated by the third component, biotin carboxylase, which does not participate in the reaction per se. The fact that biotin carboxylase activates at rate-limiting, but not at saturating, concentrations of the carrier protein, implicates a ternary complex between the protein components and suggests that the interaction between carboxyltransferase and carboxyl carrier protein is enhanced by the presence of biotin carboxylase in the complex.

Properties of Biotin Carboxylase Component and Carboxylation Reaction—Biotin carboxylase, obtained in good yield by the purification procedure outlined, has a specific activity about 2000-fold greater than that of the cell-free extract (Table I). Preparations carried through Step 6 are free of biotin (preceding section) and crystallize readily from dilute potassium phosphate buffer in the form of elongated prisms (Fig. 1, inset). Although crystallization or recrystallization leads to no further improvement in specific activity, the stability of the enzyme is markedly increased. The crystalline enzyme appears homogeneous by several criteria; such preparations give rise to a single sedimentation boundary in the analytical ultracentrifuge (s20,w = 5.7 S; Ref. 4) and to a single stained protein band by polyacrylamide gel electrophoresis (Fig. 1). Moreover, electrophoresis of the sodium dodecyl sulfate-dissociated enzyme on polyacrylamide gels containing sodium dodecyl sulfate (4) also yields a single stained protein band. On the basis of its subunit weight estimated by sodium dodecyl sulfate-dissociated enzyme on polyacrylamide gels (Fig. 1) and to a single stained protein band by polyacrylamide gel electrophoresis, is concluded (4) that biotin carboxylase is composed of two similar or identical polypeptide chains of 50,000 daltons.

In view of previous work on related oligomeric carboxylases (38, 39) which contain a covalently bound biotin prosthetic group, yet catalyze the carboxylation of free d-biotin, it was anticipated that the carboxylation product would be free carboxybiotin. This is consistent with the finding that the H[14CO3] fixation product of the biotin carboxylase reaction is acid-labile, but relatively stable at neutral pH and low temperature (Table VI), a property possessed by carboxybiotin (1). As reported in the accompanying paper (34), the pH dependence of the first order decarboxylation rate of the labeled carboxylation product is identical with that of 1'-N-carboxy-d-biotin. Methylation...
with diazomethane converted the acid-labile product to an acid-stable derivative which co-chromatographs (38-40) precisely with authentic 1'-N-methoxy carbonyl-d-biotin methyl ester (40) (results not shown). That the 1'-N position of biotin is the actual site of carboxylation is verified by the demonstration in stable derivative which co-chromatographs (38-40) precisely with [carbon-14] biotin carboxylase, as well as for the model half-reaction catalyzed by carboxyltransferase.

The stoichiometry of the biotin carboxylase-catalyzed reaction was measured in the carboxylation direction by following $^4\text{H}_2\text{CO}_3\text{O}^-$ incorporation into carboxybiotin (II; $^4\text{H}_2\text{CO}_3\text{O}^-$ fixed into a form stable to gassing with CO$_2$ at pH $^\circ$, but which is labile in acid). As shown in Table VI, approximately 1 mole of carboxybiotin (0.90 to 0.93) was synthesized per mole of ADP formed. Carboxybiotin formation as assessed by one or both of these assay methods (Table VI) is absolutely dependent upon d-biotin, ATP, and divalent cation. Although the carboxylase-catalyzed reaction is activated 5- to 6-fold by 10% ethanol (4), the stoichiometry (Table VI) and the requirement for biotin, ATP, and Mg$^{2+}$ are the same in the presence and absence of ethanol. The enzyme is highly specific in that L-biotin, the unnatural isomer, is completely inactive and a variety of other biotin derivatives are either inactive or less active than d-biotin (4, 13). The divalent cation requirement can be met by one or both of these assay methods (Table VI) is absolutely dependent upon d-biotin, ATP, and Mg$^{2+}$; Co$^{2+}$ and Mn$^{2+}$ become inhibitory at concentrations greater than 2 mM (i.e., at an ATP-metal ion concentration ratio $>$2). A comparison of the pH optima for Mg$^{2+}$ and Mn$^{2+}$ shows the optimum for the former to be pH 8.0 and, for the latter, about pH 6.5. At the pH of the standard assay, i.e., pH 8, Co$^{2+}$ is somewhat more active than Mg$^{2+}$ or Mn$^{2+}$.

