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One-Step Purification and Properties of a Two-Peptide Fatty Acid Synthetase from the Uropygial Gland of the Goose†

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ABSTRACT: Cell-free extracts from the uropygial gland of goose catalyzed the incorporation of malonyl-CoA into normal fatty acids and methylmalonyl-CoA into multimethyl branched acids with NADPH as the preferred reductant (J. S. Buckner and P. E. Kolattukudy (1975), *Biochemistry 14,* 1774). Purification of fatty acid synthetase from this extract was accomplished in one step by gel filtration with Sepharose 4B. Homogeneity of the fatty acid synthetase was shown by analytical ultracentrifugation, immunodiffusion assays, polyacrylamide disc gel electrophoresis, and sodium dodecyl sulfate polyacrylamide disc gel electrophoresis. At a pH of 7.0, apparent K_m values of 3.6×10^{-5} M and 1.5×10^{-5} M were calculated for malonyl-CoA and NADPH, respectively. The major products synthesized by the enzyme from malonyl-CoA and methylmalonyl-CoA were free hexadecanoic acid and free 2,4,6,8-tetramethyldecanoic acid, respectively, with acetyl-CoA as primer. A molecular weight value of 547 000 was determined for the goose fatty acid synthetase by sedimentation equilibrium centrifugation. The purified enzyme had an $s_{20,w}$ of 13.5 S and was partially dissociated in low-ionic strength buffer into a 9.3S species, and this dissociation was accompanied by a corresponding partial inactivation of the enzymatic activity. Reassociation and reactivation of the partially dissociated fatty acid synthetase were accomplished in either 0.2 M KCl or 200 μM NADPH. These properties of the goose enzyme are similar to those of other animal fatty acid synthetases, as was the amino acid composition. Dissociation of the purified enzyme with sodium dodecyl sulfate resulted in only two equal molecular weight polypeptides (269 000), as determined by sodium dodecyl sulfate polyacrylamide disc gel electrophoresis. Injection of labeled pantothenic acid into the uropygial gland resulted in the synthesis of labeled fatty acid synthetase in which the label appeared to be located exclusively in the 4′-phosphopantotheine moiety. Analysis of the labeled enzyme by gel filtration and polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate showed that the labeled pantothenate was contained exclusively in the half molecular weight moiety. The enzyme contained one 4′-phosphopantetheine residue per subunit (269 000), as determined by measurement of the taurine generated by hydrolysis of performic acid-treated enzyme. Sodium dodecyl sulfate-activated proteolytic activity was shown to be associated with goose fatty acid synthetase, and this proteolysis was shown to result in the formation of small-molecular-weight protein fragments (<200 000) during treatment of the enzyme with sodium dodecyl sulfate. This proteolysis could be prevented by diisopropyl fluorophosphate and *p*-chloromercuribenzoate. These results strongly suggest that the goose uropygial gland fatty acid synthetase consists of two multifunctional polypeptide subunits, each containing one covalently linked 4′-phosphopantetheine.

Fatty acid synthetases isolated from bacteria (Lennarz et al., 1962; Goldman et al., 1963), plants (Overath and Stumpf, 1964), yeast (Lynen, 1961), and animals (Martin et al., 1961; Hsu et al., 1965; Smith and Abraham, 1970) have been studied extensively. The synthetase from *Escherichia coli* consists of several enzymes readily separable from one another by standard protein fractionation techniques (Vagelos et al., 1966). However, the fatty acid synthetases from yeast and animals do not dissociate into individual enzymes. The animal enzyme is obtained as a protein of approximately 500 000 molecular weight by conventional purification procedures (Smith and Abraham, 1970; Kumar et al., 1972; Yun and Hsu, 1972). Dissociation of this synthetase into 200 000–250 000 molecular weight subunits has been demonstrated with both low ionic strength buffers (Kumar et al., 1972; Yun and Hsu, 1972) and prolonged storage at 0–4 °C (Smith and Abraham, 1971; Muesing et al., 1975). The purified enzyme from pigeon liver has been separated into nonidentical subunits, each having a molecular weight half of that of the parent. The prosthetic

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group, 4′-phosphopantetheine, was reported to be associated with only one of these subunits. A low-molecular weight protein (mol wt ~10 000) was separated from the subunit containing 4′-phosphopantetheine and it possessed properties similar to those of the acyl carrier protein (ACP) previously isolated from *E. coli* (Qureshi et al., 1974). Isolation of a similar protein (ACP) has also been reported from the fatty acid synthetase preparation from dog liver (Roncari, 1974) and yeast (Willecke et al., 1969).

In contrast to the structural concept of a multienzyme complex is the recent experimental evidence obtained with yeast and chicken liver fatty acid synthetases, suggesting that the synthetase is made up of polypeptides containing multiple active sites (Schweizer et al., 1973; Stoops et al., 1975). The animal fatty acid synthetase was recently reported to contain two polyfunctional polypeptides (250 000), and 4′-phosphopantetheine was demonstrated to be attached to such a peptide rather than to a small peptide (ACP) similar to that isolated from yeast or pigeon liver (Willecke et al., 1969; Qureshi et al., 1974).

In this paper, we describe a one-step purification and properties of the fatty acid synthetase from the goose uropygial gland. Properties of the purified enzyme such as molecular weight, amino acid composition, cofactor requirement, pH optimum, K_m values, and stability are shown to be similar to those of other animal fatty acid synthetases (Hsu et al., 1967; Smith and Abraham, 1970; Burton et al., 1968; Yun and Hsu, 1972). Results which strongly support the concept that this fatty acid synthetase consists of two multifunctional polypeptides, each containing a covalently attached 4′-phosphopantetheine, are presented.

Experimental Section

Materials. Domestic white geese were purchased from the Richards Goose Hatchery, Outlook, Washington, and were maintained on a low-energy breeder ration. Malonyl-CoA, acetyl-CoA, NADPH, NADH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase (type XI), dithioerythritol, bovine serum albumin (fraction V), urease (type VII), thyroglobulin, Sepharose 4B and 6B, sodium dodecyl sulfate, and ficoll were purchased from Sigma Chemical Co.; Dowex 50W-X and Bio-Gel P-2 were purchased from Bio-Rad Laboratories. Agar (Noble) for immunodiffusion was purchased from Difco Laboratories. [2-^{14}C]Malonyl-CoA and [methyl-^{3}H]methylmalonyl-CoA were synthesized chemically from [2-^{14}C]malonic acid and [methyl-^{3}H]methylmalonic acid, respectively, as described previously (Buckner and Kolattukudy, 1975b).

Preparation of [G-^{3}H]Pantothenic Acid. Generally labeled calcium pantothenate was prepared by catalytic exchange at New England Nuclear Corp. The calcium [G-^{3}H]pantothenate was converted into [G-^{3}H]pantothenic acid by ion-exchange chromatography and purified by thin-layer chromatography on silica gel G with 1-butanol:acetic acid:water (25:4:10) as the developing solvent. Radioactive pantothenic acid was recovered from the silica gel by elution with 1-butanol:acetic acid:water (25:4:10). The solvent was evaporated under reduced pressure and the labeled material was dissolved in the appropriate aqueous medium.

Purification of Fatty Acid Synthetase. Geese were killed by exsanguination and the uropygial glands were excised from the birds. After removing the residual fat and muscle tissues the two lobes of each gland were thinly sliced and homogenized in 100 mM phosphate buffer (pH 7.6), containing 250 mM sucrose, 0.5 mM DTE, and 1.0 mM $MgCl_2$ as previously described (Buckner and Kolattukudy, 1975b). Centrifugation of the tissue homogenate at 12 000*g* for 20 min, followed by centrifugation of the 12 000*g* supernatant at 15 000*g* for 20 min, was employed to remove cell debris, fat, and mitochondria. Centrifugation of the 15 000*g* supernatant at 105 000*g* for 90 min and subsequent centrifugation of the 105 000*g* supernatant at 105 000*g* for 60 min completely removed the microsomal fraction. The soluble supernatant was filtered through two layers of cheesecloth for removal of floating lipid materials. All of the procedures were performed at 0–4 °C.

