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Purification and Partial Characterization of Rat Liver Folate Binding Protein: Cytosol I†

Robert J. Cook and Conrad Wagner*

ABSTRACT: The high molecular weight folate binding protein of rat liver cytosol has been purified to apparent homogeneity. Purification was achieved by using a combination of gel filtration, *O*-(diethylaminoethyl)cellulose chromatography, and affinity chromatography. This folate binding protein was initially identified during purification by an in vivo labeling procedure involving intraperitoneal injection of [^{3}H]folic acid prior to sacrifice and subsequently by its ability to bind naturally reduced [^{3}H]folate polyglutamates in vitro. A molecular weight of 210 000 was estimated by gel chromatography. This is distinct from the trifunctional formyl–methenyl–methylene synthetase of rat liver which has a molecular weight of 225 000. Sodium dodecyl sulfate electrophoresis revealed a single band with a molecular weight of about 100 000 which suggests the native protein is composed of two identical subunits. The partially purified protein contains bound tetrahydropteroylpentaglutamate.

Zamierowski & Wagner (1974, 1977) reported the presence of three proteins in rat liver cytosol which contained tightly bound endogenous folates. These proteins were separated by chromatography using Sephadex G-150 and were referred to as folate binding proteins of cytosol I, II, and III (FBP-CI, FBP-CII, and FBP-CIII, respectively).[1] FBP-CII has previously been purified and characterized (Suzuki & Wagner, 1980), and its distribution in various rat tissues has been measured by radioimmunoassay (Cook & Wagner, 1981). FBP-CIII was shown to be associated with dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) activity and has an estimated molecular weight of 25 000. A fourth folate binding protein was identified in sonicated extracts of rat liver mitochondria and was referred to as mitochondrial folate binding protein (MFBP; Zamierowski & Wagner, 1974, 1977). MFBP was recently resolved into two related enzymes which have been purified and characterized. They are dimethylglycine dehydrogenase (EC 1.5.99.2) and sarcosine dehydrogenase (EC 1.5.99.1) (Wittwer & Wagner, 1980, 1981a,b).

We now report the purification to apparent homogeneity and partial characterization of FBP-CI. This protein was previously shown to have an approximate molecular weight of about 350 000 by gel filtration of liver cytosol. Purification

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[1] Abbreviations: FBP-CI, folate binding protein cytosol I; FBP-CII, folate binding protein cytosol II; FBP-CIII, folate binding protein cytosol III; MFBP, mitochondrial folate binding protein; PteGlu, pteroylglutamic acid (folic acid); H_2PteGlu, 7,8-dihydropteroylglutamic acid (dihydrofolic acid); H_4PteGlu, 5,6,7,8-tetrahydropteroylglutamic acid (tetrahydrofolic acid); CH_3, methyl; HCO, formyl; CH_2, methylene; $PteGlu_2$, pteroyldiglutamic acid; $PteGlu_3$, pteroyltriglutamic acid; $PteGlu_5$, pteroylpentaglutamic acid; $PteGlu_6$, pteroylhexaglutamic acid; *p*-ABG, (*p*-aminobenzoyl)glutamic acid; FMMS or formyl–methenyl–methylene synthetase (combined), formyltetrahydrofolate synthetase (EC 6.3.4.3), methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9), and methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5); BSA, bovine serum albumin; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; $NaDodSO_4$, sodium dodecyl sulfate; DEAE-cellulose, *O*-(diethylaminoethyl)cellulose; TEAE-cellulose, *O*-(triethylaminoethyl)cellulose; TBAP, tetrabutylammonium phosphate; Tris, tris(hydroxymethyl)aminomethane.

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has shown that FBP-CI has an apparent molecular weight of 210 000 and is composed of two subunits of molecular weight 100 000. FBP-CI also contains $H_4PteGlu_5$ as the tightly bound ligand.

Materials and Methods

Sephacryl S-200 and AH-Sepharose-4B were obtained from Pharmacia Fine Chemicals; 2-mercaptoethanol was from Eastman-Kodak Co.; DEAE-cellulose (DE-52) was from Whatman; TEAE-cellulose (Cellex-T) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDAC) were from Bio-Rad Laboratories; 5-formyltetrahydrofolic acid was from Lederle Laboratories; [G-^{3}H]PteGlu, potassium salt (5 Ci/mmol), was from Amersham-Searle. PteGlu and *p*-ABG were purchased from Sigma Chemical Co. Tetrabutylammonium hydroxide was from Fisher Chemical Co. Authentic $PteGlu_3$ and $PteGlu_5$, prepared by the solid phase synthetic method (Krumdieck & Baugh, 1969), were a generous gift of Dr. Carlos Krumdieck.

Animals. Male Sprague-Dawley rats (150–300 g) were used and were fed ad libitum on Wayne Lab Blox. Rats were injected intraperitoneally with 25 μCi of [G-^{3}H]PteGlu (0.5 mL) 48 and 24 h before sacrifice.

Preparation of Rat Liver Cytosol. Rats were injected intraperitoneally with 25 μCi of [G-^{3}H]PteGlu as described above. The animals were sacrificed, and the liver was removed and washed with 0.25 M sucrose (pH 7.0). The livers were weighed, minced with scissors, and homogenized in 4 volumes of 0.25 M sucrose (pH 7.0) containing 10 mM 2-mercaptoethanol in a Potter-Elvehjem homogenizer with a Teflon pestle. Homogenates were centrifuged at 600*g* for 10 min at 4 °C. The supernatant solution was collected and centrifuged at 100000*g* for 90 min at 4 °C. The resulting supernatant was designated cytosol and frozen at −20 °C. It was used as the starting material either for further purification of FBP-CI or for preparation of naturally occurring ^{3}H-labeled folates used in binding studies.

Preparation of ^{3}H-Labeled Folates Used in Binding Studies. The cytosol was concentrated approximately 8-fold by using an Amicon filter apparatus with a PM 30 filter. One-milliliter aliquots of the cytosol concentrate were made 1% (w/v) with respect to sodium ascorbate, protected from light, and heated in a boiling water bath for 10 min to dissociate [^{3}H]folate species from protein. Denatured protein was removed by centrifugation at 600*g* for 10 min, and the supernatant containing the [^{3}H]folate was stored at −20 °C under N_2. Alternatively, partially pure FBP-CI from the Sephacryl S-200 step (see below) was made 1 M with respect to 2-mercaptoethanol and treated as described above.