As indicated previously (Table VI and Ref. 4), biotin carboxylase-catalyzed carboxylation of free d-biotin is markedly activated by ethanol. Activation is dependent on ethanol concentration reaching a maximum of 10-fold at approximately 15 volumes % (Table VII); at higher concentrations, e.g., 20 volumes %, inactivation of the enzyme occurs. It is evident (Table VII) from the diversity of organic solvents which proved effective that activation is not a specific property of ethanol. However, at comparable concentrations, ethanol produced rate enhancements greater than any solvent tested. Among the active solvents were medanol, ethanol, 1- and 2-propanol, ethylene glycol monomethyl ether, acetone, 1,4-dioxane, and tetrahydrofuran; solvents having the higher dielectric constants were inactive. As with ethanol, several solvents caused irreversible inactivation of the enzyme above a critical concentration.

Like the carboxylation of free biotin, the biotin carboxylase-catalyzed, carbamyl phosphate: ADP phosphotransferase reaction, which is also dependent on free d-biotin, is activated by ethanol (41). However, in the latter case, the magnitude of activation (about 2.2 fold with 10 volumes % ethanol) is lower. In both instances (13), the principal kinetic effect of activation is on the maximal velocity and not on the $K_m$ values for substrates or effectors. Thus, rate enhancement appears to be due to an effect on the reaction of bound substrate. Unlike the above model reactions which utilize free biotin as HCO$_3^-$ acceptor, two other biotin carboxylase-catalyzed reactions, i.e., the carboxylation of acetyl-CoA or of carboxyl carrier protein which involve subunit interaction with the carboxyl transferase or carrier protein components, or both, respectively, are not activated by organic solvents.

Since changes in the hydrophobic character of the medium are known to alter the tertiary-quaternary structures of enzymes, the possibility was considered that a productive conformational change may be associated with aggregation of the enzyme. Thus, the effect of ethanol on the state of aggregation of the carboxylase was assessed by sucrose density gradient centrifugation under standard assay conditions, but at lower temperature. Preliminary experiments revealed that the homogeneous enzyme (10 $\mu$g per ml of assay mix) was completely stable for 24 hours in the complete assay reaction mixture containing 10 volumes % ethanol. In the density gradient experiments, avidin-[14C]biotin complex (avidin-biotin) was included as internal marker to correct for the effect of ethanol in the viscosity and density of the medium which in turn affects sedimentation velocity. Ten volumes per cent ethanol was found to decrease the sedimentation rate of both avidin-biotin complex and biotin carriers markedly.

### Table VII

**Effect of organic solvents on biotin carboxylase activity**

The biotin carboxylase-catalyzed carboxylation of free d-biotin was determined by the spectrophotometric method (see "Assay Methods"). Assays were completed within 3 min to minimize inactivation by the organic solvents; except as indicated, carboxylation rates were linear for at least 2 min.

<table>
<thead>
<tr>
<th>Addition*</th>
<th>Volumes per cent</th>
<th>Relative activity*</th>
<th>Addition*</th>
<th>Volumes per cent</th>
<th>Relative activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>%</td>
<td>Ethylene glycol monomethyl ether</td>
<td>10</td>
<td>210</td>
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</tr>
<tr>
<td>Methanol</td>
<td>10</td>
<td>330</td>
<td>20</td>
<td>480</td>
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</tr>
<tr>
<td>Ethanol</td>
<td>5</td>
<td>200</td>
<td>30</td>
<td>540</td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td>680</td>
<td>40</td>
<td>900</td>
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</tr>
<tr>
<td></td>
<td>15</td>
<td>1000</td>
<td>50</td>
<td>1200</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Inactivation*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Propanol</td>
<td>5</td>
<td>310</td>
<td>20</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Inactivation*</td>
<td>5</td>
<td>360</td>
<td></td>
</tr>
<tr>
<td>2-Propanol</td>
<td>5</td>
<td>100</td>
<td>10</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>300</td>
<td>20</td>
<td>Inactivation*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Inactivation*</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

---

* 1,2-Propylene glycol, glycerol, and dimethyl formamide did not activate.

* Activity relative to control with no addition.