A Sepharose 4B column (2.7 × 96 cm) was equilibrated with 100 mM phosphate (pH 7.6) containing 20 mM citrate and 1 mM DTE. The soluble supernatant (5–7 ml) was placed atop the column and proteins were eluted with the same buffer with a flow rate of 20 ml/h. The absorbance of the column effluent was measured at 280 nm with an Isco Model UA-5 monitor and 5-ml fractions were collected with an Isco Model 568 fraction collector.

Purified fatty acid synthetase solutions (about 40 ml) from gel filtration chromatography were usually concentrated to a volume of 5–10 ml by ultrafiltration with a stirred Amicon pressure cell fitted with a PM-30 membrane and operated under 50 psi of N_2. An extremely slow rate of stirring was used to avoid denaturation of the enzyme which was found to be readily denatured upon stirring at moderate rates. Smaller (<2 ml) protein samples were concentrated by covering these solutions, contained in dialysis tubing, with powdered Ficoll.

Enzyme Assays. Fatty acid synthetase activity of all enzyme preparations and column fractions was usually measured spectrophotometrically. Initial rates of NADPH oxidation at 30 °C were obtained by measuring the absorbance decrease at 340 nm of reaction mixtures contained in 0.5-ml microcells (10 mm path length) using a Beckmann DU spectrophotometer equipped with a Model 222 Gilford photometer. Temperature control was provided by a Model FJ Haake-constant temperature circulator. Reaction mixtures contained 15 μmol of phosphate buffer, pH 7.0, 0.1 μmol of dithioerythritol, 0.04 μmol of NADPH, 0.01 μmol of acetyl-CoA, 0.04 μmol of malonyl-CoA, and enzyme in a total volume of 0.2 ml. Reactions were usually initiated by the addition of malonyl-CoA. Specific activity for fatty acid synthetase was expressed as nanomoles of NADPH oxidized per minute per milligram of protein. Protein in solution was determined by the method of Lowry et al. (1951) after precipitation of the protein with 10% trichloroacetic acid. Bovine serum albumin served as standard.

Fatty acid synthetase activities were also determined by a radiochemical assay. Procedures for the incubation of the purified fatty acid synthetase with [2-^{14}C]malonyl-CoA and isolation and purification of the resultant labeled fatty acids have been previously described (Buckner and Kolattukudy, 1975). A stoichiometry of 2 mol of NADPH oxidized per mol of malonyl-CoA incorporated has already been established for the synthesis of palmitic acid by the goose fatty acid synthetase (Buckner and Kolattukudy, 1975b).

Chromatography. Thin-layer chromatography was performed with 20 × 20 cm plates coated with silica gel G and activated overnight at 110 °C. Free fatty acids and fatty acid butyl esters were purified by thin-layer chromatography with hexane:ethyl ether:formic acid (40:10:1) as the developing solvent. Radio gas–liquid chromatography was performed with a Perkin-Elmer Model 801 gas chromatograph equipped with a flame ionization detector and an effluent splitter, attached

[1] Abbreviations used: ACP, acyl carrier protein; CoA, coenzyme A; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

to Barber-Coleman radioactivity monitor. Fatty acid butyl esters were analyzed on a coiled copper column (0.6 × 180 cm) packed with 5% OV-1 on 80–100 mesh Gas-Chrom Q with a carrier-gas flow of 80 ml/min. The column temperature was programmed from 160 to 240 °C with a 2 °C/min rate. Authentic samples were used as standards for thin-layer and gas–liquid chromatography.

Identification of the Product of Fatty Acid Synthetase. Reaction products resulting from the incubation of fatty acid synthetase with [2-^{14}C]malonyl-CoA were recovered by chloroform extraction. In order to determine whether CoA derivatives of fatty acids were present the aqueous layer, after the chloroform extractions, was treated with alkali at 100 °C for 20 min followed by acidification and additional chloroform extraction. No radioactivity was detected in the latter chloroform extract, showing that acyl-CoA was not a product. Lipids in the first chloroform extracts were analyzed by thin-layer chromatography as described earlier. Labeled fatty acids, purified by thin-layer chromatography, were refluxed with 14% BF_3 in 1-butanol for 10 min. The labeled fatty acid butyl esters were recovered from reaction mixtures by chloroform extraction and purified by thin-layer chromatography as described earlier. The labeled fatty acids, as butyl esters, were analyzed by radio gas–liquid chromatography as described in the previous section.

Determination of Radioactivity. Radioactivity on thin-layer chromatograms was monitored with a Berthold thin-layer scanner. Solutions containing ^{14}C or ^{3}H were assayed by liquid scintillation spectrometry. Aliquots of solutions containing the ^{14}C-labeled material were transferred into counting vials and the solvent was evaporated. The residue was dissolved in 15 ml of 30% ethanol in toluene containing 4 g/l. of Omnifluor and assayed for radioactivity in a Packard Model 3003 Tri-Carb scintillation spectrometer. Portions of solutions containing tritiated materials were transferred into counting vials containing 15 ml of Aquasol and assayed for radioactivity in the same manner as for ^{14}C. Internal standards of [^{3}H]toluene and [^{14}C]toluene were used to determine counting efficiencies of 25% and 74% for ^{3}H and ^{14}C, respectively.

Radioactivity in dodecyl sulfate polyacrylamide gels was analyzed by liquid scintillation spectrometry. Following electrophoresis, stained gels were hardened by cooling them with powdered dry ice and then cut into slices with a razor blade. Gel slices were treated with 0.5 ml of hydrogen peroxide at 70 °C for 2 h in counting vials. After cooling to room temperature the contents were mixed with 15 ml of Aquasol and assayed for ^{3}H as described above.

Analytical Ultracentrifugation. Sedimentation velocity experiments were performed in a Spinco Model E ultracentrifuge equipped with schlieren optics. Protein solutions (5–7 mg/ml) were centrifuged at a rotor speed of 56 000 rpm and a rotor temperature of 20 °C. Observed sedimentation coefficients were corrected for density and viscosity at 20 °C. Equilibrium sedimentation experiments were performed on the purified protein with the high-speed meniscus depletion method of Yphantis (1964) using the AnD rotor with a double sector cell at 20 °C. All samples were run at a protein concentration of 0.5 mg/ml and a rotor speed of 12 000 rpm. The partial specific volume was calculated from the amino acid composition of the purified enzyme.

Amino Acid Analysis. The purified fatty acid synthetase was analyzed for amino acid composition at the Bioanalytical Laboratory, Washington State University, Pullman, Wash. Protein samples were dialyzed against 5 mM phosphate buffer (pH 7.6), lyophilized, dissolved in 6 N HCl, and hydrolyzed for 24 h, under vacuum, at 110 °C. The amino acid composition of each hydrolysate was determined with a Beckman Model 121 amino acid analyzer, fitted with an automatic sample injector, according to the procedure of Moore and Stein (1958). Cysteine and methionine were analyzed after oxidation of the protein samples with performic acid (Hirs, 1967) followed by acid hydrolysis for 24 h, under vacuum, at 110 °C. Tryptophan analysis was done by the method of Matsubara and Sasaki (1969). The 4′-phosphopantetheine content of the fatty acid synthetase was determined by analyzing the hydrolysate of performic acid oxidized protein for taurine and β-alanine. Samples were run on the Beckman Model 121 amino acid analyzer using a physiological buffer system (Moore and Stein, 1958).

