Stabilization and Physicochemical Properties of the Fatty Acid Synthetase of Chicken Liver*

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ABSTRACT: Chicken liver fatty acid synthetase was purified in homogeneous form with a procedure modified from the method for the isolation of the pigeon liver enzyme. It has a $s^0_{20,b}$ of 11.8 S and a molecular weight of 500,000 estimated from equilibrium studies. It absorbs light maximally at 279 mμ, giving an extinction coefficient of 0.965 for a 0.1% protein on a dry weight basis.

Palmitic acid is the major product formed by this enzyme. Purified chicken liver enzyme is completely stabilized by glycerol, and is thereby providing an excellent source for further studies on the molecular properties of the native avian enzyme complex.

The malonyl-CoA-dependent pathway for the biosynthesis of fatty acids has been well documented (Majerus and Vagelos, 1967). In yeast and higher animals enzyme components reside in multienzyme complexes which are capable of catalyzing the entire sequence of reactions while the intermediates are covalently bound to the protein complex (Lynen, 1961, 1967a,b; Brodie *et al.*, 1964). These complexes are highly organized and appropriately possess a degree of catalytic efficiency far greater than analogous dissociable systems (ratio of specific activities for crude enzymes from *Escherichia coli* and chicken liver approximately 1:1000).

Multienzyme complexes represent biochemical entities with structural complexity between that of discrete enzyme molecules and cellular organelles and lend themselves as ideal models for studies on structure–function relationship of enzymes at a high level of organization. An excellent review on these protein aggregates has been published (Reed and Cox, 1966). Fatty acid synthetase has been isolated from pigeon and rat liver, rat lactating mammary gland, as well as from yeast (Hsu *et al.*, 1965; Burton *et al.*, 1968; Smith and Abraham, 1969; Lynen, 1961). These animal enzymes represent the first protein complex isolated from the cytosol portion of the cell. They are comparable in size and are composed to two subunits approximately equal in size. Elegant studies by Lynen and colleagues (1967a,b) on the yeast complex suggested the existence of three subcomplexes, each containing a full complement of component enzymes. Analogous studies on the native avian enzyme, including critical evaluation of its many molecular properties in a quantitative and unequivocal manner (*i.e.*, active site titration, full kinetic treatment, etc.), have been difficult due to the inherent instability of the pigeon liver enzyme (Hsu *et al.*, 1965).

Chicken liver fatty acid synthetase can be prepared in quantity by modification of the procedure for the pigeon enzyme (Hsu *et al.*, 1965). Results of the present study indicate that it resembles the latter in molecular weight, catalytic activity, products formed, and other chemical properties. However, it is more stable. In the presence of glycerol stabilization was extended and full activity was retained for moret han 1 month, as compared with 4 days for the pigeon enzyme (Yang *et al.*, 1967). As an alternate source of avian enzyme, therefore, the chicken complex is more suitable for a comprehensive study on its properties beyond the scope of our present knowledge.

DEAE-cellulose chromatography of enzyme from Columbian–New Hampshire chickens yielded multiple activity peaks suggesting the presence of fatty acid synthetase isozymes.

In an accompanying paper, a study on the number and nature of TPNH binding sites is presented.

Materials and Methods

[1-^{14}C]Acetyl-CoA (New England Nuclear); dithiothreitol, ethylenediaminetetraacetic acid (tetrasodium dihydrate), and crystalline bovine albumin (Calbiochem); coenzyme A, 2-mercaptoethanol, and boron trifluoride methanol (Sigma); acetyl-CoA, malonyl-CoA, and TPNH (P-L Biochemicals); ammonium sulfate and sucrose (special enzyme grade), and Mannex DEAE-cellulose (Mann); Sephadex G-100 and G-200 (Pharmacia); crystalline alcohol dehydrogenase (Worthington); diethylene glycol succinate, Anachrom A, 60/80 mesh (Analabs); and unlabeled fatty acid methyl esters (Applied Science) were purchased from the specified sources. Calcium phosphate gel and saturated ammonium sulfate solution were prepared as previously described (Hsu *et al.*, 1965). Deionized, distilled water was used throughout these experiments. For these experiments 4–6-week-old Leghorn chickens or a crossbreed between Columbian females and New Hampshire males were used. In order to obtain maximal activity, these chickens were fasted for 3–5 days and refed for 2–3 days before slaughter (Feigenbaum and Fisher, 1962).

