The Purification and Properties of the Fatty Acid Synthetase of Pigeon Liver*

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The fatty acid synthetase of pigeon liver catalyzes the conversion of acetyl- and malonyl-coenzyme A to long chain saturated fatty acids in the presence of reduced nicotinamide adenine dinucleotide phosphate. The major product of the reaction is palmitic acid, but small amounts of other long chain fatty acids are also formed.

The over-all reaction for the synthesis of palmitic acid is: 1 acetyl-CoA + 7 malonyl-CoA + 14 NADPH + 14 H+ → 1 palmitic acid + 14 NADP+ + 7 CO2 + 8 CoA + 6 H2O (1).

The purified fatty acid synthetase effects the formation of the fatty acid synthetase system was not established, however.

In the current work, the fatty acid synthetase was purified to an activity of 1.0 µmole of NADPH oxidized per min per mg of protein. Enzyme of this activity system was not established, however.

In the current work, the fatty acid synthetase was purified to an activity of 0.3 µmole of NADPH oxidized per min per mg of protein. Enzyme of this activity behaves as a single homogeneous protein on sedimentation, on moving boundary electrophoresis, and on chromatography on diethylaminoethyl cellulose. This protein has a molecular weight of 5.4 × 10^5 g per mole. It does not contain flavin, but it does contain at least 50 sulfhydryl groups.

The purified fatty acid synthetase effects the formation of acetyl- and malonyl-enzyme from acetyl- and malonyl-CoA, respectively. It also effects the acetyl-CoA-dependent decarboxylation of malonyl-CoA.

The synthesis of fatty acids by the purified fatty acid synthetase is inhibited by low concentrations of palmityl-CoA. The mechanism and the significance of this inhibition is not known. It is possible, however, that this compound is important in the control of fatty acid synthesis within the avian liver cell.

EXPERIMENTAL PROCEDURE

Chemicals—2-14C-Malic acid and 1-14C-acetic anhydride were obtained from the New England Nuclear Corporation, and 1,3-14C-malonic acid and 3-14C-HMG were purchased from Isotope Specialties Corporation and Tracerlab, Inc., respectively. 2-14C-DL-Mevalonic acid lactone was obtained from the Volk Radiochemical Company. Coenzyme A, NAD, NADH, NADPH, FMN, FAD, and crystalline liver L-glutamic dehydrogenase were supplied by the Sigma Chemical Company, and 2-mercaptoethanol was obtained from Calbiochem. Enzyme grade ammonium sulfate was a product of Nutritional Biochemical Corporation. DEAE-cellulose (Mannex-DEAE), unlabelled HMG, and mevalonic acid were purchased from Mann Research Laboratories, and Sephadex G-25 and G-100 were obtained from the Pharmacia Company.

Other Reagents—Traces of heavy metals were removed from the distilled water used in these investigations by passage through a mixed bed ion exchange resin. All of the phosphate buffers (except the molar potassium phosphate buffer, pH 7.0, used for assays) were 1 mM in respect to EDTA and 2-mercaptoethanol. The saturated ammonium sulfate solution contained 3 mM EDTA.1 mM 2-mercaptoethanol, and it was neutralized to pH 7.0 with either potassium hydroxide or ammonium hydroxide before use.

Synthesis, Purification, and Quantitation of Coenzyme A Esters—1-14C-Acetyl-CoA and unlabeled acetyl-CoA were prepared from the corresponding anhydrides by the method of Simon and Shemin (7), and 1,3-14C-malonyl-CoA, 2-14C-malonyl-CoA, and nonradioactive malonyl-CoA were synthesized by the method of Trams and Brady (8). 3-14C-HMG-CoA and unlabeled HMG-CoA were prepared from the corresponding acids via acylation of the intramolecular anhydride (9). Nonradioactive palmityl-CoA was prepared from palmitylchloride by the method of Seubert (10). Each of the above CoA esters, except palmityl-

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CoA, was purified by chromatography in an ammonia-isobutyric acid paper chromatographic system (11).

Dilution of Enzyme Solutions—Protein determinations were made on all enzyme preparations by the biuret method of Gornall, Bardwell, and David (12). All enzyme solutions were diluted before assay with 0.25 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 2-mercaptoethanol, unless otherwise specified.

Assay for Fatty Acid Synthetase Activity—Potassium phosphate buffer, pH 7.0, 100 μmoles; 2-mercaptoethanol, 5 μmoles; EDTA, pH 7.0, 3 μmoles; malonyl-CoA, 98 μmoles; 14C-acetyl-CoA, 12.3 μmoles and 24,700 cpm; NADPH, 0.3 μ mole; and enzyme and water to make a final volume of 1.0 ml were added to a ground-glass stoppered extraction tube (1.5 x 15 cm). Enzyme was added to start the reaction. Incubations were made for 6 min at 38°, and the reaction was stopped by the addition of 0.03 ml of 60% perchloric acid. The incubation mixture was then diluted with 1 volume of absolute ethanol, and long chain fatty acids were extracted with petroleum ether and assayed for radioactivity as reported previously (3).