Preparation of Antiserum. Antiserum was prepared by immunizing rabbits with purified fatty acid synthetase. Approximately 5 mg of protein dissolved in 0.5 ml of 0.9% NaCl was emulsified by sonication (Biosonik III, needle probe, 2 × 10 s) with 0.5 ml of complete Freund's adjuvant and the mixture was subcutaneously injected into rabbits. Two weeks after the first injection, 5 mg of protein, emulsified with incomplete Freund's adjuvant, was injected into the rabbits. Two weeks after the second injection, the rabbits were bled from the ear and the antiserum was prepared and stored at −20 °C without further purification.

Immunodiffusion. Double diffusion analysis was done according to the method of Ouchterlony (1966). Immunodiffusion was performed on 1% agar in 0.9% NaCl contained in Petri dishes. Diffusion was complete after 24 h, nonagglutinated protein was removed from the agar by repeated washing with 0.9% NaCl, and the NaCl was removed by washing with distilled water. The agglutinated protein bands were stained with a solution of 0.5% Amido black, 5% $HgCl_2$ in 5% acetic acid for 30 min. Excess dye was removed from the agar with 5% acetic acid.

Molecular Weight Estimation by Sodium Dodecyl Sulfate Gel Filtration. The purified fatty acid synthetase and standard proteins were dissociated into subunits with sodium dodecyl sulfate. Protein solutions (2–5 mg in 0.5 ml) were heated in a boiling water bath for 1 min prior to the addition of 0.5 ml of 2% sodium dodecyl sulfate containing 200 μmol of DTE. After 3 min, the samples were removed from the boiling water bath and cooled. The dissociated proteins were either used immediately for column chromatography or electrophoresis, or were stored until use at −20 °C.

A Sepharose 6B column (1.8 × 58 cm) was equilibrated with 20 mM Tris-HCl buffer (pH 7.6), containing 5 mM dithioerythritol and 0.1% sodium dodecyl sulfate. The sodium dodecyl sulfate treated fatty acid synthetase and protein standards were each applied on the column in a volume of 1 ml and proteins were eluted from the column with 20 mM Tris-HCl buffer (pH 7.6) containing 0.1% sodium dodecyl sulfate and 5 mM dithioerythritol at a flow rate of 16.8 ml/h. The column effluents were monitored at 280 nm with an Isco Model UA-2 absorbance monitor and 2.8-ml fractions were collected. A void volume of 40.5 ml was determined for the column with Blue Dextran 2000. Standard proteins used for molecular weight determinations for both gel filtration chromatography and sodium dodecyl sulfate disc gel electrophoresis were thyroglobulin, myosin, urease, bovine serum albumin, and pepsin with subunit molecular weights of 335 000, 210 000, 83 000, 68 000, and 35 000, respectively.

Electrophoresis. Sodium dodecyl sulfate disc gel electrophoresis was performed in an analytical electrophoresis apparatus from Hoefer Scientific Instruments, according to the

method of Maizel (1971). The gel system containing 0.1% dodecyl sulfate consisted of a 5% polyacrylamide resolving gel (pH 8.9) and a 2.5% stacking gel (pH 7.2). The length of the resolving and stacking gels were 7 and 2.5 cm, respectively, with a gel tube inner diameter of 0.45 cm. About 10–50 μg of each protein (dodecyl sulfate treated) was applied to the gel in a volume of 0.1 ml. Electrophoresis was performed at a constant current of 2 mA per tube over a period of 3 h. Protein bands were fixed and stained by immersion of the gels in a solution of 0.05% Coomassie brilliant blue in 45% methanol and 9% acetic acid for 24 h. The excess dye was removed from the gels electrophoretically using an Ames Model 1801 Quick Gel Destainer with a destaining solution of 7.5% acetic acid and 5% methanol.

Standard disc gel electrophoresis was performed with 4% polyacrylamide gels according to the procedure of Ornstein and Davis (1964). In order to keep the 4% gel from sliding out of the gel tubes during the electrophoretic run, short columns of 7.5% acrylamide were polymerized in each tube. The length of the resolving gel was 5 cm with a gel tube inner diameter of 0.45 cm. After electrophoresis, protein bands were fixed and stained by immersion in a solution of 1% Amido Schwartz in 7.5% acetic acid for 1 h. The excess dye was removed from the gel by electrophoresis in 7.5% acetic acid.

Incorporation of [G-^{3}H]Pantothenic Acid into Fatty Acid Synthetase. The in vivo incorporation of purified [G-^{3}H]pantothenic acid into goose uropygial gland fatty acid synthetase was accomplished by injection of labeled pantothenic acid directly into the glandular tissue of a goose. Since the uropygial gland of a goose lies directly beneath the skin at the base of the tail, a syringe needle could be inserted into the two glandular lobes with little, if any, difficulty provided the small feathers covering the skin above the gland were removed. [G-^{3}H]Pantothenic acid (3.4 μmol, 1800 μCi) in 0.2 ml of 0.9% NaCl was injected into the gland (two injections of 50 μl each into each lobe of the gland). Forty-eight hours after the injection of the labeled pantothenic acid the bird was killed by exsanguination and the uropygial gland was excised. The fatty acid synthetase was isolated as described above.

Results and Discussion

Enzyme Purification. Purification of the fatty acid synthetase was accomplished in one step by gel filtration of the soluble high-speed supernatant fraction obtained by ultracentrifugation of the goose uropygial gland homogenate (see Experimental Section). The soluble supernatant (105 000g) was filtered through a column of Sepharose 4B and the resulting elution profile for protein is shown in a later section (Figure 6). The fatty acid synthetase activity coincided with the second major protein peak. The fractions with high fatty acid synthetase activity were combined and the pooled fraction was assayed for enzymatic activity and protein concentration. Fractions eluted from the column immediately after the synthetase contained malonyl-CoA decarboxylase activity and to avoid the decarboxylase only the fractions of highest synthetase activity were pooled. As a result of this single gel filtration a three- to sixfold purification of the fatty acid synthetase was achieved and the resulting preparations had specific activity values which ranged from 700 to 1100 nmol of NADPH oxidized per min per mg of protein. This specific activity compares favorably with the specific activities reported for other animal fatty acid synthetases (Hsu et al., 1965; Burton et al., 1968; Smith and Abraham, 1970).

Pooled fractions (2–4 mg of protein/ml) from the gel filtration columns were usually concentrated fivefold by ultrafiltration with no appreciable loss in enzymatic activity, providing the rate of stirring in the ultrafiltration cell was slow. Reaction rates for the purified fatty acid synthetase, measured either spectrophotometrically or by radiochemical assay, were linear with respect to time and protein concentration up to 15 min and 0.02 mg/ml, respectively.

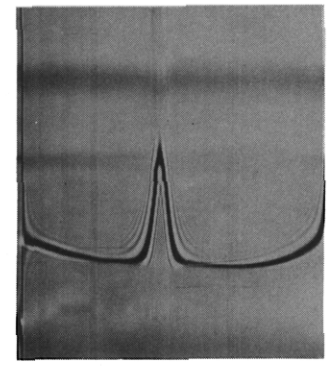

FIGURE 1: Sedimentation velocity pattern of the purified fatty acid synthetase (5 mg/ml) in 100 mM phosphate buffer, pH 7.6, containing 20 mM citrate and 1 mM dithioerythritol. Centrifugation was performed at 56 000 rpm at 20 °C. The pattern was taken approximately 24 min after the rotor had attained full speed.

Since the uropygial gland is a highly specialized organ, whose major function is synthesis of lipids, it is not surprising to find that fatty acid synthetase constitutes a large (1/3–1/6) portion of the total soluble protein of this gland. However, it is quite rare in animal tissues to find a single enzyme which constitutes as high a proportion of the total soluble protein as observed in the present case. Thus the simple procedure described here is adequate to prepare highly purified (homogeneous) fatty acid synthetase. A similar procedure does not provide homogeneous synthetase from the goose liver or rat liver.