Preparation of Standard Folate Derivatives. $H_4PteGlu$, $H_4PteGlu_3$, and $H_4PteGlu_5$ used as standards for TEAE-cellulose chromatography of the endogenous folate ligand were prepared from the corresponding oxidized folates by reduction with sodium borohydride as previously described by Wittwer & Wagner (1980). Other folates used as standards for TEAE-cellulose chromatography and high-pressure liquid chromatography (HPLC) were prepared as follows: *dl*-5-CH_3-$H_4PteGlu$ was synthesized by the method of Gupta & Huennekens (1967) and purified according to Horne et al. (1978). $H_2PteGlu$ was synthesized byZn plus NaOH reduction of PteGlu (Zakrewski & Sansone, 1971). $H_4PteGlu$ was made by catalytic hydrogenation (Blakely, 1957). 10-HCO-$H_4PteGlu$ was synthesized according to Rabinowitz (1963). All solutions of standard folates were stored at −20 °C in Thunberg tubes under N_2 with 0.2 M 2-mercaptoethanol and 1% (w/v) sodium ascorbate.

Measurements of Folate Binding Activity. Binding of naturally occurring [^{3}H]folates was measured by a centrifugal procedure which rapidly separates macromolecules and small molecules by using Bio-Gel P-2 columns. Protein samples and ^{3}H-labeled ligand (400–800 cpm of ^{3}H-labeled naturally occurring folate) were incubated at 4 °C for 10 min and then separated from free ligand by centrifugation. Competition studies with purified FBP-CI using naturally occurring [^{3}H]folates isolated from the DEAE-cellulose step of protein purification and various unlabeled folates were performed as described above with the exception that incubations were at room temperature for 30 min.

Hydrolysis of Folate Polyglutamates with Conjugase. Hog kidney conjugase was prepared as described by Iwai et al. (1964). The incubation mixture contained 1.0 volume of conjugase, 0.5 volume of folate sample, 0.5 volume of 0.06 M 2-mercaptoethanol, and 0.2 volume of 1 M sodium acetate buffer, pH 4.7. The mixture was gassed with N_2 and incubated in the dark at 37 °C for various times. After incubation, the pH was adjusted to the required value for either chromatography or chemical modification. The mixture was then heated in a boiling water bath for 10 min. Denatured protein was removed by centrifugation at 600*g* for 10 min.

Alternatively, folate polyglutamates were treated with rat serum conjugase. Rat blood was collected and allowed to clot overnight at 4 °C, and serum was prepared by centrifugation. The incubation mixture contained 1.0 volume of sample folate adjusted to pH 7.0, 0.5 volume of rat serum, and 2-mercaptoethanol to a final concentration of 1 M. The mixture was incubated either at room temperature for 16–18 h or at 37 °C for 90–180 min. This latter method omitted ascorbate which interfered with HPLC analysis of folates and also avoided acid pH which accelerates oxidative breakdown of $H_4PteGlu$.

HPLC Analysis of Folate Monoglutamates. [^{3}H]Folate dissociated from partially purified FBP-CI was treated with rat serum conjugase and analyzed by HPLC. HPLC was performed on a Waters system with a model 440 absorbance detector working at 280 nm and using a μBondapak C_{18} column (39 mm × 300 mm, 10 μm). The reverse phase, ion-pairing technique used a mobile phase of methanol–acetonitrile–water (9:6:85 v/v/v) with tetrabutylammonium phosphate (TBAP; 3:100 mL of mobile phase). TBAP reagent was prepared from tetrabutylammonium hydroxide according to Horne et al. (1981). Samples (100–200 μL) containing the standards *p*-ABG, 10-HCO-$H_4PteGlu$, $H_4PteGlu$, 5-HCO-$H_4PteGlu$, $H_2PteGlu$, 5-CH_3-$H_4PteGlu$, and PteGlu were separated at a flow rate of 2 mL/min (2500 psi). The elution of standards was monitored at 280 nm, and 1-mL fractions were collected for determination of radioactivity.

Assay of 10-Formyltetrahydrofolate Synthetase Activity. 10-Formyltetrahydrofolate synthetase (EC 6.3.4.3) was assayed by the modified method of MacKenzie & Tan (1980). The reaction mixture contained 0.1 mL of 1 M triethanolamine hydrochloride buffer, pH 8.5, 0.1 mL of 1 M KCl, 0.1 mL of 0.1 M $MgCl_2$, 0.1 mL of 1 M sodium formate, 0.1 mL of 2 M 2-mercaptoethanol, 0.05 mL of 0.1 M ATP, 0.025 mL of 22 mM $H_4PteGlu$, and water to 1 mL. The reaction was started by addition of sample (0.05–0.1 mL) and incubated for various times at 37 °C. Blanks were prepared without ATP. Following incubation, the reaction was stopped by acidification with 1 mL of 0.72 M HCl. Protein precipitates were removed by centrifugation at 5000*g* for 10 min at 4 °C. The absorbance was determined at 350 nm, and activity is expressed as ΔA_{350nm}.

Assay of 5,10-Methylenetetrahydrofolate Reductase Activity. 5,10-Methylenetetrahydrofolate reductase (EC 1.1.1.68) was assayed by the modified method of Kutzback & Stokstad (1971). The reaction mixture contained 0.5 mL of 0.2 M potassium phosphate buffer, pH 7.2, 5 μL of 1 mM FAD, 0.2 mL of 2 mM NADPH, 0.25 mL of 0.1 M 2-mercaptoethanol, and 0.5 mL of enzyme sample. Blanks contained 0.25 mL of 20 mM formaldehyde while test cuvettes contained 30 μL of 10 mM 5,10-CH_2-H_4PteGlu. Distilled water was added to a total volume of 2.5 mL. Reactions were started by the addition of NADPH, and initial rates were recorded at 366 nm on a Gilford ultraviolet–visible spectrophotometer.

Preparation of 5-HCO-H_4PteGlu Affinity Gel. 5-HCO-H_4PteGlu was linked to AH-Sepharose-4B by the water-soluble EDAC method (Waxman & Schreiber, 1975) as described by Wittwer & Wagner (1981a).

Chemical Modification of Conjugase-Treated Folates with Formaldehyde and Sodium Borohydride. The method of Blair & Saunders (1970) for the synthesis of 5-CH_3-H_4PteGlu was adapted as follows. The conjugase-treated folate material was adjusted to pH 7.8, equilibrated with nitrogen gas, and mixed with 0.1 mL of 37% formaldehyde. Aqueous sodium borohydride (50 mg/0.4 mL) was then added dropwise with stirring and the solution incubated at 45 °C for 60 min. The solution was then adjusted to pH 7.6 with 6 N HCl, diluted with an equal volume of 0.2 M 2-mercaptoethanol, and chromatographed. The addition of formaldehyde was omitted when borohydride treatment alone was required.