Units of Enzyme Activity—A unit of enzyme activity is defined as the amount of enzyme required to catalyze the incorporation of 1 μmole of 14C-acetyl-CoA into fatty acid (i.e. equivalent to the formation of 1 μmole of palmitic acid) per min under the conditions of the standard assay described above. Specific activity of the enzyme is expressed as units of enzyme activity per mg of protein.

Preparation of Pigeon Liver Supernatant Solution—Pigeon liver supernatant solution was prepared according to the method of Wakil, Porter, and Gibson (13), except that the homogenizing buffer was made to pH 8.0, and the 100,000 g centrifugation step was extended to 45 min. Usually 10 dozen pigeons were killed for each preparation. The resulting supernatant solution was sealed under N2 in 40-ml portions in plastic tubes, and stored at -15°C. This preparation is stable for 1 or 2 months under the above conditions.

Purification of Fatty Acid Synthetase—All purification steps reported in the following paragraphs were carried out at room temperature in an air conditioned laboratory. The purification method was similar to that described by Bressler and Wakil (1), but it was extensively modified in detail, and other steps were added.

First Ammonium Sulfate Fractionation—Two tubes of pigeon liver supernatant solution (80 ml) were thawed, warmed to room temperature, and stirred gently with a magnetic stirrer under a stream of N2. Saturated ammonium sulfate was added slowly to 25% saturation. Stirring was continued for 15 min, and the suspension was centrifuged at 18,000 g for 10 min. The precipitate was discarded. The supernatant liquid was brought to 40% saturation with saturated ammonium sulfate, and centrifuged as before. The precipitate was dissolved in 0.005 M potassium phosphate buffer, pH 7.0, to a volume of 40 ml (R1). The R1 fraction contains most of the original fatty acid-synthesizing activity, and it is stable for 1 to 2 hours.

Calcium Phosphate Gel Adsorption—R1 enzyme (40 ml) was diluted with 360 ml of 0.005 M potassium phosphate buffer, pH 7.0. An amount of calcium phosphate gel (prepared by the procedure of Keilin and Hartree (14)) equal to half of the weight of protein was added and mixed with the solution with a glass rod. The suspension was immediately centrifuged for 3 min at 4000 g. The gel precipitate was washed with 10 ml of 0.05 M potassium phosphate buffer, pH 7.0, and the supernatant solutions were combined (R2). Since the enzyme is unstable at low ionic strength, the gel adsorption and the subsequent DEAE-cellulose chromatography were carried out without delay.

DEAE-cellulose Chromatography—Two columns of DEAE-cellulose (10.3 x 3.5 cm) were made with Mannex-DEAE previously equilibrated with 0.05 M potassium phosphate buffer, pH 7.0. R2 enzyme was divided into two 200-ml portions, and each portion was added to a column. The major portion of the protein was eluted with 0.05 M potassium phosphate buffer, pH 7.0. Elution of the protein was continued until the optical density at 280 μm was below 0.2. Usually this occurred at a total elution volume of less than 500 ml. Further elution was carried out with 0.25 M potassium phosphate buffer, pH 7.0. The protein peak containing active enzyme usually appeared at 60 ml of elution volume, and 30 to 40 ml of this peak was collected (DEAE Fraction II). The latter fraction had a protein concentration of about 3 mg per ml and was stable for several hours.

Second Ammonium Sulfate Fractionation—Saturated ammonium sulfate was slowly added to DEAE Fraction II, with stirring under N2, to 25% saturation. The suspension was centrifuged as before, and the precipitate was discarded. The supernatant solution was brought to 32% saturation with saturated ammonium sulfate, stirred, and centrifuged. The precipitate contained the active enzyme (II AS). Enzyme II AS was dissolved in about 1 ml of 0.8 M potassium phosphate buffer, pH 7.0. It was stable overnight if stored at -15°C. If Sephadex G-100 chromatography was made the same day, Enzyme II AS was usually taken up in a minimum volume of 0.2 M potassium phosphate buffer, pH 7.0, prior to this step.

Sephadex G-100 Chromatography—A column of Sephadex G-100 (22.0 x 1.0 cm) (bed volume, 18.0 ml) was made, and then it was equilibrated for several days (15) with 0.25 M potassium phosphate buffer, pH 7.0. A flow rate of 15 ml per hour was maintained by restricting the hydrostatic head to less than 10 cm. Enzyme II AS, in a volume of not more than 1.0 ml, was added and eluted with 0.25 M potassium phosphate buffer, pH 7.0. Fractions, 0.5 ml, were collected. The enzyme was retained slightly. It had a hold up volume of 6.0 to 6.2 ml (0.15 to 0.35 ml larger than the void volume of 5.85 ml obtained with crystalline L-glutamate dehydrogenase, mol. wt. 1 million). A single protein peak of high specific activity for fatty acid synthesis was obtained. Usually the 11th to 13th 0.5 ml fractions were pooled and used as "purified enzyme" for the following studies. The entire operation of purification of the fatty acid synthetase, starting with pigeon liver supernatant solution, was accomplished in about 6 hours, and the purity of the final product was very reproducible.