Radioactivity was determined by the Nuclear-Chicago 723 or the Beckman LS-150 liquid scintillation counters. The

* From the Department of Biochemistry and Microbiology, Rutgers, The State University, New Brunswick, New Jersey 08903, and the Department of Biochemistry, State University of New York, Upstate Medical Center, Syracuse, New York 13210. *Received September 3, 1969.* This investigation was supported by the Institute of Arthritis and Metabolic Diseases, National Institutes of Health (AM 13390), the Research Foundation of the State University of New York (0337-04-011-68), and the General Research Support Grant from the National Institutes of Health (5 S01 FR-05402-08).

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counting efficiencies for a nonaqueous system were 79 and 94%, respectively. Protein concentration was determined by the biuret method (Hsu and Lardy, 1967), by its 279-mμ extinction coefficient of 0.965 for a 0.1% protein concentration (see Results), or alternatively by 280-mμ measurements with an ultraviolet absorption monitoring and recording system during chromatography and sucrose density gradient centrifugation experiments. Fatty acid synthetase assay was carried out according to Hsu *et al.* (1965). Incorporation of radioactivity into long-chain fatty acids was proportional to protein concentration. DEAE-cellulose was washed by suspending a large batch (80–100 g) of Mannex-DEAE in distilled water. After 20 min, the fines were removed by suction. This procedure was repeated 2–3 times. The anion exchanger was then washed with 3–4 l. of 1 M potassium phosphate buffer (pH 7.0) until the effluent was at the same pH. The suspension was kept in the cold before use. Sephadex gel column was prepared according to instructions from Pharmacia. For each experiment, a new Sephadex column was used.

Experimental Section

Purification of Enzyme. Chicken liver fatty acid synthetase was prepared essentially according to the procedure described for the pigeon enzyme (Hsu *et al.*, 1965), with modifications to assure complete purification with high yield. Usually 45–50 chickens were killed. The high-speed supernatant fraction was frozen in 80-ml aliquots under N_2 and was stable indefinitely. Deviations from the above procedure are described below.

R_2 fraction from ammonium sulfate fractionation was dialyzed against buffer I[1] for 30 min (R_2D) and diluted to 500 ml with the same buffer before gel treatment. The supernatant fraction (R_2DG) from calcium phosphate gel treatment was immediately chromatographed on a Mannex-DEAE cellulose column (2 × 37 cm) previously equilibrated with 2 l. of buffer I. Stepwise elution was carried out with buffers I, II, and III (enzyme fractions DEAE I, II, and III, respectively). The effluent was monitored by its absorption at 280 mμ. During each step sufficient buffer was allowed to pass through the column until the optical density was below 0.05. Elution with buffer III was collected in 10-ml portions and continued until active enzyme was eluted. Usually 50 ml of this fraction was pooled and precipitated by saturated ammonium sulfate (33% saturation, fraction III-AS). III-AS was dissolved *carefully* in a minimum volume of buffer III (1.2–2.0 ml), centrifuged at 5000*g* for 10 min to remove small amounts of insoluble matter, and chromatographed on a Sephadex G-200 column (1.6 × 32 cm) preequilibrated with the same buffer. A constant flow rate of up to 10 ml/hr was maintained by the reverse flow technique and by limiting the hydrostatic head to under 15 cm at all times. Portions (1 ml) were collected. Usually 10 ml of active enzyme was pooled which represented the purified enzyme (G-200 fraction). In early purification experiments Sephadex G-100 was used instead of G-200 (G-100 fraction). Enzyme prepared in this manner, however, was not always pure. Only homogeneous preparations (as measured by specific activity and sedimentation patterns) were used for further experimentation.

Identification of Fatty Acids. [^{14}C]Fatty acids were methylated with boron trifluoride methanol (Morrison and Smith, 1964) and chromatographed on a F & M Model 720 gas chromatograph equipped with dual columns packed with 12% diethylene glycol succinate on Anachrom A. [^{14}C]Fatty acid methyl esters were identified by their retention time. Fractions were collected and quantitated by radioactivity measurements. Further separation on the basis of saturation was carried out by thin-layer chromatography of these [^{14}C]methyl esters in the presence of cold carriers on plates coated with silicic acid (75%) and $AgNO_3$ (25%). The methyl esters were separated according to their degree of unsaturation, with the unsaturated esters giving lower R_F values. After chromatography, the plate was developed with a 0.2% solution of 2,7-dichlorofluorescein in ethanol and the compounds were visualized under an ultraviolet light source. The fluorescent bands corresponding to saturated and unsaturated fatty acid derivatives were scraped off and eluted on Florisil columns (1 × 3 cm) each with 20 ml of ether. The ether extracts were evaporated to dryness and radioactivity was counted in the scintillation counter.