Estimation of Molecular Weight by Gel Filtration. The M_r of native FBP-CI was estimated by gel filtration using a Sephacryl S-200 column (87 × 5 cm). The column was calibrated according to the method of Andrews (1966) by using the following proteins: BSA fraction V (Sigma), yeast alcohol dehydrogenase (Sigma), catalase (Sigma), thyroglobulin (gift of Dr. Leon Cunningham), and FBP-CII. Columns were eluted with 10 mM potassium phosphate buffer, pH 7.0, containing 10 mM 2-mercaptoethanol. Catalase was assigned an M_r of 195 000 as described by Andrews (1966).

Polyacrylamide Gel Electrophoresis. Denaturing electrophoresis in the presence of sodium dodecyl sulfate (NaDod-SO_4) was conducted according to the method of Laemmli (1970) by using a 3% stacking gel at pH 6.8 and a 9% separating gel at pH 8.8. Gels were stained for protein in 0.05% Coomassie Blue, 14% methanol, and 8% acetic acid and destained by diffusion in methanol–acetic acid–water (1:1:8). The method of Zacharius et al. (1969) was used to stain for the presence of glycoprotein. Destained gels were dried onto cellulose acetate sheets with a Bio-Rad Laboratories Model 224 gel slab drier. Dried gels were scanned at 550 nm on a Corning Model 740 densitometer.

Liquid Scintillation Counting. Samples were dissolved in ACS (Amersham) and counted in a Searle Isocap or Tracor scintillation counter. Corrections for quenching and efficiency were made by the external standard ratio method or by the addition of an internal standard of [^{3}H]toluene.

Measurements of Protein. Protein was measured by the method of Bradford (1976) using BSA (Sigma) as a standard. Three milliliters of dye reagent was mixed with 0.1-mL samples, and the absorbance was determined at 595 nm.

Results

Purification of Folate Binding Protein—Cytosol I Chromatography on Sephacryl S-200. Zamierowski & Wagner (1977) previously showed that Sephadex G-150 chromatography of liver cytosol prepared from rats injected with [^{3}H]-PteGlu resulted in three peaks of protein-bound radioactivity. The first peak emerged soon after the breakthrough volume of the column, and its molecular weight was estimated to be about 350 000. When a maximum of 50 mL of concentrated cytosol was applied to a 87 × 5 cm column of Sephacryl S-200 and eluted at 5 °C (all subsequent steps were at 5 °C) with 10 mM potassium phosphate, pH 7.0, containing 10 mM 2-mercaptoethanol, the elution profile (Figure 1) showed three protein-bound radioactive peaks. The first peak was designed as FBP-CI, peak 2 as FBP-CII (Suzuki & Wagner, 1980; Cook & Wagner, 1981), and peak 3 as FBP-CIII. FBP-CI clearly falls within the resolving power of the Sephacryl S-200 column and has an elution position equivalent to a molecular weight of about 210 000 (Figure 1, insert).

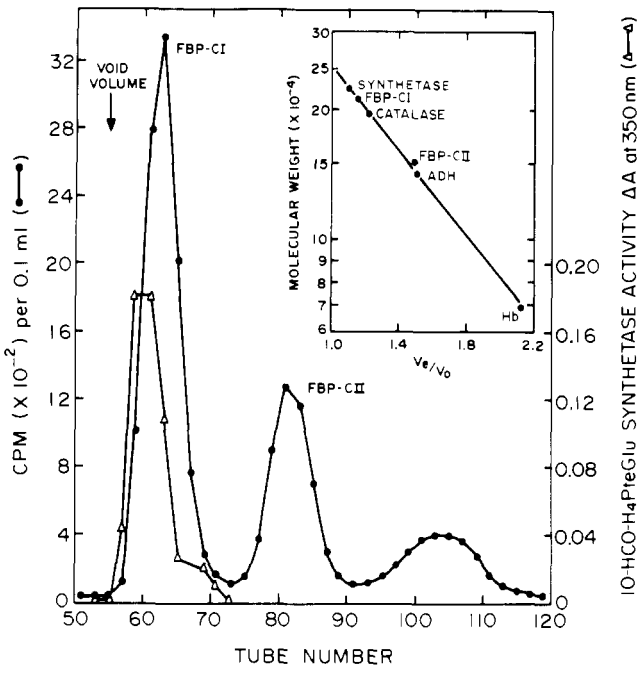

FIGURE 1: Sephacryl S-200 chromatography of rat liver cytosol. Cytosol (50 mL) was applied to a 5 × 87 cm column of Sephacryl S-200 equilibrated with 10 mM potassium phosphate, pH 7.0, containing 10 mM 2-mercaptoethanol. The column was eluted with the same buffer by using upward flow at 1.6 mL/min. Fractions of 10 mL were collected. 10-Formyltetrahydrofolate synthetase activity was determined as described under Materials and Methods. Inset: Calibration of Sephacryl S-200 column for molecular weight. The S-200 column was calibrated for molecular weight as described under Materials and Methods. Synthetase refers to 10-formyltetrahydrofolate synthetase.

The native M_r of FBP-CI suggested it might be the trifunctional enzyme formyl–methenyl–methylene synthetase (FMMS; EC 6.3.4.3, 3.5.4.9, and 1.5.1.5) which has been characterized from pig liver (M_r 150 000), yeast (M_r 201 000), and sheep liver (M_r 226 000). Assay for 10-formyltetrahydrofolate synthetase activity showed that peak enzyme activity eluted three tubes (30 mL) before the FBP-CI (Figure 1). On this basis, the previously uninvestigated rat liver FMMS has a native M_r of approximately 225 000.

5,10-Methylenetetrahydrofolate Reductase Activity in Sephacryl S-200 Eluate. Kutzback & Stokstad (1971) reported the partial purification of 5,10-methylenetetrahydrofolate reductase (EC 1.1.1.68) from rat liver which has a native M_r of 160 000–200 000 which is similar to FBP-CI. Assays for 5,10-methylenetetrahydrofolate reductase activity were performed on the fractions obtained from chromatography on a Sephacryl S-200 column. The peak of enzyme activity (not shown) eluted between FBP-CI and FBP-CII at a position equivalent to a molecular weight of 160 000–170 000.