Assay for Properties and Components of Purified Fatty Acid Synthetase—Sedimentation studies were made in a Spinco model E ultracentrifuge on freshly prepared enzyme, and moving boundary electrophoresis analysis was carried out in a Spinco model H instrument. The purity of the enzyme was also assayed by gradient elution from DEAE-cellulose and by starch-gel electrophoresis. Prior to these assays, enzyme was passed through Sephadex G-100 equilibrated with 1 mM EDTA and dithiothreitol in 0.25 M potassium phosphate buffer, pH 7.0.
Further conditions of the above assays are detailed in later sections of the paper.

The amino acid composition of the purified protein was determined on an acid hydrolysate with the Spinco model 120 amino acid analyzer. The number of sulfhydryl groups per mole of the purified enzyme was determined by the method of Ellman (16), with 5,5'-dithiobis(2-nitrobenzoic acid) as the chromophore. The flavin content of the enzyme was determined spectrophotometrically at 450 μm. A molar extinction coefficient of 1.03 × 10³ was used. In this assay, 50% trichloroacetic acid was added to a 0.2-ml aliquot of purified enzyme containing 0.5 mg of protein. The suspension was centrifuged, and the spectrum of the supernatant solution was determined in the visible range before and after the addition of small amounts of sodium hydroxide.

**Binding of Acetyl and Malonyl Moieties to Enzyme**—Potassium phosphate buffer (1 m), pH 7.0, 0.05 ml, was preincubated at 38° with acetyl- or malonyl-CoA, and then it was mixed and incubated at 38° with a prewarmed aliquot of enzyme solution. At specified time intervals, a portion of this mixture was pipetted into 0.2 ml of 60% perchloric acid, and centrifuged. The precipitate was washed three times with 5-ml portions of 0.2 N acetic acid. The residue was dissolved in 1 N NaOH and made to a final volume of 1.0 ml. Aliquots were taken for determinations of protein and radioactivity.

**Assay for Condensation-Decarboxylation Reaction**—The acetyl-CoA-dependent decarboxylation of malonyl-CoA was determined by a modification of the method of Bressler and Wakil (1). Incubations were made in 10-ml Erlenmeyer flasks fitted with injection-type rubber stoppers. A center well, containing 0.3 ml of 1 N KOH, made from glass tubing sealed at one end, was supported by a piece of wire wound spirally around one end of the well and pierced through the rubber stopper at the other end. A reaction mixture containing potassium phosphate buffer, pH 7.0, 100 μmoles; 2-mercaptoethanol, 5 μmoles; EDTA pH 7.0, 3 μmoles; acetyl-CoA, 102 mmoles; 1,3-14C-malonyl-CoA, 98 mmoles and 98,200 cpm; enzyme and water to a final volume of 1.0 ml was incubated at 38° for 10 min. Blanks contained no acetyl CoA. The reaction was started by the addition of enzyme, and it was stopped by the addition of 0.2 ml of 4 N HCl. Each solution was added with a syringe through the rubber stopper. After the addition of acid, the flasks were shaken for an hour on a Fisher clinical rotator. The entire contents of the center well were assayed for radioactivity in dioxane-phosphor in a liquid scintillation spectrometer.

**Assay for Synthesis of HMG-CoA and 3,5-Diketoheaxanoic Acid**—A methanol extract of a lyophilized incubation mixture was chromatographed on Whatman No. 3 MM paper in an ammonium-isobutyric acid system (11). HMG-CoA was identified by coincidence in Rf value with that of an authentic sample of HMG-CoA chromatographed on the same paper. 3,5 Diketoheaxanoic acid was identified by agreement in Rf value (0.86) with the value reported earlier from this laboratory (3). Tracings of radioactivity on chromatograms were made with a Nuclear-Chicago chromatographic strip counter.

**Assay for Formation of Mevalonic Acid**—Each incubation tube contained potassium phosphate buffer, pH 7.0, 50 μmoles; 2-14C-malonyl-CoA, 141,000 cpm and 56 mmoles; acetyl-CoA, 56 mmoles; NADPH, 0.2 μmole; purified fatty acid synthetase, 1.05 mg of protein; and water to make a final volume of 1.0 ml. The tubes were incubated at 38° for 3 hours under an atmosphere of nitrogen. At the end of the incubation period, the contents of three identical tubes were combined, and 10 μmoles of mevalonic acid were added as carrier. The combined incubation mixture was acidified, extracted with ethyl ether, and then the extract was chromatographed as described previously (6). Fractions of 0 to 25 ml, 25 to 50 ml, and 50 to 90 ml from the Celite column were each evaporated to a small volume. Each sample was chromatographed on a butanediol succinate column in a Barber-Colman gas-liquid chromatograph. The emerging peaks were trapped and assayed for radioactivity. 2-14C- and unlabeled mevalonic acid lactone were used as controls.