* Biotin carboxylase inactivated in "mock" preliminary incubation under these conditions, but without substrate.
carboxylase. Using the mobilities ratio method of Martin and Ames (42) and 4.58 S as the $s_{20,w}$ for avidin (1), the reference protein, sedimentation coefficients of 5.8 and 5.9 S, respectively, were calculated for biotin carboxylase in the presence and absence of ethanol. Therefore, ethanol at a concentration capable of promoting a 5-fold activation causes neither polymerization nor depolymerization of the enzyme under assay conditions.

Properties of Carboxyltransferase Component—The procedure outlined for purifying the transferase results in a 500-fold increase in specific enzyme activity from the ammonium sulfate stage (Table II, Step 2). The presence in the cell-free extract of an inhibitor which is removed by ammonium sulfate fractionation (Step 2), precludes use of the standard transferase assay until after this point in the fractionation. Based on a modified carboxyltransferase assay (pH 4.5 instead of pH 8.0 and the use of d-biotin in place of its methyl ester as substrate), it is estimated that the ammonium sulfate fractionated enzyme is purified approximately 3-fold over the cell-free extract. Thus, the overall purification is approximately 1500-fold.

As indicated earlier, two peaks of carboxyltransferase activity are resolved by phosphocellulose chromatography (Fig. 2). Since Peak I comprises greater than 80% of the total transferase activity, this fraction is used for further purification. A narrow cut of Peak I fractions is made to avoid cross-contamination with Peak II; hence, the yield at this step is not maximal. Kinetic studies at pH levels of 4.5, 6.8, and 8.0 revealed no differences in the ratios of activities nor in the ratios of carboxyltransferase to malonyl-CoA decarboxylase activities of the two fractions (Peaks I and II). Both fractions are free of biotin carboxylase and carboxyl carrier protein and possess equal activity in reconstituting acetyl-CoA carboxylation in the presence of biotin carboxylase and carboxyl carrier protein. The significance of the occurrence of two forms of the transferase remains obscure.

Like the avian liver acetyl-CoA carboxylase (43), the carboxyltransferase component of the E. coli acetyl-CoA carboxylase system catalyzes a biotin-independent decarboxylation of malonyl-CoA. As illustrated in Table II, the ratio of carboxyltransferase to malonyl-CoA decarboxylase activities of the two fractions (Peaks I and II) is 2:1. Both fractions are free of biotin carboxylase and carboxyl carrier protein and possess equal activity in reconstituting acetyl-CoA carboxylation in the presence of biotin carboxylase and carboxyl carrier protein. The significance of the occurrence of two forms of the transferase remains obscure.

The molecular weight of the native transferase was assessed by gel filtration and sedimentation equilibrium methods. Prior to the molecular weight determinations, the enzyme was dialyzed for at least 30 hours against 50 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol. Gel filtration was carried out using a Sephadex G-200 column previously equilibrated with dialysis buffer and calibrated with proteins of known molecular weight. A molecular weight of 145,000 was estimated from plots of molecular weight against $K_{av}$ (45). This value agrees reasonably well with that ($M_{R} = 130,000 \pm 3,000$) determined by the more accurate sedimentation equilibrium method. Sedimentation equilibrium runs were conducted in duplicate at 10,000 and 12,000 rpm with a Spinco model E analytical ultracentrifuge equipped with electronic speed control. Experiments were performed at 20° with protein concentrations of 0.5 to 0.7 mg per ml; calculations were made according to Roark and Yphantis (46) from Rayleigh interference patterns obtained after 24 hours at 10,000 rpm and after an additional 12 hours at 12,000 rpm using an estimated partial specific volume of 0.73. Base-line corrections of interference patterns for cell distortion were made at speed after replacing the sample with the dialysis buffer without disassembling the cell.

The dissociation of homogeneous transferase with sodium dodecyl sulfate followed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels gives rise to two stained protein bands (Bands A and B, left and right, respectively) as shown in Fig. 3 (Gel A). This indicates that the enzyme is composed
of two polypeptide chains of different size. The intensities of the stained bands were analyzed using a Gilford gel scanner and the relative mass ratio estimated from the areas under the two peaks on gel scan traces. The mass ratio estimated in this manner was approximately 1 to 0.78 for Peaks A and B, respectively. Comparison of the electrophoretic mobilities of the two transferase polypeptides on sodium dodecyl sulfate-10% acrylamide gels with those of marker polypeptides (see Fig. 3) shows their molecular weights to be 35,000 (Band A) and 30,000 (Band B), respectively. In view of the relatively good agreement between the mass ratio of 1:0.78 (A:B) and the polypeptide weight ratio of 1:0.86 (A:B), it appears that the two subunits are present in nearly equivalent amounts. Thus, a subunit composition for native carboxyltransferase (130,000 daltons) of A2B2 is suggested.