Table I: Purification of FBP-CI

step	vol (mL)	total protein (mg)	[^{3}H]folate binding activity[a] (cpm/mg of protein)	x-fold purification
cytosol	138	1070	500	1
Sephacryl S-200 chromatography	86	186	4250	8
DEAE-cellulose chromatography	80	40	28000	56
affinity chromatography I	25	3.53	39900	80
affinity chromatography II	30	1.53	90900	182

[a] Measured as described under Materials and Methods.

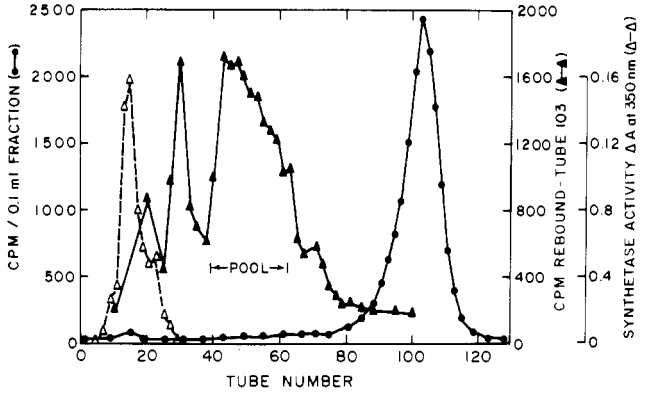

FIGURE 2: DEAE-cellulose chromatography of FBP-CI. Tubes 59–66 from the Sephacryl S-200 column containing protein-bound ^{3}H were combined and concentrated in an Amicon filtration apparatus with a PM 30 filter to a volume of 19 mL. The concentrate was made 20% (v/v) with respect to glycerol and applied to a 2 × 10 cm column of DEAE-cellulose, equilibrated with 0.1 M potassium phosphate, pH 6.1, containing 0.1 M 2-mercaptoethanol and 20% glycerol (v/v). The column was washed with 50 mL of equilibration buffer, and then a linear gradient of KCl (0–0.5 M) in equilibration buffer was applied (400 mL total volume). The column was eluted at 0.5 mL/min, and 4-mL fractions were collected.

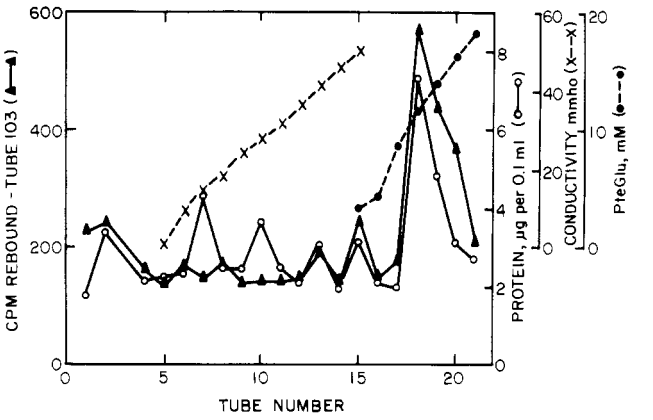

FIGURE 3: Affinity chromatography of FBP-CI. The affinity column (1 × 3 cm) was prepared as described under Materials and Methods. The rebinding peak from the first affinity column was pooled, concentrated, and dialyzed in an Amicon filter (PM 30) against 10 mM potassium phosphate, pH 7.0, containing 10 mM 2-mercaptoethanol and 1 mM sodium azide until the conductivity was equal to that of the dialyzing buffer (24 h). A concentrated sample (5 mL) was applied to the column which was equilibrated with dialysis buffer. The column was then washed with 45 mL of dialysis buffer followed by a linear 0–1.0 M KCl gradient in dialysis buffer (total volume 100 mL). This was followed by a linear 0–20 mM PteGlu gradient in dialysis buffer containing 1 M KCl (total volume 50 mL). The flow rate was 0.4 mL/min, and 10-mL fractions were collected.

Chromatography on DEAE-cellulose. The ^{3}H-labeled FBP-CI peak from the S-200 column was pooled and concentrated to approximately 20 mL with an Amicon filter apparatus equipped with a PM-30 filter. In this experiment, the concentrate was made 20% with respect to glycerol in order to stabilize FMMS activity. The concentrate was applied to a 2 × 10 cm column of DEAE-cellulose equilibrated with 0.1 M potassium phosphate, pH 6.0, containing 0.1 M 2-mercaptoethanol and 20% glycerol (v/v). The column was washed with 40 mL of buffer before a 0–0.5 M KCl gradient in equilibration buffer was started. The results (Figure 2) show that FMMS activity does not coelute with any of the three folate binding peaks.

The radioactive material eluting at tube 103 was not protein bound. It represented natural folate polyglutamates which had been bound to FBP-CI after elution from the Sephacryl column and which dissociated during chromatography on DEAE-cellulose. When fractions eluted from this column were tested for their ability to rebind in vitro the radioactive folate contained in tube 103, three peaks were obtained (Figure 2). The tubes containing the major folate binding peak were pooled and purified further by affinity chromatography.

5-HCO-H_4PteGlu Affinity Chromatography. The rat liver MFBP has been purified by using affinity chromatography with 5-HCO-H_4PteGlu as the immobilized ligand (Wittwer & Wagner, 1980, 1981a). Preliminary binding studies with partially purified FBP-CI showed that 5-HCO-H_4PteGlu was able to inhibit the binding of natural [^{3}H]folates which suggested that a 5-HCO-H_4PteGlu affinity column could be used to purify FBP-CI.

The major folate rebinding peak from DEAE-cellulose chromatography (Figure 2) was concentrated and then dialyzed at 5 °C against 10 mM potassium phosphate, pH 7.0, containing 10 mM 2-mercaptoethanol and 1 mM sodium azide. When the conductivity of the sample was equal to that of the dialysis buffer, the sample was concentrated to approximately 5 mL. A maximum of 5 mL of sample was applied to a 5-HCO-H_4PteGlu affinity column followed by a 45-mL wash with dialysis buffer. After the column was washed, a 0–1 M KCl gradient in dialysis buffer was applied followed by a 0–20 mM PteGlu gradient in dialysis buffer containing 1 M KCl. Fractions were collected and tested for [^{3}H]folate binding activity and protein concentration. The results showed a folate binding peak eluting between tubes 17 and 20. Usually, NaDodSO$_4$–polyacrylamide gel electrophoresis of the folate binding peak showed a single protein band with an M_r of 100000. Occasionally, several minor bands were also seen, and the affinity chromatography step was repeated. The results are shown in Figure 3. NaDodSO$_4$–polyacrylamide gel electrophoresis of tubes 18–20 showed a single protein band with an M_r of 100000, suggesting that native FBP-CI exists as a dimer. Figure 4 shows a densitometer scan of the NaDodSO$_4$–polyacrylamide gel of tube 19. Staining of gels for the presence of carbohydrate was negative.