Homogeneity Tests and Molecular Weight Determination. All sedimentation studies were carried out at 20° in the Spinco Model E analytical ultracentrifuge with the An-H rotor. Velocity sedimentation measurements were made according to Schachman (1957) using schlieren optics. For equilibrium studies, interference optics was used. Photographs were measured with a Nikon profile projector with a 20× enlargement factor.

Molecular weight was determined by the high-speed equilibrium method of Yphantis (1964) using the multichannel centerpiece with a column height of 3 mm. The speed was chosen to give effective reduced molecular weight (σ_w) of about 5 cm^{-2}. Equilibrium time was estimated by the equation $t_{eq} = 2.3(b - a)/w^2sr$, where $b - a$, r, s, and w are, respectively, the solution column height, radius, sedimentation coefficient, and angular velocity. For a molecule of 12 S sedimenting at 10,000 rpm, approximately 20 hr are required to reach equilibrium. Weight-average molecular weight was calculated according to

$$\bar{M}_w = \frac{2\,RT}{(1 - \bar{v}\rho)w^2} \times \frac{\mathrm{d} \ln C_{(r)}}{\mathrm{d}(r^2)} \qquad (1)$$

where $R, T, \bar{v}, \rho$, and $C_{(r)}$ are, respectively, gas constant, absolute temperature, partial specific volume, solution density, and concentration at r.

Gradient centrifugation was performed according to the method of Martin and Ames (1961) with a Spinco SW-39 swing-out head, or alternatively with the SW-50L head in the Spinco Model L preparative ultracentrifuge under refrigeration. All gradients were made with 2.2 ml of 25% sucrose and 2.4 ml of 10% sucrose, each containing potassium phosphate buffer (0.05 M, pH 7.0), EDTA (1 mM), and 2-mercaptoethanol (2 mM) in a 5-ml Büchler linear gradient device. Crystalline alcohol dehydrogenase was used as the reference standard. Average revolutions per minute were calculated by dividing the odometer reading by total centrifugation time.

Cellulose acetate electrophoresis was performed with the

[1] Buffers used during enzyme purification were: (I) potassium phosphate (0.005 M)–EDTA (1 mM)–dithiothreitol (1 mM)–(pH 7.0); (II) potassium phosphate (0.05 M)–EDTA (1 mM)–dithiothreitol (1 mM) (pH 7.0); (III) potassium phosphate (0.25 M)–EDTA (1 mM)–dithiothreitol (1 mM) (pH 7.0).

TABLE I: Purification of Fatty Acid Synthetase from Chicken Liver.

Fraction	Volume (ml)	Total Units		Total Protein (mg)	Specific Activity (units/mg)	Yield (%)	Purification Factor
High-speed supernatant fraction	78	15,600	(9,050)[a]	3,401	4.6 (4.6)	100	
R_2	40	11,200	(8,940)	1,168	9.7	72	2.1
R_2D	42	13,440	(10,460)	1,168	11.5		
R_2DG	545	10,900	(8,800)	807	13.5	70	2.9
DEAE-I		Nil	(1,480)				
DEAE-II		Nil	(2,440)				
DEAE-III	49	6,680	(3,160)	96	69.5	43	15.1
III-AS	1.9	4,750		77	61.7	30	13.4
G-200	7.6	3,344	(1,280)	416	80.0 (78.0)	22	17.4

[a] Figures in parentheses are values obtained from Columbian–New Hampshire chickens. Other figures are from Leghorn chickens.

Gelman apparatus using Colab cellogel electrophoresis strips at 4°. For each protein sample two strips were electrophoresed. One strip was stained for protein and the other cut into 0.5-cm portions and incubated in 1.0 ml of buffer solution containing 100 μmoles of potassium phosphate (pH 7.0). After 20 min, an assay mixture for fatty acid synthetase containing all reagents (except buffer) was added and incubated at 38° for 30 min. Radioactive fatty acids were extracted and counted in the usual manner.