Criteria of Purity

Analytical Ultracentrifugation. The purity of the fatty acid synthetase from the Sepharose 4B gel filtration column was examined by analytical ultracentrifugation. The purified enzyme appeared as a single sharp peak as shown by the sedimentation pattern in Figure 1. The $s_{20,w}$ for the fatty acid synthetase (5 mg/ml) was 13.5×10^{-13} s. The purified fatty acid synthetase was also analyzed by the sedimentation equilibrium method of Yphantis (1964), and plots of ln c vs. r^2 were found to be linear (data not shown). From this method, a molecular weight value of 547 000 ± 3500 was calculated for the fatty acid synthetase. This molecular weight value is similar to the molecular weight values obtained with other animal synthetases which range between 450 000 and 540 000 (Burton et al., 1968; Smith and Abraham, 1970; Yun and Hsu, 1972).

Immunological Tests. Immunodiffusion assays were performed with the purified fatty acid synthetase from goose uropygial glands and the antisera obtained from injections of the purified enzyme into two rabbits. As shown in Figure 2, only one precipitin line was formed for each antiserum. Purity of the enzyme preparation is indicated by the presence of only one precipitin line and fusion of precipitin lines when antigen was placed in adjacent wells. Increasing amounts of the rabbit antiserum caused a rapid and near linear decrease in enzymatic activity, finally resulting in almost complete inhibition of the enzyme. As a control, enzyme was assayed for activity in the presence of serum obtained from rabbits not injected with fatty acid synthetase. The control serum had no effect on the fatty

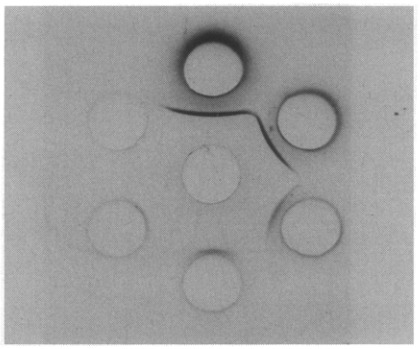

FIGURE 2: Immunodiffusion analysis (Ouchterlony) of the purified fatty acid synthetase. The top most outer well contained 2.00 mg/ml of fatty acid synthetase and the remaining five wells in a clockwise direction contained 1.00, 0.50, 0.25, 0.10, and 0.05 mg/ml. The center wells contained undiluted rabbit antiserum.

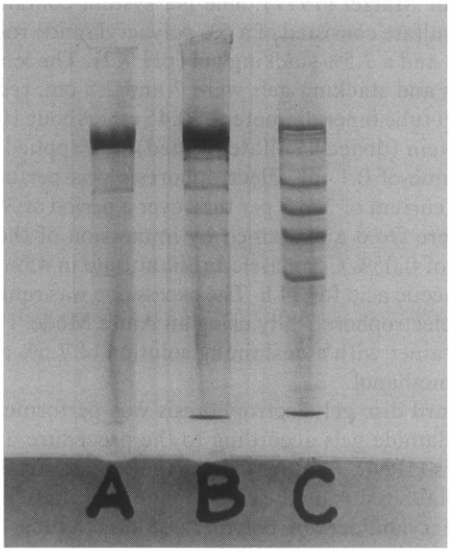

FIGURE 3: Sodium dodecyl sulfate polyacrylamide disc gel electrophoresis of the purified fatty acid synthetase. Experimental conditions included: A, heat treatment of the enzyme (2.5 mg/ml) at 100 °C for 1 min prior to treatment with 1% dodecyl sulfate in 100 mM dithioerythritol at 100 °C for 3 min; B, incubation of the enzyme (5 mg/ml) with 1 mM diisopropyl fluorophosphate and 1 mM *p*-chloromercuribenzoate for 10 min at 40 °C prior to treatment with 1% dodecyl sulfate in 100 mM dithioerythritol at 40 °C for 3 h; C, treatment of the enzyme (5 mg/ml) with 1% dodecyl sulfate in 100 mM dithioerythritol at 40 °C for 3 h.

acid synthetase activity.

Electrophoresis. The purity of the fatty acid synthetase from goose uropygial glands was also examined by electrophoresis. Since the fatty acid synthetase has a molecular weight of over 500 000, standard disc electrophoresis was performed with a polyacrylamide gel concentration of 4%. Electrophoresis of 10–20 μg of the purified fatty acid synthetase showed only two major bands of equal intensity with R_f values of approximately 0.2 and 0.35. Since previous reports on animal fatty acid synthetase have suggested that the enzyme complex is composed of nonidentical subunits of equal molecular weight of about 250 000 (Kumar et al., 1972; Yun and Hsu, 1972), it is possible that the two bands in the 4% polyacrylamide gel were either the two subunits with significantly different net charges or that the faster moving band constituted the unresolved subunits and the slower band the associated enzyme. The two subunits of the fatty acid synthetase from chicken liver have been resolved by polyacrylamide gel electrophoresis and the difference in mobilities was shown to be due to difference in net charge and not the molecular weight of the subunits (Yun and Hsu, 1972). Attempts to recover the enzyme from the gels were not successful as no enzymatic activity could be detected with either the isolated protein bands or a combination of the two bands.

In order to examine the subunit composition of the goose fatty acid synthetase purified enzyme was treated with 1% sodium dodecyl sulfate containing 0.1 M dithioerythritol, at 100 °C for 3 min. The dodecyl sulfate treated sample was then analyzed by dodecyl sulfate polyacrylamide disc gel electrophoresis. The result of a typical electrophoretic run is shown in Figure 3A. Only one major band was detected in the polyacrylamide gel upon staining with Coomassie brilliant blue. This result provided further evidence for the homogeneity of the enzyme preparation.

If the single protein band in the dodecyl sulfate polyacrylamide gel does represent two equal molecular weight subunits of the fatty acid synthetase complex, the migration of the dodecyl sulfate treated protein should be that of a 274 000 molecular weight peptide. From a linear plot of V_e/V_0 vs. the log of the subunit molecular weight of several protein standards, a molecular weight of 269 000 was calculated for the dodecyl sulfate treated fatty acid synthetase. This value is in agreement with the molecular weight of 547 000 for the native enzyme as determined by the sedimentation equilibrium method, and was similar to the values reported for the subunits of fatty acid synthetases from pigeon liver (Kumar et al., 1972), chicken liver (Yun and Hsu, 1972; Stoops et al., 1975), and rat liver (Stoops et al., 1975).

Properties of Fatty Acid Synthetase

Cofactor Requirement. The purified fatty acid synthetase required reduced pyridine nucleotide for activity and showed an absolute specificity for NADPH as previously reported for crude fatty acid synthetase of the uropygial gland of goose and for fatty acid synthetases from other animals. NADH was not oxidized by the purified enzyme, and the addition of NADH to assay mixtures containing enzyme and NADPH had no effect on the activity. The rates of NADPH oxidation catalyzed by the purified enzyme in the absence of added acetyl-CoA were only 10–20% lower than the rates obtained when acetyl-CoA was included in the reaction mixtures. The purified fatty acid synthetase preparation could contain traces of malonyl-CoA decarboxylase which might generate nearly adequate amounts of acetyl-CoA from malonyl-CoA. An extremely active malonyl-CoA decarboxylase is known to be present in this gland and since the molecular weight of the decarboxylase is about 300 000 it is eluted from the Sepharose 4B column immediately after fatty acid synthetase (Buckner and Kolattukudy, 1975a). An alternate explanation for a lack of acetyl-CoA dependency could be that the synthetase itself generates the acetyl moiety from malonyl-CoA. The purified fatty acid synthetase from pigeon liver has been shown to catalyze the synthesis of fatty acids from only malonyl-CoA and NADPH (Katiyar et al., 1974). The experimental results indicated that the malonyl moiety was decarboxylated when covalently linked to 4′-phosphopantetheine of the enzyme to give the bound acetyl moiety. In a similar manner acetyl-CoA might be generated by the goose fatty acid synthetase.

pH Dependence. The rates of NADPH oxidation were highest between pH 6.5 and 7.5, and the rates decreased sharply below pH 6.5 and above pH 7.5. This pH optimum is slightly higher than that reported earlier for the soluble supernatant from the goose uropygial gland (Buckner and Kol-

attukudy, 1975). This pH dependence is similar to that observed with the purified fatty acid synthetases obtained from other animals (Hsu et al., 1965; Burton et al., 1968; Yun and Hsu, 1972).