A summary of the above purification procedure is shown in Table I.

Identification of the Endogenous Folate Ligand: Nature of the Pteridine Moiety. Based upon the growth response of

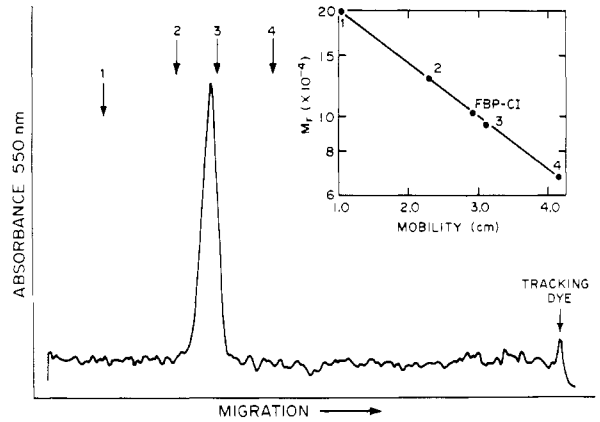

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of purified FBP-CI. Five micrograms of protein was treated with 1% $NaDodSO_4$ and 1% 2-mercaptoethanol, heated in a boiling water bath for 5 min, and electrophoresed in a 1.5 mm × 10 cm × 14 cm slab gel. The gels were stained, dried, and scanned at 550 nm as described under Materials and Methods. The arrows indicate the positions to which the following marker proteins migrated: (1) myosin; (2) β-galactosidase; (3) phosphorylase *b*; (4) BSA. The inset shows the M_r standard curve.

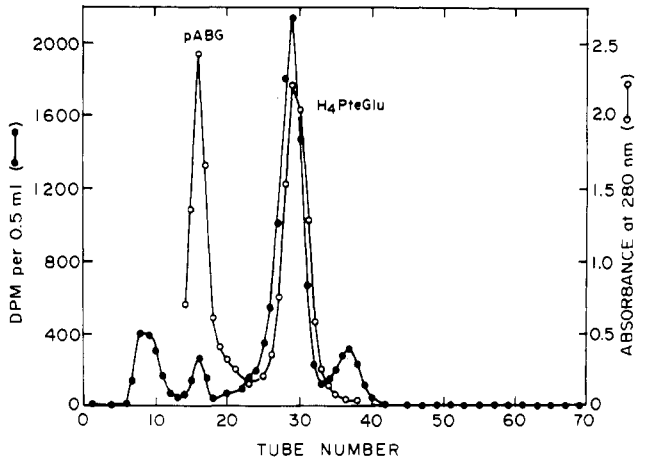

FIGURE 5: TEAE-cellulose chromatography of the endogenous folate ligand after treatment with conjugase. The sample was prepared as described under Materials and Methods. A 0.9 × 30 cm column of TEAE-cellulose was equilibrated with 0.05 M Tris-HCl, pH 7.5, containing 0.1 M 2-mercaptoethanol and 0.1 M NaCl. The sample, containing *p*-ABG and H_4PteGlu standards, was applied to the column and eluted with a 0.1–0.4 M NaCl gradient in equilibration buffer. The column was eluted at 4 °C in the dark, and 4-mL fractions were collected at 0.5 mL/min. The *p*-ABG and H_4PteGlu standards were identified by their UV spectra and relative elution positions.

Lactobacillus casei and *Streptococcus faecium*, Zamierowski & Wagner (1977) had noted that the folate ligand bound to FBP-CI was a polyglutamate but was not a 5-CH_3-H_4PteGlu derivative. FBP-CI was isolated by Sephacryl S-200 chromatography and concentrated to a small volume by using an Amicon filtration apparatus with a PM 30 filter. The bound [^{3}H]folate was released from the protein by boiling in the presence of 1% sodium ascorbate and 0.1 M 2-mercaptoethanol. The extract was treated with hog kidney conjugase (as described under Materials and Methods) and then applied to a TEAE-cellulose column equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.1 M 2-mercaptoethanol. Standard markers, *p*-ABG and H_4PteGlu, were added to the conjugase-treated mixture before application to the column. The column was eluted with a 0–0.4 M NaCl gradient. The elution profile (Figure 5) shows that the major radioactive peak eluted ahead of H_4PteGlu. There were also detectable ^{3}H-labeled

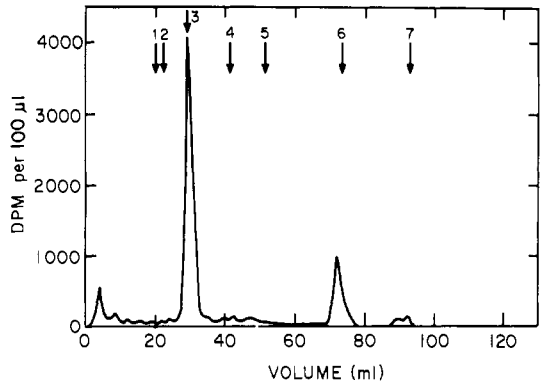

FIGURE 6: HPLC analysis of the endogenous folate ligand after conjugase treatment. Samples were prepared as described under Materials and Methods and in the text, were mixed with the following standards, and were injected onto a C-18 Bondapak column (Waters): (1) *p*-ABG; (2) 10-HCO-H_4PteGlu; (3) H_4PteGlu; (4) 5-HCO-H_4PteGlu; (5) H_2PteGlu; (6) 5-CH_3-H_4PteGlu; (7) PteGlu. The column was eluted with methanol–acetonitrile–H_2O (9:6:85 v/v/v), 100 mL of which was mixed with 3 mL of TBAP, at 2 mL/min (2500 psi) as described under Materials and Methods.

peaks at tubes 8–10 which are probably due to ^{3}H-labeled pterins and H_2O and at tube 16 (*p*-ABG), all products of H_4PteGlu decomposition. The ^{3}H-labeled peak eluting after H_4PteGlu is probably H_4PteGlu$_2$, a result of incomplete conjugase action on the original polyglutamate since it was not seen when conjugase was omitted (Figures 7 and 8).