Determination of Extinction Coefficient. Light absorption spectrum was determined with a Cary Model 16 spectrophotometer equipped with an automatic wavelength scanning accessory. Extinction coefficient at the absorption maximum was determined as follows: During purification, Sephadex G-200 column chromatography was carried out with buffer III in the absence of dithiothreitol. This step was taken to assure accurate measurement of ultraviolet light absorption without interference from oxidized dithiothreitol which absorbs at 283 mμ (Cleland, 1964). The G-200 enzyme prepared in this manner was dialyzed in the cold exhaustively against either buffer I or buffer II without dithiothreitol. After dialysis, a portion of the enzyme solution was appropriately diluted with buffer III without dithiothreitol, and its optical density at 279 mμ was determined on a Beckman DU spectrophotometer using the dilution buffer as blank in matched cuvets. Dry weights of the enzyme solution and the dialysis medium were determined by pipetting known volumes of these solutions using calibrated volumetric pipets into tared weighing bottles and dried overnight at room temperature *in vacuo*. Further drying was carried out at 105° until constant weight was obtained.

Extinction coefficient of the enzyme was calculated according to the relationship: $\epsilon_{(0.1\%)}$ = optical density at 279 mμ/concentration of protein in mg/ml on a dry weight basis. Dry weight of the protein was determined by dividing (dry weight of enzyme plus buffer) − (dry weight of buffer) by (volume of aliquot in milliliters taken for dry weight determination).

Results

Purification Experiments. Table I represents results of a typical purification experiment on crude enzyme from Leghorn chickens. Modifications from the procedure for the pigeon enzyme, although not extensive, are significant. Tenacious adherence of this procedure produced purified enzyme with consistent homogeneity. It is significant to note that with a longer Sephadex column and improved technique in the present method, larger amounts of purified enzyme (40–60 mg) can be obtained in one preparation. The depression of activity in the R_2 and III-AS fractions must be due to inhibition by ammonium sulfate, since this effect was removed by dialysis (R_2D).

High-speed supernatant fraction prepared from Columbian–New Hampshire chickens (Table I, numbers in parentheses) gave comparable specific activity, and yielded purified enzyme according to this procedure. However, the elution pattern from DEAE-cellulose chromatography was significantly different and fractions I, II, and III all contained fatty acid synthetase activity. It is, however, conceivable that insufficient lowering of ammonium sulfate concentration of R_2 fraction by short dialysis and dilution prior to the chromatography step could result in early elution of the enzyme in DEAE-I and -II fractions. To minimize this uncertainty, DEAE-cellulose chromatography was carried out with a steep phosphate gradient (0.005–0.5 M) as shown in Figure 1. This chromatogram clearly establishes the presence of two adjacent, but distinct activity peaks. DEAE-III fractions isolated from livers of Leghorn and Columbian–New Hampshire chickens are identical and are used for the preparation of purified enzyme for further studies.

Products of the Reaction. Purified chicken liver fatty acid synthetase yields the same fatty acids found for the pigeon enzyme (Brodie *et al.*, 1964). During gas chromatographic analysis, major portions of the radioactivity were found in methyl palmitate (60%) and methyl stearate (19%). No unsaturated fatty acids were found by the above technique or by thin-layer

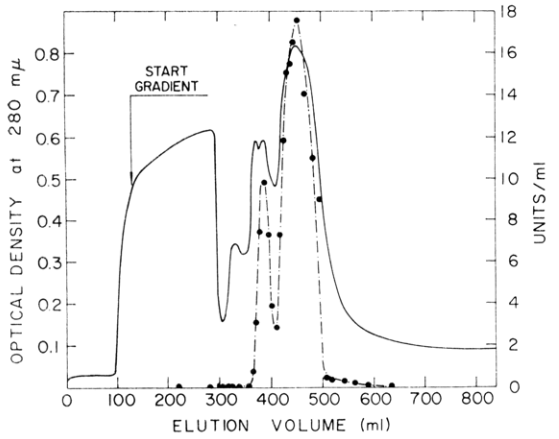

FIGURE 1: Gradient DEAE-cellulose chromatography of fatty acid synthetase from Columbian–New Hampshire chickens. R_2DG fraction (210 ml, 2840 units) was chromatographed on a 2 × 30 cm DEAE-cellulose column. Linear concentration gradient consisted of 500 ml each of buffer I and potassium phosphate (0.5 M)–EDTA (1 mM)–dithiothreitol (1 mM) (pH 7.0). (—) 280 mμ optical density; (—·—) fatty acid synthetase activity.