Effects of Substrate Concentration. Typical saturation curves were obtained for both substrates and the double reciprocal plots were linear. From these data, apparent K_m values of 3.6×10^{-5} M and 1.8×10^{-5} M were calculated for malonyl-CoA and NADPH, respectively. These K_m values are similar to those reported for the fatty acid synthetase activities of rat mammary gland (Smith and Abraham, 1970), rat adipose tissue (Martin et al., 1961), and pigeon liver (Katiyar et al., 1974).

Product Identification. In order to identify the major reaction product(s) of the fatty acid synthetase, radiochemical assays were performed. Thin-layer chromatographic analysis of labeled products resulting from [2-^{14}C]malonyl-CoA incorporation into lipids by the purified enzyme showed that all the radioactivity was located in free fatty acids. Analysis of the labeled fatty acid, as butyl esters, by radio gas–liquid chromatography revealed that the major product of the goose uropygial gland fatty acid synthetase was n-C_{16} fatty acid (>80%) with n-C_{18} fatty acid as a variable minor component (Figure 4). On the other hand, the purified fatty acid synthetase gave rise to 2,4,6,8-tetramethyldecanoic acid and 2,4,6,8-tetramethylundecanoic acid as the major products from methylmalonyl-CoA when acetyl-CoA and propionyl-CoA, respectively, were used as primers (Figure 4). Thus this synthetase presents an interesting case of specificity of the chain termination process. Presumably the thioesterase which releases the free acid from the synthetase has a high degree of preference for a 16-carbon straight chain, whereas with four methyl branches the thioesterase prefers chains of 10 or 11 carbon atoms. A direct study of the specificity of the thioesterase has yet not been conducted.

Stability. The fatty acid synthetase from the gel filtration step could be stored in 100 mM phosphate, pH 7.6, containing 1 mM dithioerythritol at a protein concentration of 2–5 mg/ml for at least 1 week at 0–4 °C without an appreciable loss of enzymatic activity. Prolonged storage of the goose uropygial gland fatty acid synthetase at protein concentration below 0.5 mg/ml or a lack of thiol-protecting reagents in the solution resulted in a rapid loss of activity. Losses in activity due to a lack of thiol protection could be reversed by the addition of freshly prepared solutions of dithioerythritol (1 mM). In comparison, the fatty acid synthetase activity from rat mammary gland was shown to decrease as a result of storage of the enzyme at 0 °C, both with and without dithiothreitol, although a more rapid decrease in activity was observed in the absence of dithiothreitol (Smith and Abraham, 1971).

The goose fatty acid synthetase activity was also affected by changes in the ionic strength of buffers used for spectrophotometric assays. The rates of NADPH oxidation by the purified enzyme decreased progressively at phosphate buffer concentrations above 10 mM, and the synthetase activity in low-ionic strength buffer (1 mM phosphate) was only about 40% of that observed with 10 mM phosphate. Increasing the ionic strength of the reaction mixture containing 1 mM phosphate buffer with 200 mM KCl resulted in the highest rate of NADPH oxidation. Additions of either 200 mM KCl to reaction mixtures containing 10–500 mM phosphate buffers or 500 mM KCl to mixtures containing 1 mM phosphate had no stimulating effect on the fatty acid synthetase activity. The inactivation of the fatty acid synthetase at low ionic strength was also demonstrated in the presence of 5 mM Tris, 35 mM glycine, pH 8.3. Although the addition of KCl to the reaction mixture increased the rate of NADPH oxidation, the enzymatic activity was only about one-half of that of reaction mixtures containing 1 mM phosphate and 200 mM KCl.

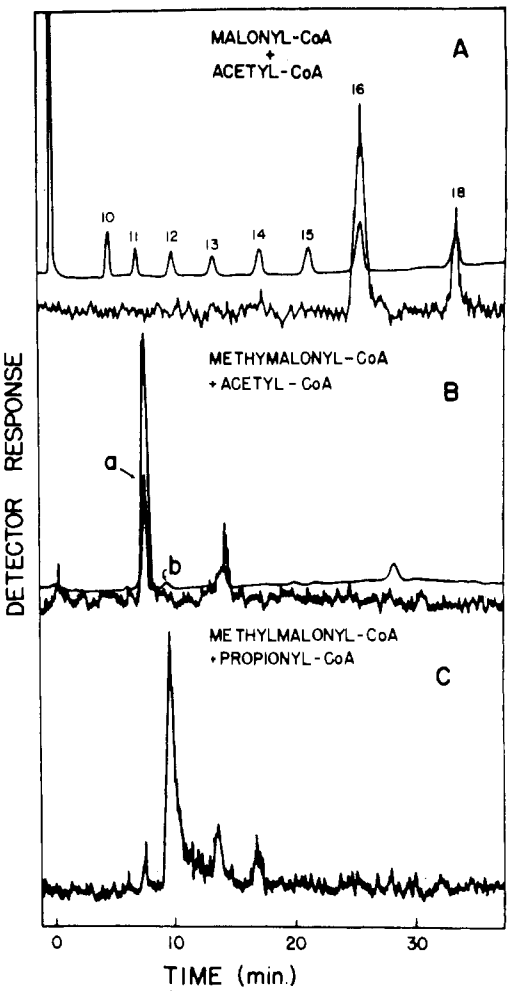

FIGURE 4: Radio gas–liquid chromatograms of fatty acids (as butyl esters) synthesized from malonyl-CA or methylmalonyl-CoA by the purified fatty acid synthetase with acetyl-CoA or propionyl-CoA as primers. In chromatogram A the top tracing is flame ionization detector response for normal fatty acid butyl ester standards (10–18 carbons) and the bottom tracing is radioactivity. In chromatogram B the top tracing is flame ionization detector response for the fatty acids (as butyl esters) from the goose uropygial gland. The letters a and b above the mass peaks denote the two major fatty acids from the gland. Reaction mixtures contained 0.26 μmol of NADPH, 3.9 μmol of glucose 6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 0.54 μmol of [methyl-^{3}H]methylmalonyl-CoA (0.63 Ci/mol) of 0.16 μmol of [2-^{14}C]malonyl-CoA (1.85 Ci/mol), 0.05 μmol of acetyl-CoA or propionyl-CoA, 0.25 μmol of dithioerythritol, and 2.8 mg of enzyme in a total of 0.5 ml of 100 mM phosphate buffer (pH 7.0). Procedures for the recovery of fatty acids, preparation of fatty acid butyl esters, an conditions for radio gas–liquid chromatography are as described in the Experimental section.

Reaction mixtures containing 10 mM phosphate and 1 mM ethylenediaminetetraacetic acid (EDTA) gave rates of NADPH oxidation which were about 25% higher than those without EDTA. On the other hand, addition of 1 mM EDTA to reaction mixtures in 100 mM phosphate did not stimulate the synthetase activity. Presumably EDTA was effective in chelating trace metal ions in the 10 mM phosphate buffer which were inhibitory to the synthetase activity. However, the 1 mM concentration of EDTA was not sufficient to remove all the inhibitory metal ions present in the 100 mM buffer.