The elution of the major radioactive peak one tube prior to unlabeled standard H_4PteGlu has been previously noted for the conjugase-treated H_4PteGlu ligand of mitochondrial folate binding protein (Wittwer & Wagner, 1981a) and may reflect isotope effects which have previously been noted for other compounds on ion-exchange columns (Steinberg & Udenfriend, 1957). It has also been reported that 5,10-CH_2-H_4PteGlu elutes slightly ahead of H_4PteGlu on DEAE-Sephadex columns (Nixon & Bertino, 1971). Conjugase-treated material was chemically reduced in the presence and absence of formaldehyde to distinguish between H_4PteGlu and 5,10-CH_2-H_4PteGlu. It is known that H_4PteGlu reacts with formaldehyde to form 5,10-CH_2-H_4PteGlu, and this may be reduced with sodium borohydride to 5-CH_3-H_4PteGlu. Treatment of conjugase-hydrolyzed material with formaldehyde and borohydride produced 5-CH_3-H_4PteGlu, while borohydride alone yielded only H_4PteGlu (data not shown), confirming that the folate ligand was H_4PteGlu after conjugase treatment.

Further analysis of rat serum conjugase-treated ligand by ion-paired, reverse-phase HPLC showed the majority of the ^{3}H-labeled material coeluted with H_4PteGlu at 29 mL (Figure 6). A second radioactive peak eluted at 72 mL slightly ahead of 5-CH_3-H_4PteGlu. This may have been H_4PteGlu$_2$ produced as a result of incomplete hydrolysis by the rat serum conjugase. A small radioactive peak at 4 mL was also recorded and is probably pterin. Treatment of the ligand with boiled conjugase (not shown) resulted in only two very small ^{3}H-labeled peaks appearing at 4 mL (pterins) and 29 mL (H_4PteGlu) but none at 72 mL. The remainder of the radioactive material bound tightly to the column and was eluted when the column was stripped with a mixture of methanol–H_2O (8:2 v/v), consistent with the behavior of a polyglutamate. This result indicates that the ^{3}H-labeled peak eluting at 72 mL slightly ahead of 5-CH_3-H_4PteGlu (Figure 6) was the result of incomplete conjugase treatment. The ability of the HPLC system to produce base-line separations of reduced folate derivatives strongly indicates that the conjugase-treated ligand is

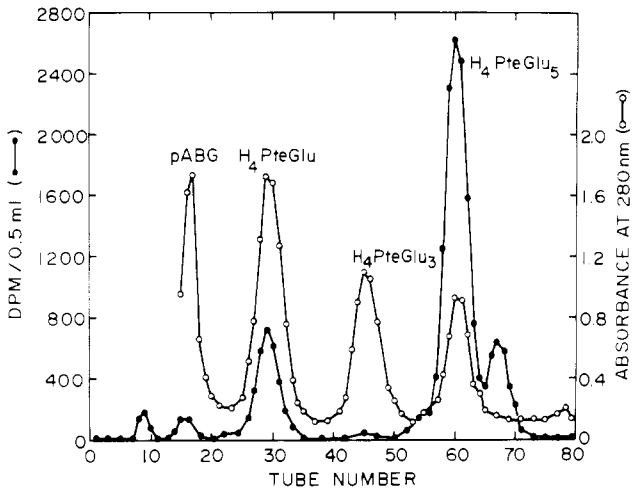

FIGURE 7: TEAE-cellulose chromatography of the endogenous folate ligand. The sample was prepared as described in the text and chromatographed on a TEAE-cellulose column exactly as described for Figure 5. Standards of *p*-ABG, H_4PteGlu, H_4PteGlu$_3$, and H_4PteGlu$_5$ were mixed with the sample and applied to the column.

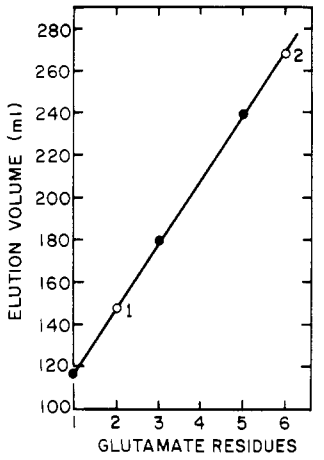

FIGURE 8: Relationship between elution position and H_4PteGlu polyglutamate chain length by TEAE-cellulose chromatography. Data are taken from Figures 5 and 7. Solid circles are the elution volumes of the H_4PteGlu, H_4PteGlu$_3$, and H_4PteGlu$_5$ standards. Open circle 1 corresponds to the elution volume of the minor peak appearing at tube 37 of Figure 5. Open circle 2 corresponds to the elution volume of the minor peak appearing at tube 67 of Figure 7.

H_4PteGlu. Horne et al. (1981) have shown that 5,10-CH_2-H_4PteGlu, which elutes close to H_4PteGlu on TEAE-cellulose (not shown in Figure 5), elutes between 5-CH_3-H_4PteGlu and PteGlu in this HPLC system. Since no major radioactive peak elutes in this region (Figure 6), the major folate ligand cannot be 5,10-CH_2-H_4PteGlu.

Additional studies on DEAE-cellulose with potassium phosphate buffer (0.05 M), pH 6.2, and on TEAE-cellulose with sodium acetate (0.05 M), pH 5.0, or Tris-HCl (0.05 M), pH 8.3, all using NaCl gradients to elute folates, confirmed that the major radioactive peak obtained after conjugase treatment was H_4PteGlu.

Identification of the Endogenous Folate Ligand: Polyglutamate Chain Length. The above results show the pteridine moiety to be H_4PteGlu after conjugase treatment. For determination of the number of glutamate residues in the ligand, FBP-CI, partially purified by Sephacryl S-200 chromatography, was treated as described under Materials and Methods and the protein-free extract chromatographed with chemically synthesized H_4PteGlu, H_4PteGlu$_3$, and H_4PteGlu$_5$ as markers on TEAE-cellulose (Figure 7). The results show that H_4PteGlu$_5$ was the predominant folate species in the absence of conjugase treatment. H_4PteGlu was also present and a minor ^{3}H-labeled peak which eluted after H_4PteGlu$_5$.

Examination of the elution positions of H_4PteGlu, H_4PteGlu$_3$, and H_4PteGlu$_5$ on TEAE-cellulose chromatography shows a linear relationship between elution volume and glutamate chain length (Figure 8). When this relationship is used, the radioactive peak eluting after H_4PteGlu$_5$ (tube 67) in Figure 7 corresponds to the position one would expect to find H_4PteGlu$_6$ (Figure 8). The ^{3}H-labeled peak eluting after H_4PteGlu (tube 37) in Figure 5 corresponds to the position of H_4PteGlu$_2$. It should be noted that the ^{3}H-labeled putative H_4PteGlu$_2$ seen in Figure 5 does not appear in Figure 7, which indicates that it arises after conjugase action and supports the hypothesis that it is H_4PteGlu$_2$.