**Determination of Radioactivity** The radioactivity of all of the compounds was determined with a liquid scintillation spectrometer as reported previously by Brodie, Wesson, and Porter (3).

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**RESULTS**

**Assay for Fatty Acid Synthetase Activity**—The dependence of fatty acid formation on time and protein concentration is shown in Fig. 1. A linear response to time and protein concentration was obtained.

**Purification and Stability of Enzyme**—The preparation of pigeon liver supernatant solution is shown in Table I, and the results of the subsequent purification of the fatty acid synthetase of this preparation are shown in Table II. The over-all purification is about 70-fold, with a yield of about 10% in enzyme activity.

Early experiments on the purification of this enzyme were performed at 4°, but later this practice was abandoned when it
was found that the enzyme was not inactivated when held at room temperature during purification. The stability of the enzyme was markedly reduced, however, at low salt concentrations (Fig. 2). At a concentration of 0.005 M potassium phosphate and 0%, a condition used for the dialysis of this enzyme in earlier studies, 50% of the activity was lost in 2 hours. In the present purification method, the dialysis step is eliminated. The low salt concentration required for gel adsorption and DEAE-cellulose chromatography is achieved by rapid dilution of the enzyme solution with buffer of low ionic strength.

The enzyme is susceptible to heavy metal inactivation, as shown by the fact that synthetase activity was always increased when 3 μmoles of EDTA were added to the standard assay. The stimulation by EDTA was as high as 200% over that of a control with ordinary distilled water. 2-Mercaptoethanol was also found to be essential for the protection of enzymatic activity during purification. This compound was also essential for maximum activity of the enzyme during assay.

The Enzyme II AS (50 to 60 mg) can be dissolved in about 1 ml of ice-cold 0.8 M phosphate buffer. However, when the solution was allowed to warm to room temperature, some of the active enzyme precipitated, thus indicating a marked decrease in solubility of the protein with an increase in temperature.

The enzyme appeared, at later stages of purification, to be susceptible to surface denaturation. If the side of the glass tube containing the enzyme solution was scratched with a glass rod, pieces of thread-like particles (denatured protein) formed immediately on or near the glass rod.

Properties and Components of Purified Fatty Acid Synthetase—The pigeon liver supernatant solution is stable over 1 to 2 months of storage at -15°, but more purified preparations of the fatty acid synthetase are not stable for more than 1 or 2 days under any of the methods of storage that have been tried.3 A high salt concentration (0.8 M potassium phosphate buffer, pH 7.0, or 20% saturated ammonium sulfate) stabilized the enzyme for the longest period of time. Enzyme II AS was stable overnight when dissolved in a minimum volume of 0.8 M potassium phosphate buffer, pH 7.0, and stored at -15°. However, only 50% of the activity remained after a week's storage. Other experiments have shown that the enzyme is stable to freezing and thawing.

Properties and Components of Purified Fatty Acid Synthetase—

The purified fatty acid synthetase sediments as a single homogeneous protein in the Spinco model E ultracentrifuge in a 0.25 M phosphate buffer, pH 7.0, containing 1 mM EDTA and 1 mM 2-mercaptoethanol (Fig. 3). The sedimentation coefficient is 12.0 (uncorrected). Based on a determination of the ratio of sedimentation and diffusion coefficients at menisci by Archibald's approach to equilibrium method (17), the molecular weight is estimated as 5.4 x 10^9 g per mole.4 When freshly prepared enzyme is allowed to stand for 1 day in 0.25 M phosphate buffer, pH 7.0, containing 1 mM EDTA and 1 mM 2-mercaptoethanol, a new component appears which sediments at a slower rate. The activity of this aged enzyme preparation for fatty acid synthesis declines during the 24-hour aging period.

Homogeneity of the purified enzyme was also demonstrated by moving boundary electrophoresis in a potassium phosphate buffer at 0.14 ionic strength, pH 7.0, with a Spinco model II instrument. A single peak was obtained in the descending and ascending boundaries (Fig. 4). The electrophoretic mobility was found to be -3.12 x 10^-6 cm²/sec per volt, indicating that the protein is negatively charged at pH 7.0.

When the fatty acid synthetase was subjected to chromato-
FIG. 3. Sedimentation patterns of freshly prepared fatty acid synthetase. Measurements were made at 20° in a medium that was 0.25 M in phosphate, pH 7.0, 1 mM in 2-mercaptoethanol, and 1 mM in EDTA. The centrifugal speed was 32,640 rpm and the interval between photographs was 8 min. The protein concentration was 4 mg per ml. Sedimentation proceeds from right to left.

Log \( k = \frac{\Delta H_a}{2.303R T} + \log s \)

where \( \Delta H_a \) is the heat of activation for denaturation, \( R \) is the gas constant, and \( s \) is a constant. The heat of activation for denaturation of this protein was calculated from the slope of the line. A value of \( 1.6 \times 10^5 \) calories per mole was obtained.