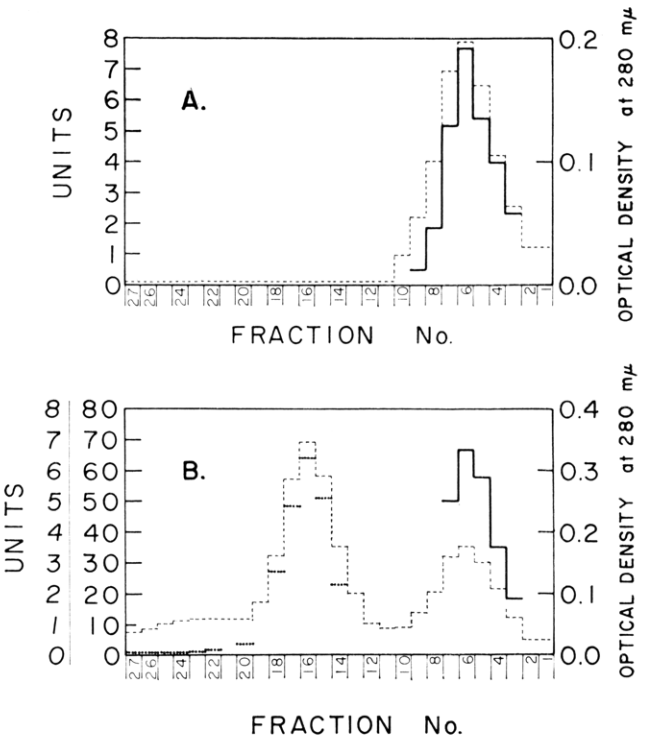

FIGURE 3: Sucrose density centrifugation of fatty acid synthetase: average speed, 37,300 rpm; centrifugation time, 625 min. Each fraction contained 12 drops: (A) 1.3 mg of purified enzyme in 0.2 ml of buffer III; (B) 1.3 mg of purified enzyme and 2.0 mg of crystalline yeast alcohol dehydrogenase in 0.2 ml of buffer III. (—) Fatty acid synthetase activity (units); (– – –) 280 mμ optical density; (. . . .) alcohol dehydrogenase activity.

analysis of [^{14}C]methyl esters. Gas chromatographic analysis of the [^{14}C]fatty acids synthesized by DEAE-II fraction prepared from Columbian–New Hampshire chickens also yielded identical distribution patterns, indicating that these enzymes catalyze the same sequence of reactions.

Homogeneity Tests. The freshly purified enzyme gave a single, symmetrical peak in the ultracentrifuge (Figure 2). The sedimentation coefficient was slightly concentration dependent and decreased at higher protein concentrations in a linear manner. The extrapolated $s_{20,b}$ value at zero protein concentration was found to be 11.8 S. Sucrose density centrifugation and cellulose acetate electrophoresis permit the measurement of enzymatic activity and therefore yield additional information on homogeneity. Figure 3a shows a typical centrifugation pattern for the purified enzyme. Again a single, symmetrical protein peak was obtained which exhibited constant specific activity. When crystalline yeast alcohol dehydrogenase (s = 7.6) was used as a marker, as shown in Figure 3b, a sedimentation coefficient of 12.8 S was calculated on the basis of the ratio of distances traveled by these two enzymes.

Cellulose acetate electrophoresis patterns are presented in Figure 4. Figure 4a shows the distribution of proteins in the high-speed supernatant fraction of Leghorn chickens. At least eight protein bands can be seen. Only a single activity peak was found (arrow). Purified enzyme (Figure 4b) gave a single activity peak matching the protein band.

Stabilization by Glycerol. Chicken liver fatty acid synthetase is more stable than the pigeon enzyme. In the presence of buffer III (0.25 M potassium phosphate, 1 mM each of EDTA and dithiothreitol (pH 7.0)), the purified chicken enzyme re-

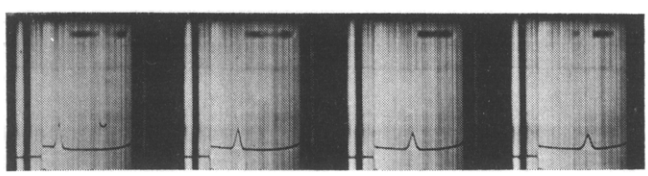

FIGURE 2: Sedimentation pattern of purified chicken liver fatty acid synthetase: speed, 44,770 rpm; protein concentration, 4.1 mg/ml in buffer III; bar angle 75°. The pictures were taken at 16-min intervals.