Relative Binding Affinities of Folate Derivatives for FBP-CI. The binding of naturally occurring [^{3}H]folate polyglutamates to pure FBP-CI could be inhibited by the addition of various unlabeled folate derivatives to the binding assay. Table II summarizes these data in terms of the concentration of derivative required for 50% inhibition of [^{3}H]folate polyglutamate binding. The results show that the tetrahydro derivatives, i.e., H_4PteGlu, 10-HCO-H_4PteGlu, and 5-CH_3-H_4PteGlu, had a greater affinity for FBP-CI than PteGlu or H_2PteGlu, indicating that reduction of the pteridine ring is an imporatnt requirement for binding. The oxidized polyglutamates, PteGlu$_3$ (100 μM) and PteGlu$_5$ (80 μM), had no effect on the binding of [^{3}H]folate polyglutamates when tested on FBP-CI at the DEAE-cellulose stage of purification.

Table II: Relative Affinities of Folate Derivatives for FBP-CI

folate derivative	concn for 50% inhibition of naturally occurring [^{3}H]folate polyglutamate[a] (μM)
H_4PteGlu	13.5
10-HCO-H_4PteGlu	9.4
5-CH_3-H_4PteGlu	13.2
PteGlu	260
H_2PteGlu	350

[a] Naturally occurring [^{3}H]folate polyglutamates were obtained during purification of FBP-CI at the DE-52 chromatography stage.

Discussion

Previous studies in this laboratory have identified four folate binding proteins from rat liver (Zamierowski & Wagner, 1974, 1977). The binding protein in the cytosol, FBP-CII, has been purified and characterized in some detail (Suzuki & Wagner, 1980; Cook & Wagner, 1981). This protein contains 5-CH_3-H_4PteGlu$_5$ as the form of folate which is tightly bound. It has no enzymatic activity and together with its primary localization in the liver has prompted the suggestion that it serves as a storage protein for folate (Cook & Wagner, 1981; Wagner, 1982). On the other hand, the folate binding protein of rat liver mitochondria was recently shown to consist of two closely related enzymes, dimethylglycine dehydrogenase (EC 1.5.99.2) and sarcosine dehydrogenase (EC 1.5.99.1) (Wittwer & Wagner, 1980, 1981a,b). Both of these enzymes contain tightly bound H_4PteGlu$_5$ which has been shown to participate in the enzymatic mechanism by combining with formaldehyde generated during the reaction to form 5,10-CH_2-H_4PteGlu$_5$ (Wittwer & Wagner, 1981b).

Previous studies by Zamierowski & Wagner (1977) indicated that FBP-CI contained a tightly bound folate polyglutamate which was not a 5-CH_3-H_4PteGlu derivative. In these studies, we have identified the principal form of folate

which is bound as $H_4PteGlu_5$. Small amounts of $H_4PteGlu$ and $H_4PteGlu_6$ were also found in the material which was not treated with conjugase. The monoglutamate may have arisen as a result of endogenous conjugase action. The identification of the monoglutamate form of the polyglutamate as $H_4PteGlu$ appears to be accurate since it was carried out after conjugase treatment by DEAE-cellulose, TEAE-cellulose, and HPLC. The latter method clearly separates $H_4PteGlu$ and 5,10-CH_2-$H_4PteGlu$. A possibility remains, however, that the form of the ligand bound to FBP-CI is a polyglutamate form of 5,10-CH_2-$H_4PteGlu$. This compound is in equilibrium with formaldehyde and $H_4PteGlu$ (Blakely, 1960), and if 5,10-CH_2-$H_4PteGlu_5$ were the natural ligand, one would expect it to have dissociated to $H_4PteGlu_5$ during release from the binding protein and subsequent chromatography.

The observation that FBP-CI contains tightly bound $H_4PteGlu_5$, similar to the mitochondrial folate binding proteins which are in fact enzymes, led us to explore the possibility that FBP-CI has an enzymatic function as well. Of the enzymes known to require $H_4PteGlu$ as a cofactor, only the trifunctional FMMS is of a size similar to FBP-CI. The enzyme from sheep liver has an M_r of 226 000 (Paukert et al., 1976), and the one from pig liver has an M_r of 150 000 (Tan et al., 1977). A dimeric structure has been demonstrated for the sheep liver enzyme. Measurement of the 10-formyltetrahydrofolate synthetase activity of the trifunctional enzyme in both the Sephacryl S-200 (Figure 1) and the DEAE-cellulose (Figure 2) purification steps showed that FBP-CI clearly separated from the rat liver trifunctional enzyme, which had an approximate M_r of 225 000. The possibility that FBP-CI was 5,10-methylenetetrahydrofolate reductase was also considered. This enzyme was shown by Kutzback & Stokstad (1971) to have an M_r of between 160 000 and 200 000. In confirmation of this, we estimated the M_r of this enzyme of Sephacryl S-200 to be about 160 000–170 000, and it was clearly separated from FBP-CI.

Previous studies (Zamierowski & Wager, 1977) have eliminated serine transhydroxymethylase (EC 2.1.2.1), glutamate formiminotransferase (EC 2.1.2.5), and tetrahydropteroylglutamate methyltransferase (EC 2.1.1.13) as possible candidates for FBP-CI on the basis of size. On the same basis, cyclodeaminase (EC 8.3.2.2) (Drury et al., 1975) and thymidylate synthetase (EC 2.1.1.45) (Blakely, 1969) were also eliminated, although the latter two enzymes were not studied in rat liver but in other mammalian species. Two other enzymes, involved in the de novo synthesis of the purine ring, phosphoribosylglycinamide formyltransferase (EC 2.1.2.2) and phosphoribosylaminoimidazolecarboxamide formyltransferase (EC 2.1.2.3), were also considered. They, too, have not been purified from rat liver. In chicken liver (Smith et al., 1980), phosphoribosylglycinamide formyltransferase is composed of two M_r 56 000 subunits, and phosphoribosylaminoimidazolecarboxamide formyltransferase is composed of two M_r 68 000 subunits. Both enzymes use 10-HCO-$H_4PteGlu_n$ as one substrate and form $H_4PteGlu_n$ as one of the products. After conjugase treatment, we found no evidence for the presence of 10-HCO-$H_4PteGlu$ as the bound ligand (Figure 6). Only $H_4PteGlu$ was found. It would be extremely unlikely to find the product of an enzymatic reaction, $H_4PteGlu$, bound to an enzyme in preference to the substrate, 10-HCO-$H_4PteGlu$. Also purine biosynthesis is not a major pathway in mammals, and these enzymes are present in very small quantities, making it unlikely that FBP-CI is one of them.