The purified enzyme was assayed for acid dissociable flavin and found to contain no more than 0.012 mole of flavin per mole of protein. The addition of sodium hydrosulfite also had no effect on the visible spectrum. The acid-precipitated protein was colorless, and therefore, it could not be expected to contain more than traces of flavin as impurities. In addition, the enzyme gave no observable light absorption in the visible spectrum, thus indicating the absence of heme compounds.

The ultraviolet light absorption spectrum of the purified enzyme is shown in Fig. 8. The protein has an absorption maximum at 279 nm and a molar extinction coefficient of \( 4.64 \times 10^5 \). A small shoulder at 290 nm is also evident. The protein has no absorption peak at 260 nm, thus indicating the absence of nucleotides and nucleic acids.

The amino acid composition of an acid hydrolysate of the purified fatty acid synthetase is reported in Table IV. The enzyme has an excess of acidic amino acids as expected from its electrophoretic behavior. The tryptophan content of this protein was not determined since this compound is destroyed by acid hydrolysis.

Characteristics of Fatty Acid Synthetase Reaction—The dependence of fatty acid synthetase activity upon pH is reported in Fig. 9. The pH optimum was found to be 6.7. An identical set of assays (not shown) with double reaction time demonstrated the linearity of the reaction with time at all pH values used in this study, thus indicating that no time-dependent enzyme inactivation occurred during the reaction period.

The activity of Enzyme II AS for NADPH oxidation and for
FIG. 5. Gradient elution of fatty acid synthetase from a DEAE-cellulose column. Fatty acid synthetase (20 mg of protein), prepared as reported under "Experimental Procedure," was diluted to a phosphate buffer concentration of 0.05 M with deionized water containing 1 mM EDTA and 1 mM dithiothreitol, and then it was adsorbed on a Mannex-DEAE-cellulose column (1.4 X 10 cm). The column was previously equilibrated with 0.05 M potassium phosphate buffer containing 1 mM EDTA and 1 mM dithiothreitol. The column was then subjected to a concentration gradient of 0.05 M to 0.3 M phosphate buffer. The buffer solutions were 1 mM in respect to EDTA and dithiothreitol. A total of 120 fractions (1.5 ml each) was collected. These fractions were assayed for total protein (calculated from $E_{279}$ and a molar extinction value of $4.64 \times 10^5$) and for total units of fatty acid synthetase activity.

FIG. 6. The heat stability of fatty acid synthetase. Aliquots of 0.05 ml each of purified enzyme, containing 1 mg of protein in 0.25 M potassium phosphate buffer, pH 7.0, were heated in test tubes (1.5 X 12.5 cm) at the indicated temperatures for 2 min, cooled, and assayed for fatty acid synthetase activity by the standard method.

**TABLE III**
First order rate constant and half-time for heat denaturation of fatty acid synthetase

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Temperature (absolute)</th>
<th>$k$</th>
<th>$t$</th>
</tr>
</thead>
<tbody>
<tr>
<td>50$^\circ$</td>
<td>323$^\circ$</td>
<td>$10^4$</td>
<td>37.6</td>
</tr>
<tr>
<td>52</td>
<td>325</td>
<td>0.307</td>
<td>7.5</td>
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<tr>
<td>54</td>
<td>327</td>
<td>1.54</td>
<td>1.5</td>
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<tr>
<td>56</td>
<td>329</td>
<td>7.84</td>
<td>0.3</td>
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FIG. 7. Graph of $\log k$ against $1/T \times 10^3$ for the heat denaturation of the fatty acid synthetase of pigeon liver.

The purified pigeon liver fatty acid synthetase is quite specific for NADPH. The substitution of NADH (0.3 mM) as the hydrogen donor in the standard assay for fatty acid synthesis resulted in the incorporation of only one-seventh to one-eighth as much $1-{^{14}}C$-acetyl-CoA into fatty acids as obtained when NADPH was the hydrogen donor.

The effect of CoA, palmitic acid, and palmityl-CoA on fatty acid synthesis by the purified enzyme is reported in Table V.
malonyl-moieties were bound per mg of enzyme. In addition, comparable losses of acetate and malonate from protein with time were observed. There was no decline, however, in the binding of acetyl- and malonyl-moieties to aged enzyme even though the enzyme had lost most of its activity for fatty acid synthesis. The deacylation of acetate and malonate from the aged protein was markedly reduced.

The inhibition of acetate and malonate binding by sodium arsenite is shown in Table VI. Inhibition of 40 to 50% was obtained in the binding of each of these substrates when 6 mg of enzyme were preincubated with 4 μmoles of sodium arsenite and 0.5 μmole of β-mercaptoethanol in a volume of 0.6 ml.