Inactivation of the fatty acid synthetases from pigeon liver

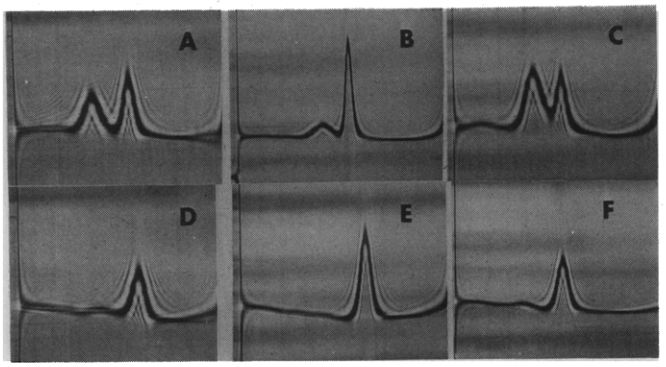

FIGURE 5: Sedimentation velocity patterns for the dissociated and reassociated fatty acid synthetase. Solutions for centrifugation included: A, fatty acid synthetase (5 mg/ml) dialyzed 24 h at 4 °C against 1 mM phosphate, 1 mM dithioerythritol, pH 8.0; B, fatty acid synthetase (7.5 mg/ml) dialyzed 24 hr at 4 °C against 0.44 mM Tris, 35 mM glycine, 1 mM dithioerythritol, pH 8.3; and C, fatty acid synthetase (5.4 mg/ml) dialyzed 48 h at 4 °C against 0.44 mM Tris, 35 mM glycine, 1 mM dithioerythritol, pH 8.3. Solutions D, E, and F were fatty acid synthetase solutions A, B, and C, respectively, incubated with 200 mM KCl for 2 h at room temperature. Solutions D, E, and F contained fatty acid synthetase at protein concentrations of 3.3, 5.0, and 3.6 mg/ml, respectively. Centrifugations were performed at 56 000 rpm at 20 °C and all schlieren patterns (A–F) were taken at approximately 32 min after attaining full rotor speed.

(Kumar et al., 1972) and chicken liver (Yun and Hsu, 1972) in low ionic strength buffers was accompanied by dissociation of the fully active enzyme into inactive subunits. Since the goose fatty acid synthetase was inactivated in 1 mM phosphate and 5 mM Tris, 35 mM glycine buffers, the extent of dissociation of the purified enzyme was examined by analytical ultrafiltration. Portions of the enzyme solution (in 100 mM phosphate, pH 7.6, 5–10 mg/ml) were dialyzed for 24 h at 4 °C against an excess of either 1 mM phosphate or 0.44 mM Tris, 35 mM glycine. This concentration of Tris was shown to be more effective than 5 mM Tris in dissociating the pigeon liver enzyme (Muesing et al., 1975). Sedimentation patterns for the enzymes dialyzed against 1 mM phosphate and 0.44 mM Tris, 35 mM glycine are shown in Figures 5A and 5B, respectively. The results showed that approximately 50% of the native 13.5S fatty acid synthetase was converted into a lower molecular weight subunit with a $s_{20,w}$ of 9.3. On the other hand, dialysis against 0.44 mM Tris, 35 mM glycine gave only 10–20% dissociation of the goose synthetase (Figure 5B). Dialysis of the enzyme against 0.44 mM Tris, 35 mM glycine for 48 h (instead of 24 h) did result in a more complete dissociation (60–70%) of the enzyme (Figure 5C).

Since the fatty acid synthetase was dissociated in low ionic strength buffer, the reassociation of the enzyme might be effected by increasing the ionic strength of the solution. To test this possibility, the enzyme dialyzed against either 1 mM phosphate or 0.44 mM Tris, 35 mM glycine (24 h and 48 h dialysis) was incubated with 200 mM KCl for 2 h at room temperature and then examined by analytical ultracentrifugation. The extent of reassociation of the dissociated fatty acid synthetase by the addition of KCl to the enzyme in 1 mM phosphate, 0.44 mM Tris + 35 mM glycine (24 h), and Tris-glycine (48 h) is shown in Figures 5D, 5E, and 5F, respectively. The reassociation was virtually complete in all cases. Reassociation of the fatty acid synthetase was also effected by incubating the dissociated enzyme with 0.2 mM NADPH. These dissociation and reassociation properties of the goose fatty acid synthetase are similar to those shown for other animal fatty acid synthetases (Kumar et al., 1972; Yun and Hsu, 1972; Muesing et al., 1975).

The fatty acid synthetase, partially dissociated by dialysis with 0.44 mM Tris, 35 mM glycine for 24 and 48 h, was assayed for enzymatic activity in Tris-glycine buffer, pH 8.3. The rate of NADPH oxidation for the synthetase sample dialyzed for 48 h was only about one-third of that of the 24-h dialyzed enzyme. Assuming that only the associated enzyme (13.5 S) is active, the observed activity difference between the 24-h and 48-h dialyzed enzyme is consistent with the relative amounts of associated enzyme as shown by the sedimentation velocity patterns (Figures 5B and 5C). Reassociation of the Tris-glycine dialyzed enzyme (both 24 h and 48 h) with 200 mM KCl and 0.2 mM NADPH also resulted in stimulations of enzymatic activity. These results strongly suggest that inactivation of the goose fatty acid synthetase at low ionic strength is due to the dissociation of the fully active fatty acid synthetase (13.5 S) to form inactive subunits (9 S). Reassociation and reactivation was effected by the addition of either KCl (200 mM) or NADPH (0.2 mM) to the dissociated enzyme.

Inactivation of the purified fatty acid synthetase was also shown by incubation of the enzyme with palmitoyl-CoA. Incubation of the enzyme with either 25 or 50 μM palmitoyl-CoA had virtually no effect on the enzymatic activity, whereas treatment with 100 μM palmitoyl-CoA resulted in 80% decrease in activity. Treatment with 200 μM palmitoyl-CoA completely inactivated the fatty acid synthetase. Inactivation of the fatty acid synthetase resulting from the incubation of palmitoyl-CoA (100 μM) with fatty acid synthetase (0.25 mg/ml) could be decreased by increasing the concentration of the synthetase. Incubation of the purified synthetase with deoxycholate (200 μM) had no effect on the enzymatic activity, suggesting that the inhibition of the fatty acid synthetase by palmitoyl-CoA was not due to a simple detergent effect. The inactivation of fatty acid synthetase by palmitoyl-CoA has been shown for the purified fatty acid synthetases from pigeon liver (Hsu et al., 1965) and from rat liver (Burton et al., 1968). In *Mycobacterium smegmatis,* the inactivation of fatty acid synthetase by palmitoyl-CoA was shown to be due to the dissociation of the enzyme into inactive subunits (Flick and Bloch, 1975).

Molecular Structure of the Fatty Acid Synthetase

Amino Acid Analysis. The purified enzyme was hydrolyzed in 6 N HCl for 24 h and the amino acid analysis of the protein hydrolysate is shown in Table I. Leucine was the most abundant amino acid, followed by glutamic acid and aspartic acid, consistent with the anionic nature of the protein. The sulfhydryl content (as cysteic acid) of the fatty acid synthetase was 105 residues per mol (547 000) of protein. This amino acid composition agrees favorably with the amino acid composition of other animal fatty acid synthetases such as those from rat mammary gland (Smith and Abraham, 1970), chicken liver (Yun and Hsu, 1972), rat liver, and pigeon liver (Burton et al., 1968). From the amino acid composition, a value of 0.73 ml/g was calculated for the partial specific volume.