**Purity and Properties of Carboxyl Carrier Protein—Affinity chromatography on Sepharose-avidin (monomer) of E. coli carboxyl carrier protein preparations that had been partially purified by fractionation with ammonium sulfate, calcium phosphate gel, and guanidine HCl yielded several fractions of high purity (Table III).** Since Sepharose-avidin (monomer) possesses multiple types of binding sites which differ in their affinities for avidin, it was anticipated that the extent of release of the carrier protein from the affinity column would depend upon the nature of the eluent; thus three fractions of carrier protein were obtained (Fractions II B, C, and D, Table III). The fraction eluted with acidic guanidine HCl (Fraction II D) and employed in most experiments described in the accompanying paper (13), possessed the highest specific activity in the malonyl-CoA-[14C]acetate-CoA exchange assay and contained approximately 30 nanomoles of active site (biotin prosthetic groups) per mg of protein. There is good agreement between these parameters (malonyl-CoA-acetate-CoA exchange activity and biotin content) when applied to preparations of greatly differing purity (Table III). Moreover, the number of biotinyl prosthetic groups determined by direct carboxylation of the carrier protein preparation in the presence of MgATP and [14C]bicarbonate (29 nanomoles per mg of protein; Table III, Fraction II D) closely approximates the biotin content assessed by the differential avidin-biotin binding method (32 nanomoles per mg of protein; Table III, Fraction II D). Electrophoresis of Fraction II D on 14.5% polyacrylamide gels (Fig. 4, Gel E) revealed two stained protein bands which coincided precisely with carboxyl carrier protein activity measured by fractionation with ammonium sulfate, calcium phosphate gel, and guanidine HCl and [14C]bicarbonate incorporated into acid-stable form (malonyl-CoA) determined as previously described (8). Staining was accomplished with Amido black. In approximately equal amounts as judged by the intensity of staining. Presumably the two carboxyl carrier proteins correspond to the 10,000- and 22,000-dalton biotin-containing proteins described by Vagelos (14-16), the smaller of the two being derived from the larger via proteolysis by a protease in the cell-free extract. This is also consistent with the finding (15, 16) that the larger and apparently native form of carboxyl carrier protein is considerably more active than the smaller form in the acetyl-CoA carboxylation assay. As will be demonstrated in the accompanying paper (13) the purified carboxyl carrier protein (s) in addition to being essential for acetyl-CoA carboxylation (Table IV) and malonyl-CoA-[14C]acetate-CoA exchange (Table V), is required for ATP-[14C]ADP and ATP-[32P]P-exchange catalyzed by biotin carboxylase.

Although Fraction II B and C (Table III) have somewhat lower specific activities than Fraction II D, both exhibit the same relative amounts of the two major biotin-containing bands upon polyacrylamide gel electrophoresis. In addition, several other protein bands of slower mobility which do not contain biotin (results not shown) are detectable.

**DISCUSSION**

The acetyl-CoA carboxylases of animal cells (10) and E. coli (2-6), are multisubunit enzymes whose component polypeptide...
chains differ. Thus, Gregolin et al. (9) found that the basic 410,000 dalton protomer of the avian liver carboxylase to be comprised of four subunits of similar molecular weight, i.e. about 110,000. However, it appeared that these subunits were non-identical since only one biotinyl prosthetic group, one covalent bicarbonate-loading site, one acetyl-CoA binding site, and one tight citrate binding site were present per protomer (410,000 daltons). Moreover, it was established by affinity chromatography (47) that each protomeric unit possessed a single biotinyl prosthetic group; therefore, the protomers per se are most likely identical. It became evident using electrophoresis of sodium dodecyl sulfate-dissociated carboxylase on lightly loaded acrylamide gels (5 μg of protein per gel) that two subunit weight classes differing by only about 5,000 to 10,000 daltons could be observed with the rat liver carboxylase (12). More recently, with high resolution acrylamide gel electrophoresis in 6 M urea-0.1% sodium dodecyl sulfate, the avian liver carboxylase has been resolved into subunits of three weight classes (48), the approximate mole ratios of the three species, i.e. 117,000, 130,000, and 140,000 daltons, being 2:1:1, respectively. The covalently bound biotin prosthetic group is associated with the 117,000-dalton subunit(s) (48). This and cross-linking analysis of the avian liver carboxylase protomer by the dimethylsuberimidate method (49) show that this enzyme is composed of four different types of polypeptide chains (48). Unfortunately, attempts to assign function to the individual polypeptide chains of the animal carboxylases have met with little success; in no case has it been possible to detect or restore catalytic activity for any partial reaction following dissociation or resolution, or both.