Condensation-Decarboxylation Reaction—The purified fatty acid synthetase catalyzes an acetyl-CoA-dependent decarboxylation of malonyl-CoA. The release of CO₂ from substrate was time dependent, as demonstrated by Bressler and Wakil (1). A rate of 7.6 μmoles of CO₂ released per min per mg of protein was obtained under the standard conditions of assay.

Synthesis of HMG-CoA and 3,5-Diketohexanoic Acid by Purified Enzyme—The purified pigeon liver enzyme forms small amounts of HMG-CoA and 3,5-diketohexanoic acid from acetyl- and malonyl-CoA (Fig. 11).

Formation of Mevalonic Acid by Purified Enzyme—Gas-liquid chromatographic analysis of the fractions from 0 to 25, 25 to 50, and 50 to 90 ml, obtained on chromatography of extracts of incubation mixtures on Celite columns, showed the presence of only negligible amounts of ¹⁴C-mevalonic acid.

Only palmityl-CoA almost completely inhibited the reaction at a concentration of 20 μM. In another experiment, sodium arsenite at 2 mM gave 67% inhibition of fatty acid synthesis in the standard assay.

**Table IV**

<table>
<thead>
<tr>
<th>Amino acid composition of purified fatty acid synthetase</th>
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<tbody>
<tr>
<td>The amino acid composition was determined on an acid hydrolysate of the protein in the Spinco model 120 amino acid analyzer.</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Histidine</td>
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<tr>
<td>Ammonia</td>
</tr>
<tr>
<td>Arginine</td>
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<tr>
<td>Aspartic acid</td>
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<tr>
<td>Threonine</td>
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<tr>
<td>Serine</td>
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<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Proline</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>Half-cystine</td>
</tr>
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<td>Valine</td>
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</tr>
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<tr>
<td>Tyrosine</td>
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<tr>
<td>Phenylalanine</td>
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**Table V**

<table>
<thead>
<tr>
<th>Inhibition of fatty acid synthetase</th>
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<tr>
<td>The conditions of assay are reported in the text.</td>
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<tr>
<td></td>
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<tr>
<td>Palmitoyl-CoA</td>
</tr>
<tr>
<td>Palmitoyl-CoA</td>
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<tr>
<td>Potassium palmitate</td>
</tr>
<tr>
<td>CoA</td>
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<td>CoA</td>
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</table>
Fig. 10. The binding of acetate and malonate moieties of acetyl- and malonyl-CoA to purified fatty acid synthetase. Acetate binding is reported at the left and malonate binding is reported at the right. The binding of acetate and malonate by a freshly prepared enzyme, ○—○, and the same enzyme aged 11 days at 4°C, ●—●, are shown. The aged enzyme retained about 1% of its original fatty acid synthesizing activity. The purified enzyme was prepared as described under “Experimental Procedure,” except that chromatography on Sephadex G-100 was made with 0.1 M potassium phosphate buffer, pH 7.0. The incubation mixtures contained, in addition to potassium phosphate, pH 7.0, 9.8 mg of purified protein and 0.05 ml of 1-14C-acetyl-CoA (90 mpmoles and 350,000 cpm) or 0.15 ml of 2-14C-malonyl-CoA (98 mpmoles and 249,000 cpm).

**Table VI**

Inhibition of acetate and malonate binding to purified fatty acid synthetase by sodium arsenite

Aliquots of 6 mg of purified enzyme and 0.5 μmole of 2-mercaptoethanol were preincubated for 6 min at 38°C in a total volume of 0.6 ml with the following amounts of sodium arsenite: A, none; B, 1.2 μmoles; C, 4.0 μmoles. The incubation mixture for acetate and malonate binding contained potassium phosphate buffer, pH 7.0, 12 μmoles, and 1-14C-acetyl-CoA, 51,000 cpm and 17.1 mpmoles, or 2-14C-malonyl-CoA, 50,000 cpm and 19.7 mmoles, respectively, in a volume of 0.4 ml. Preincubated enzyme, 0.2 ml, was added to each incubation mixture, and incubations were made at 38°C for 12 sec. The reaction was stopped by the addition of 0.2 ml of 60% perchloric acid. The protein precipitate was washed and then assayed for radioactivity as reported in the text.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sodium arsenite concentration in preincubation mixture</th>
<th>Amount of substrate bound</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>mpmoles/mg protein</td>
<td>%</td>
</tr>
<tr>
<td>1-14C-Acetyl-CoA</td>
<td>0</td>
<td>2.18</td>
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</tr>
<tr>
<td></td>
<td>2.0</td>
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<td>2.0</td>
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<td>6.7</td>
<td>1.04</td>
<td>47</td>
</tr>
<tr>
<td>2-14C-Malonyl-CoA</td>
<td>0</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.48</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>6.7</td>
<td>1.04</td>
<td>47</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The specific activities reported in this paper for the crude enzyme systems for fatty acid synthesis, Tables I and II, are much higher than those reported by Bressler and Wakil (1). Our preparations of the pigeon liver supernatant solution had an activity of 2 to 3 mmoles of fatty acid synthesized per min per mg of protein (equivalent to 28 to 42 mmoles of NADPH oxidized per min per mg of protein). Bressler and Wakil (1) reported a value of 0.1 mmoles of NADPH oxidized per mg of protein. It is not possible to establish completely the reasons for the above discrepancies. It is possible, however, to report several factors which have marked effect upon the activity of an enzyme preparation for fatty acid synthesis. Fatty acid synthetase is very susceptible to loss in activity. The enzyme rapidly loses activity on standing in low ionic strength buffer solution, even in the presence of 2-mercaptoethanol, or on exposure to heavy metal ions in the absence of EDTA. In addition the enzyme appears to be easily surface-denatured. Through a combination of adjustment of the pH of the medium for extraction of enzyme from pigeon liver to pH 8.0 and the use of EDTA, 2-mercaptoethanol, and deionized distilled water in all extraction and purification steps and in incubation mixtures, an increase of 10- to 20-fold was obtained in fatty acid synthetase activity. A second factor that may affect the activity of a crude extract of pigeon liver for fatty acid synthesis is the nutritional status of the bird. Only preliminary studies are available on the effect of this factor on fatty acid synthetase activity of pigeon liver. However, it has been demonstrated previously by several workers that the level of fatty acid synthetase of rat liver can be varied greatly (10-fold or more) through starvation or through changes in the diet. A third factor affecting the fatty acid synthetase activity of pigeon liver may be the method of assay. In our experience, much more reliable results are obtained for fatty acid synthetase activity of crude enzyme preparations by radiochemical analysis than by spectrophotometric assay. Presumably a combination of the above factors is responsible for the major discrepancies between our results and those of Bressler and Wakil (1).