Proteolysis of the Fatty Acid Synthetase. In preparation for dodecyl sulfate disc electrophoresis, the purified fatty acid synthetase was heated at 100 °C for 1 min and then dissociated with 1% dodecyl sulfate in 100 mM dithioerythritol at 100 °C for 3 min. Electrophoresis of this preparation consistently showed only one major band representing the 269 000 molecular weight subunit (Figure 3A). However, if the purified enzyme was mildly treated with 1% dodecyl sulfate in 100 mM dithioerythritol at room temperature (22–25 °C) for 12–15 h or at 50 °C for 2 h prior to electrophoresis, the results were

TABLE I: Amino Acid Composition of Fatty Acid Synthetase.[a]

Amino Acid	No. of Residues per Molecule[b]	Mol %
Asx	428	8.7
Thr	217	4.4
Ser	345	7.0
Glx	549	11.2
Pro	236	4.8
Gly	402	8.2
Ala	390	8.0
Cys	105	2.1
Val	352	7.2
Met	74	1.5
Ile	238	4.9
Leu	591	12.1
Tyr	120	2.5
Phe	159	3.2
Lys	281	5.7
His	138	2.8
Arg	213	4.3
Trp	64	1.3

[a] The values reported were determined after 24-h hydrolysis of fatty acid synthetase with 6 N HCl at 110 °C. Cysteine and methionine were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation. [b] Calculations for the number of residues per molecule were based on a molecular weight value of 547 000 for the purified fatty acid synthetase.

variable. The stained gels usually showed, in addition to the protein band at about 269 000, a number of faster moving major peptide bands. Dodecyl sulfate activated proteolysis of the fatty acid synthetase could explain the additional peptide bands which resulted from the mild heat treatment of the purified enzyme with dodecyl sulfate. The absence of low-molecular-weight fragments in samples heated for 1 min at 100 °C prior to the dodecyl sulfate treatment is consistent with this notion. The purified fatty acid synthetase from chicken liver has recently been shown to undergo dodecyl sulfate activated proteolysis (Stoops et al., 1975). The extent of proteolysis was dependent on protein concentration and incubation time in the presence of dodecyl sulfate.

In order to optimize the conditions for the suspected proteolytic activity, portions of the purified synthetase were treated with dodecyl sulfate for different periods of time at various temperatures and the incubation mixtures were analyzed by dodecyl sulfate polyacrylamide disc gel electrophoresis. The greatest extent of proteolysis of the fatty acid synthetase subunits occurred with a 3-h incubation time at 40 °C. A typical pattern resulting from such an incubation condition is shown in Figure 3C. The proteolysis of the fatty acid synthetase subunits was extensive as indicated by the weak band at 269 000 and the presence of the several major peptides smaller than 269 000. That proteolysis occurred during the dodecyl sulfate treatment was further demonstrated by the observation that treatment of the synthetase with diisopropyl fluorophosphate and *p*-chloromercuribenzoate prior to dodecyl sulfate treatment prevented formation of multiple bands (Figure 3B). Therefore, these results from dodecyl sulfate electrophoresis strongly suggest that the fatty acid synthetase from the goose uropygial gland, with a molecular weight value of approximately 547 000, consists of two equal molecular weight peptides (269 000). The formation of any smaller molecular weight peptides most probably results from dodecyl sulfate activated proteolysis.

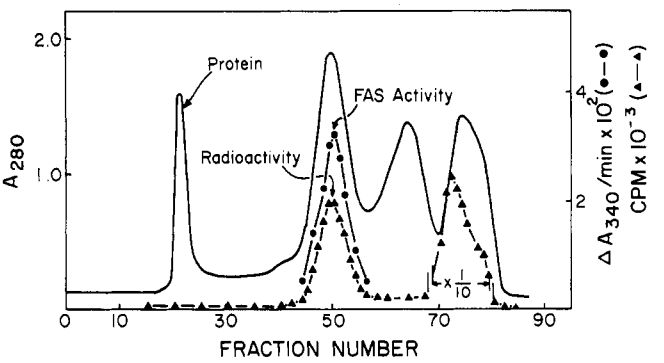

FIGURE 6: Sepharose 4B chromatography of the 105 000*g* supernatant prepared from the uropygial gland, 48 h after injection of [G-^{3}H]pantothenic acid into the gland. Synthetase activity was determined spectrophotometrically as described in the experimental section; 0.5 ml of each fraction was assayed for radioactivity in 15 ml Aquasol by liquid scintillation spectrometry.

Incorporation of [G-^{3}H]Pantothenic Acid into Fatty Acid Synthetase. The possibility that animal fatty acid synthetases contain acyl carrier protein (ACP) has been suggested by the presence of the prosthetic group 4′-phosphopantetheine in the fatty acid synthetase of several animal systems (Chesterton et al., 1968; Smith and Abraham, 1970; Stoops et al., 1975). The 4′-phosphopantotheine in the fatty acid synthetase from pigeon liver was reported to be associated with one of two equal molecular weight subunits (Lornitzo et al., 1974). A small molecular weight protein containing 4′-phosphopantetheine has been isolated from dog liver (Roncari, 1974) and pigeon liver (Qureshi et al., 1974) fatty acid synthetases. In order to determine if the fatty acid synthetase of goose uropygial gland contained such an ACP, experiments were conducted with [G-^{3}H]pantothenic acid.

About 48 h after injecting the labeled pantothenic acid directly into the gland, the high-speed supernatant prepared from the gland was fractionated by gel filtration on a Sepharose 4B column (Figure 6). A major peak of radioactivity coincided precisely with both the protein peak for fatty acid synthetase and the enzymatic activity. Although a much larger portion of the radioactivity was located in fractions eluted much later than the synthetase, dialysis of these pooled fractions resulted in nearly complete loss of the radioactivity, suggesting that the bulk of the label in these fractions was not bound to protein, but most probably constituted free pantothenic acid and small-molecular-weight derivatives. Thus fatty acid synthetase was the only labeled protein derived from pantothenic acid. If the [G-^{3}H]pantothenic acid was incorporated into the fatty acid synthetase, as 4′-phosphopantotheine, then the binding of this prosthetic group to the protein should be by a phosphodiester linkage to a hydroxy amino acid, presumably serine (Vagelos et al., 1966). To test this possibility, the labeled fatty acid synthetase was incubated with NaOH at pH 12 for 20 min at 100 °C. Following the alkaline hydrolysis, protein solutions were dialyzed overnight against buffer. Dialysis of the alkali-treated labeled synthetase resulted in the removal of most (80–85%) of the radioactivity. These results strongly suggest the presence of 4′-phosphopantotheine in the goose fatty acid synthetase.

In order to determine whether the labeled pantothenic acid was associated with the fatty acid synthetase subunits or with a small-molecular-weight protein (ACP), the labeled enzyme was heat treated at 100 °C for 1 min and dissociated with 1% sodium dodecyl sulfate in 100 mM dithioerythritol at 100 °C for 3 min. The dissociated protein was subjected to dodecyl

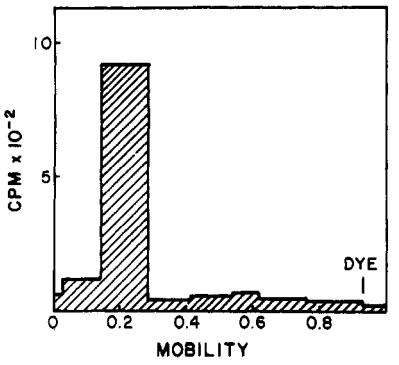

FIGURE 7: Distribution of radioactivity in dodecyl sulfate polyacrylamide gels. Approximately 0.1 mg of labeled fatty acid synthetase (1400 cpm/mg) was applied to each of 12 polyacrylamide gels. After electrophoresis and staining of the gels for 1 h in Coomassie blue the stained gels were cut into approximately 10-mm pieces and assayed for radioactivity as described in the Experimental Section.