It remains possible that FBP-CI is an enzyme containing tightly bound polyglutamate forms of $H_4PteGlu$ which functions to combine with enzymatically generated formaldehyde, like dimethylglycine dehydrogenase, but if so, it must be an enzyme not yet identified to carry out such a function.

The binding studies showed that the fully reduced monoglutamate derivatives were most effective in competing for the binding of the labeled natural folate polyglutamate $H_4PteGlu_5$ (Table II). Oxidized polyglutamates, $PteGlu_3$ and $PteGlu_5$, did not inhibit binding of the ^3H-labeled ligand. Although the actual concentration of the natural ligand, $H_4PteGlu_5$, used in these studies is not known, the apparent affinity of FBP-CI for $H_4PteGlu_5$ must be very high because high relative concentrations of reduced folate monoglutamates were required for 50% inhibition of binding to the natural ligand. The results indicate that both the reduced pteridine moiety and the pentaglutamate side chain are required for optimum binding. Radioactive folate polyglutamates of high specific activity are unavailable so that direct binding studies using the polyglutamates have not been done.

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Photoaffinity Labeling of *Klebsiella aerogenes* Citrate Lyase by *p*-Azidobenzoyl Coenzyme A†

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ABSTRACT: *p*-Azidobenzoyl coenzyme A functions as a linear competitive inhibitor for (3*S*)-citryl-CoA in the citryl-CoA oxaloacetate-lyase reaction catalyzed by the *Klebsiella aerogenes* deacetylcitrate lyase complex (K_i = 80 μM; (3*S*)-citryl-CoA K_m = 67 μM). Inactivation is irreversible on photolysis of *p*-azidobenzoyl-CoA in the presence of the deacetylcitrate lyase complex. Mg^{2+} is not required for the inactivation. Inactivation is blocked by (3*S*)-citryl-CoA in the presence of ethylenediaminetetraacetic acid. *p*-Azidobenzoyl-CoA has no effect on the acetyl-CoA:citrate CoA transferase activity of both the deacetylcitrate lyase complex and its isolated transferase subunit. The stoichiometry of the CoA ester binding has been investigated by the use of *p*-azido[^{14}C]benzoyl-CoA as a photoaffinity reagent. The labeling is exclusively on the lyase β subunit of the citrate lyase complex.

The citrate lyase multienzyme complex (EC 4.1.3.6) from *Klebsiella aerogenes* is assembled from three dissimilar subunits α, β, and γ of molecular weights 54 000, 32 000, and 10 000, respectively (Carpenter et al., 1975; Dimroth & Eggerer, 1975a). The γ subunit functions as an acyl-carrier protein (ACP)[1] with an essential acetyl function in thioester linkage (Buckel et al., 1971) on a covalently bound CoA-like prosthetic group (Oppenheimer et al., 1979). The α subunit acts as an acyl transferase involved in the formation of (3*S*)-citryl-ACP[1] in the presence of citrate with the release of acetate, while the β subunit functions as an acyl lyase catalyzing the cleavage of the citryl-ACP intermediate to oxaloacetate and acetyl-ACP[1] (Dimroth & Eggerer, 1975b). Acyl coenzyme A (acyl-CoA) derivatives such as acetyl-CoA and propionyl-CoA have been shown to serve also as substrates for the transferase reaction, while (3*S*)-citryl-CoA acts as a substrate for the lyase reaction (Buckel et al., 1973; Dimroth et al., 1977). The mechanism of action of the citrate lyase complex requires the oscillation of the prosthetic group (or of acyl-CoA in the acetyl-CoA-mediated reaction) between the active sites on the α and β subunits (Srere & Singh, 1974).

The paper reports the use of *p*-azidobenzoyl-CoA as a photoaffinity reagent for probing the acyl-CoA binding sites of the complex.

Experimental Procedures

Products. NADH, *N*-hydroxysuccinimide, sodium dodecyl sulfate ($NaDodSO_4$),[1] citrate synthase, and malate dehydrogenase were from Sigma. DTNB[1] was from Calbiochem. Sepharose CL-6B, Sephadex G-10, and Sephadex G-25 were from Pharmacia Fine Chemicals. DEAE-cellulose (DE-52) was from Whatman. *p*-Amino[*carboxyl*-^{14}C]benzoic acid (21 Ci/mol) was obtained from Radiochemicals Centre. All other products were of the highest purity obtainable commercially.

Preparation of CoA Esters. Acetyl-CoA was prepared by acetylation of CoA with acetic anhydride (Simon & Shemin, 1953). (3*S*)-Citryl-CoA was prepared enzymatically from acetyl-CoA and citrate in the presence of *K. aerogenes* deacetylcitrate lyase and EDTA[1] (Dimroth et al., 1977) and assayed with citrate synthase in the presence of DTNB.

p-Azidobenzoyl-CoA was synthesized as described by Lau et al. (1977). *p*-Azido[^{14}C]benzoyl-CoA was synthesized from *p*-amino[*carboxyl*-^{14}C]benzoic acid without excessive dilution of the radioactivity. The concentration of the reagent was determined by using ϵ_{265nm} = 22.2 mM^{-1} (Lau et al., 1977).

All acyl-CoA preparations were purified as described by Moffat & Khorana (1961) by chromatography on a DEAE-cellulose (Cl^- form) column using a linear gradient from 0 to 0.2 M LiCl containing 0.003 N HCl. Samples of the CoA esters were concentrated at 25 °C in a rotary evaporator and desalted by filtration through a Sephadex G-10 column (1.0 × 50 cm).

Purification of Citrate Lyase. Citrate lyase complex from *Klebsiella aerogenes* (NCTC 418) was purified by a modification of the procedure described earlier (Mahadik & SivaRaman, 1968), sonic extracts being treated with ATP and acetate at final concentrations of 0.3 and 1 mM, respectively, for reactivating any deacetylcitrate lyase (Leena, 1979).

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[1] Abbreviations: ACP, acyl-carrier protein; (3*S*)-citryl-ACP, (3*S*)-citrylacyl-carrier protein; acetyl-ACP, acetylacyl-carrier protein; $NaDodSO_4$, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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