It had been shown previous to the present paper that the *Escherichia coli* and *Clostridium kluyveri* fatty acid-synthesizing systems could each be separated into two or more fractions (18, 19). Earlier (4) it had been shown that the yeast fatty acid synthetase behaves as a single complex protein on moving boundary electrophoresis and sedimentation analysis. The results reported in the present paper provide evidence that the pigeon liver fatty acid synthetase is also a multifunctional protein complex. This enzyme system behaves as a single protein...
entity on sedimentation analysis and on moving boundary elec-
trophoresis. In addition, it behaves as a single protein of
constant specific activity on DEAE chromatography. More
recently we have found that this protein yields a single band on
starch gel electrophoresis. 5

The molecular weight of the above protein, as determined
by the Archibald method (17), was found to be 5.1 × 10^6 g per
mole. This is about one-fourth the molecular weight of the
yeast fatty acid synthetase. The molecular activity (i.e.,
number of molecules of palmitic acid formed per min per mole-
cule of enzyme) of the pigeon liver fatty acid synthetase is 38.
The percentage of fatty acid synthetase in the crude liver
homogenate, calculated from the 70-fold purification, is 1.4%.
Considering the fact that liver is the chemical factory of the
body and that it probably contains more than a thousand en-
ymes, this concentration represents a remarkably high level for
a single enzyme.

Analyses of the pigeon liver fatty acid synthetase showed an
excess of acidic amino acids and a negligible quantity of flavin.
Fifty —SH groups were also found. The finding of an excess
of acidic amino acids is in agreement with the behavior of the
protein as a negatively charged molecule at pH 7.0 during moving
boundary electrophoresis. The absence of flavin in the pigeon
liver fatty acid synthetase is 38.

The rate of deacylation of acetyl- and malonyl-enzyme by
aged enzyme retains the capacity to bind amounts of acetate and
malonate comparable to that bound by the fresh enzyme.

Hence, the loss of overall activity for fatty acid synthesis is at
a site other than those responsible for the initial binding of
acetyl- and malonyl-enzyme (3). The enzyme was subjected to starch gel electrophoresis in the
standard boric acid-NaOH system containing 1 mEq EDTA and
dithiothreitol at pH 8.5 and 300 volts for 36 hours. A single band
of protein was obtained on staining. Further details on this
system will be published later by Butterworth, Yang, Bock, and
Porter.

The pigeon liver fatty acid synthetase has a pH optimum of
6.7 and this enzyme is relatively specific for NADPH. The
rate of synthesis with this nucleotide was 7 or 8 times that
with NADH. A comparison of the rate of NADPH oxidation to
1-14C-acetyl-CoA incorporation into fatty acids by the puri-
ified synthetase yielded a ratio of 14.5, in good agreement with
the ratio of 14 predicted from the equation for the over-all
reaction. A negligible amount of NADPH was oxidized by
the purified enzyme in the absence of acetyl- and malonyl-CoA.

Two of the products of the over-all reaction, CoA and palmitic
acid, gave little or no inactivation of the enzyme at the concentra-
tions that might be reached during the assay. In contrast,
palmitoyl-CoA at the same concentration almost completely
inhibited the reaction. This result suggests that the rate of
fatty acid synthesis might be controlled by the level of palmitoyl-
CoA in the cell.