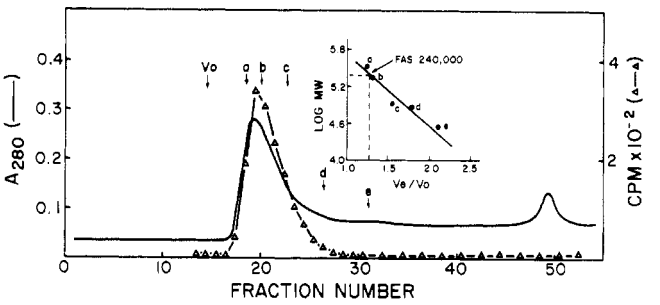

FIGURE 8: Gel filtration of dodecyl sulfate treated fatty acid synthetase containing labeled pantothenic acid. A Sepharose 6B column (1.8 × 58 cm) was equilibrated with 0.1% dodecyl sulfate in 20 mM Tris-HCl buffer, pH 7.6, containing 5 mM dithioerythritol. A portion of each fraction (0.5 ml) was assayed for radioactivity as described in Figure 6. The following protein standards were used for molecular weight determination: a, thyroglobulin (335 000); b, myosin (21000); c, urease (83 000); d, bovine serum albumin (6000); e, pepsin (35 000).

sulfate polyacrylamide disc gel electrophoresis and the stained gels showed essentially the same electrophoretic pattern as the dodecyl sulfate treated unlabeled fatty acid synthetase (see Figure 3A). The stained gels were assayed for radioactivity, and the results are shown in Figure 7. The radioactivity was associated exclusively with the 269 000 molecular weight subunits of the synthetase, and no ^3H was found in any small-molecular-weight ($\simeq$10,000–20,000) proteins. These results show that 4′-phosphopantotheine is covalently linked with one or both of the subunits (269 000) of the goose fatty acid synthetase and not with what might be termed ACP.

The effect of dodecyl sulfate on the labeled goose fatty acid synthetase was also analyzed by gel filtration chromatography with Sepharose 6B. The elution profile (Figure 8) showed only one major protein peak near the void volume of the column. Column fractions were assayed for radioactivity and the distribution of label resulted in a peak that coincided exactly with the protein peak for the fatty acid synthetase subunits. Fractions that would represent lower-molecular-weight peptides contained no radioactivity. In addition to the fatty acid synthetase, several dodecyl sulfate treated protein standards were filtered through the Sepharose 6B column. From a linear plot of V_e/V_0 vs. log molecular weight, a molecular weight value of 240 000 was determined for the subunit of the fatty acid synthetase (Figure 8). This value is slightly lower than the subunit molecular weight value (269 000) as determined by dodecyl sulfate polyacrylamide gel electrophoresis. In any case, all of the ^3H was associated with this large peptide, but not with any small-molecular-weight protein which might be referred to as ACP in the classical sense.

As described earlier in this section, the dissociation of the labeled goose fatty acid synthetase resulted in two equal molecular weight polypeptides as determined by dodecyl sulfate disc electrophoresis. Although 4′-phosphopantetheine was shown to be covalently bound to those large peptide(s) these experiments did not provide evidence at to whether the 4′-phosphopantetheine was associated with just one or both of the polypeptides. In order to determine whether each peptide contained 4′-phosphopantetheine, the purified protein was hydrolyzed after treatment with performic acid and the product was analyzed for taurine and β-alanine with an amino acid analyzer. The results showed that the fatty acid synthetase contained 2.07 ± 0.20 moles of taurine per mole of fatty acid synthetase (based on a molecular weight value of 547 000). Samples analyzed for taurine and β-alanine contained a ninhydrin-reacting material that was not resolved from β-alanine and, therefore, the content of β-alanine in the fatty acid synthetase could not be determined quantitatively. Since taurine was clearly resolved from other components, reproducible values were obtained. On the basis of the amount of taurine obtained from the fatty acid synthetase, it must be concluded either that one of the peptides contained two residues of 4′-phosphopantetheine per mol or that each peptide contained 1 mol of 4′-phosphopantetheine per mole; the latter possibility is the more likely one. These results differ from those obtained with the rat mammary gland fatty acid synthetase in that only 1 mol of taurine and 1 mol of β-alanine per mole of protein could be found with this enzyme (Smith and Abraham, 1970). Also, in pigeon liver fatty acid synthetase, 4′-phosphopantetheine was found in only one of the two equal molecular weight subunits (Lornitzo et al., 1974).

The experimental evidence summarized in this paper strongly suggests that the goose fatty acid synthetase consists of two equal molecular weight polypeptides, each containing covalently linked 4′-phosphopantetheine. A similar molecular architecture was recently proposed for the fatty acid synthetase of chicken liver (Stoops et al., 1975), although it was not clear whether this enzyme contained 1 or 2 mol of phosphopantetheine per mol (500 000) of the synthetase. These findings are contrary to the proposals that animal and yeast fatty acid synthetases are multienzyme complexes tightly bound by noncovalent interactions (Yang et al., 1967; Willecke et al., 1969). Consistent with the concept of a multienzyme complex was the experimental evidence showing the dissociation of animal fatty acid synthetase into several proteins smaller than the two subunits of the synthetase (250 000), and the isolation of a small-molecular-weight protein (ACP) containing 4′-phosphopantetheine (Yang et al., 1967; Qureshi et al., 1974; Roncari, 1974). Stoops et al. (1975) have demonstrated the occurrence of sodium dodecyl sulfate activated proteolysis in the fatty acid synthetase of chicken liver and have suggested that the presence of protein fragments of low molecular weight (<200,000), including the ACP, previously isolated by others, could be due to exposure of fatty acid synthetase to proteolysis during experimental procedures. Our results showed the occurrence of proteolysis in goose fatty acid synthetase preparations upon denaturation with dodecyl sulfate and this proteolysis was prevented by either heat treatment or exposure to inhibitors of proteolytic enzymes.

It is not known if and how both of the 4′-phosphopantetheine residues of each mole of fatty acid synthetase (assuming that the 540 000 moiety is the functional unit) participate in fatty

acid synthesis. It appears probable that each of the two peptides contain multiple active sites and the active sites of both of these peptides participate in a sequential manner in the calaysis of the various steps involved in fatty acid synthesis. Our present interpretation is that the growing aliphatic chain is bound to the 4′-phosphopantetheine of the first peptide during the steps catalyzed by that peptide and it is transferred to the 4′-phosphopantetheine of the second peptide which then catalyzes the remaining steps of that cycle. It is also quite possible that each peptide constitutes a complete enzyme, capable of catalyzing all of the reactions involved in fatty acid synthesis. If such is the case, the requirement of dimerization for activity represents only a regulatory feature.

The results presented in this paper show that normal fatty acids and multibranched fatty acids are synthesized from different precursors by the same enzyme system. This is the first report on the isolation of a fatty acid synthetase the major function of which is synthesis of multibranched fatty acids. The structure and properties of this enzyme, however, are very similar to those of other fatty acid synthetases. The presence of the large quantity of this enzyme in the gland probably reflects the relatively low capacity of the enzyme to synthesize multibranched acids which are the major products of the gland. It is noteworthy that the dramatic difference between the structure of the fatty acids of this sebaceous gland and those of the other organs is not due to the specificity of the synthetase. It is the presence of an ancillary enzyme, namely malonyl-CoA decarboxylase, which regulates the concentration of the precursors and thus bring about the production of multibranched fatty acids (Buckner and Kolattukudy, 1975a). It is probable that the uniqueness of the sebaceous gland lipids of animals is the result of the occurrence of similar ancillary enzymes.

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