In contrast to the avian liver carboxylase whose subunits are dissociated only by drastic treatment (6 M guanidine HCl or sodium dodecyl sulfate) (9, 10, 12), those of the E. coli acetyl-CoA carboxylase dissociate readily. Moreover, unlike the associated subunits of the avian enzyme which lose activity irreversibly by this treatment, the resolved components of the E. coli system (i.e. biotin carboxylase, carboxyltransferase, and carboxyl carrier protein) retain enzymatic activity allowing the assignment of function to each. The three protein components of the E. coli acetyl-CoA carboxylase have been obtained in homogenous form and their respective molecular characteristics determined: biotin carboxylase and carboxyltransferase in our laboratory (Refs. 4 and 5, and this paper) and carboxyl carrier protein in Vagelos’ laboratory (14-17). These properties are summarized in Table VIII. Biotin carboxylase, the catalytic element responsible for the first half-reaction (Reaction 1), is a dimer composed of apparently identical 51,000 dalton subunits. The catalyst for the second half-reaction (Reaction 2), carboxyltransferase, appears to be a tetramer having an A4I6 structure and subunits with molecular weights of 30,000 and 35,000. Finally, the carboxyl carrier peptide which possesses the covalently bound prosthetic group has been found in Vagelos’ laboratory (15-17) to have a molecular weight of 22,000. Thus, at least four polypeptide chains are implicated in the carboxylation of acetyl-CoA and perhaps the regulation of this process in E. coli (21). This is interesting in view of the finding that four apparently nonidentical subunits comprise the basic protomeric unit of the avian liver acetyl-CoA carboxylase (10). In the case of the carboxylase system from E. coli, it has not been possible to demonstrate unequivocally the existence of a complex of the protein components (biotin carboxylase, carboxyltransferase, and carboxyl carrier protein), whereas all of the acetyl-CoA carboxylases from animal cells have stable quaternary structures (1, 10).

Tentative evidence for an interaction of the three components of the E. coli carboxylase system, i.e. in a ternary complex, derives from the observation (Table V) that biotin carboxylase, although not an essential participant in the carboxyl carrier protein and carboxyltransferase dependent malonyl-CoA [PC] \textsuperscript{+} acetyl-CoA exchange, activates this reaction. This suggests that the binding of biotin carboxylase to either carboxyltransferase or the carboxyl carrier protein stabilizes a ternary complex of the three components.

Acknowledgment—We thank Mr. Eberhard Zwergel for outstanding technical assistance.

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Table VIII

| Molecular weight | Biotin content | Carboxyl carrier protein
<table>
<thead>
<tr>
<th></th>
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</tr>
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<tbody>
<tr>
<td>44,000 (or higher order aggregates)</td>
<td>22,000</td>
<td></td>
</tr>
<tr>
<td>51,000</td>
<td>35,000</td>
<td></td>
</tr>
<tr>
<td>30,000</td>
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<td></td>
</tr>
<tr>
<td>A2</td>
<td>A2</td>
<td></td>
</tr>
<tr>
<td>A2B2</td>
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</tr>
<tr>
<td>1.0 per 22,000 daltons</td>
<td></td>
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</tbody>
</table>

* Results presented in this paper or Refs. 4 and 5.

b Results from Vagelos’ laboratory (14-16).
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Acetyl Coenzyme A Carboxylase System of *Escherichia coli* : PURIFICATION AND PROPERTIES OF THE BIOTIN CARBOXYLASE, CARBOXYLTRANSFERASE, AND CARBOXYL CARRIER PROTEIN COMPONENTS
Ras B. Guchhait, S. Efthimios Polakis, Peter Dimroth, Erwin Stoll, Joel Moss and M. Daniel Lane


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