The purified enzyme independently binds acetate and malonate
moieties of acetyl- and malonyl-CoA, and the activities for
binding (a minimum of 2 mmoles of acetate and malonate
bound per mg of protein) are greater than those previously re-
ported by Brodie, Wasson, and Porter (3) for a less purified
enzyme. This enzyme also catalyzes a time-dependent decaya-
lization of acetyl- and malonyl-enzyme (3).

Aged enzyme preparations retain the capacity to bind amounts of acetate and
malonate comparable to that bound by the fresh enzyme.

Hence, the loss of over-all activity for fatty acid synthesis is at
a site other than those responsible for the initial binding of
acetate and malonate.

The rate of deacylation of acetyl- and malonyl-enzyme by
aged enzyme preparations is much slower than the rate obtained
with fresh enzyme. This loss in activity is not responsible, how-
ever, for the loss in fatty acid-synthesizing capacity of aged
enzyme preparations since some aged preparations retain a
slow rate of deacylase activity after they have lost all activity
for fatty acid synthesis.

It is thought that acetyl- and malonyl-binding sites of the
enzyme are close to one another, either in fixed positions or in
positions that can be brought into close proximity with each
other. Such an arrangement would be necessary for conversion

Fig. 11. The formation of HMG-CoA and 3,5-diketoheptanoic
acid by purified fatty acid synthetase. Incubation mixtures con-
tained potassium phosphate buffer, pH 7.0, 25 mmoles; malonyl-
CoA, 71 mmoles; 1-14C-acetyl-CoA, 72 mmoles and 280,000 cpm;
enzyme protein, 1 mg; water to a final volume of 1 ml. The re-
action mixture was incubated at 38° for 60 min, and the reaction
was stopped by the addition of an equal volume of methanol.
The solution was chilled, and the protein was removed by cen-
trifugation. The precipitate was washed with water, and the
supernatant solutions from three identical runs were combined
and lyophilized to dryness. The product was dissolved in a mini-
imum amount of 99% methanol and chromatographed on paper.
of acetyl- and malonyl-enzyme to acetoacetyl-enzyme. The inhibition of both fatty acid synthesis and acetyl- and malonate-binding by sodium arsenite is consistent with the proposed involvement of closely associated —SH groups of enzyme. In addition these findings are consistent with earlier reports (20, 21) of the inhibition of fatty acid synthesis by arsenite, but they appear to be inconsistent with the report of Brady, Trams, and Bradley (20) that the condensation reaction (acetoacetate formation) catalyzed by a rat liver preparation is not inhibited by arsenite.

The demonstration of the acetyl-CoA-dependent decarboxylation of malonyl-CoA by the purified enzyme confirms this reaction as an integral part of fatty acid synthesis (1, 3). The time dependence of the reaction indicates the formation of nonenzyme bound products (3,5-diketohexanoic acid and acetoacetic acid (3)).

Previous reports from this laboratory (3, 22) have identified butyryl-, β-ketohexanoyl-, and palmityl-enzyme, as well as acetyl-, malonyl-, and acetoacetyl-enzyme as intermediates in fatty acid synthesis. In the present study, it has been demonstrated that at least 1 mole of acetate and 1 mole of malonate are bound per mole of enzyme when acetyl-CoA or malonyl-CoA are incubated separately with enzyme. In the present experiments, it has also been shown that the acetyl-CoA-dependent decarboxylation of malonyl-CoA occurs. In this reaction, a decarboxylation of malonyl-enzyme might occur with the formation of a carbanion, as shown in Fig. 12, or a proton might be removed from malonyl-enzyme with the formation of a carbanion at carbon 2. The carbanion would then react with the carbonyl carbon of the acetyl-enzyme compound, thus resulting in the concomitant decarboxylation and transfer of the acetyl-moiety and the formation of acetoacetyl-enzyme on the original malonyl-binding site. Subsequent reactions (Fig. 12) of fatty acid synthesis by the pigeon liver enzyme are indicated from the report.

![Figure 12: The reactions catalyzed by the purified pigeon liver fatty acid synthetase](http://www.jbc.org/)
by coenzyme A and palmityl-coenzyme A at a concentration at least 50 sulfhydryl groups per mole. The enzyme also has as with reduced nicotinamide adenine dinucleotide. The activation energy for the heat denaturation of the enzyme is 1.6 × 10^6 cal/mole.

The enzyme also catalyzes the acetyl-CoA-dependent decarboxylation of malonyl-CoA. The purified enzyme synthesizes very small amounts of the CoA ester of β-hydroxy-β-methylglutaric acid and 3,β-diketohexanoic acid in the absence of NADPH. Negligible amounts of mevalonic acid are formed in the presence of NADPH.

A proposal is made of the pathway of biosynthesis of fatty acids by the pigeon liver enzyme. This pathway is very similar to one proposed earlier by Lyen for the biosynthesis of fatty acids by yeast fatty acid synthetase.

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