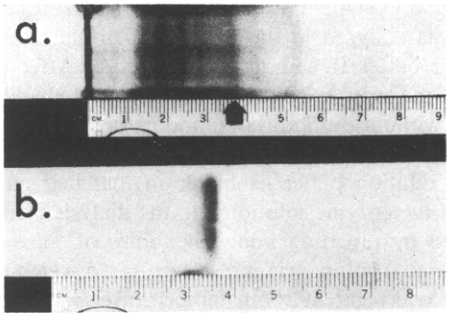

FIGURE 4: Cellulose acetate electrophoresis of chicken liver fatty acid synthetase. (a) Electrophoretic pattern of high-speed supernatant fraction from Leghorn livers; buffer, 0.022 M potassium phosphate (pH 7.5). Electrophoresis was carried out at 200 V for 2.5 hr. A single activity peak was found between 3.5 and 4.0 cm from the origin, which coincides with the protein peak at that position (see arrow). (b) Electrophoretic pattern of purified enzyme; buffer, 0.022 M potassium phosphate (pH 7.0). Electrophoresis was carried out at 200 V for 2 hr. The single activity peak coincides with the single protein peak.

TABLE II: Stabilization of Chicken Liver Fatty Acid Synthetase by Glycerol.[a]

	Activity Remaining (% of original)					
Tube	Day 4	Day 6	Day 17	Day 24	Day 30	Day 37
1	40	13	8	1	0.5	0
2	100	100	100	100	100	92

[a] Purified enzyme was used in both treatments. Protein concentration, 1.75 mg/ml. Tube 1 contained potassium phosphate buffer (0.2 M)–EDTA (1 mM)–dithiothreitol (6 mM) (pH 7.0) and enzyme. Tube 2 contained 20% glycerol in addition to the above compounds. Both tubes were stored at 4° under N_2. Aliquots were diluted and assayed at the designated time intervals.

tained full activity for at least 7–8 days (as compared with 4 days for the pigeon enzyme), and resisted dissociation for at least 3 weeks. Further stabilization of overall enzymatic activity was achieved by the addition of glycerol. Table II shows the results of such an experiment. Under the conditions of the experiment, loss of activity was accelerated in the absence of glycerol. Only 40% activity remained after 4 days, and complete inactivation was affected after 24 days. In the presence of 20% glycerol, however, full activity was retained for a period of 37 days.

Molecular Weight of the Chicken Liver Enzyme. Further studies on the molecular properties of an avian enzyme rely heavily on accurate determination of its molecular weight. For the present study, sedimentation was carried out for 100 hr, far beyond the time required for attainment of equilibrium in order to examine for time-dependent dissociation. Pictures were taken at convenient time intervals and measured. In one experiment the three channels were filled with purified enzyme in buffer III at protein concentrations of 3.68, 1.51, and 0.58 mg per ml. Figure 5a shows a plot of net fringe displacement against r^2 for the highest protein concentration after a centrifugation time of 87 hr. The experimental points conformed to a straight line. Plots for other protein concentrations were also linear. The reduced weight-average molecular weights, σ_w, were calculated from the slopes of these plots and were used in the computation of $\bar{M}_w$ according to eq 1. Further evaluation of the experimental data was made by a plot of the effective reduced weight-average molecular weight, $\sigma_{w(r)}$, *vs.* $b^2 - r^2$ according to Yphantis (1964), with the $\sigma_{w(r)}$ values obtained by the least-squares method from linear fringe displacement *vs.* X plots. As shown in Figure 5b, plots for three different protein concentrations do not deviate significantly from a continuous flat line, therefore indicating lack of observable concentration dependency or dissociation after lengthy centrifugation time. From a plot of $\sigma_{w(r)}$ against fringe displacement (concentration), the size of the smallest species was found to be 460,000. Density of the medium (buffer III) was determined by weighing and found to be 1.0292 at 20°. Partial specific volume of the protein was assumed to be 0.744 (Yang *et al.*, 1965). From the above values, the weight-average and z-average molecular weights were calculated for the three protein concentrations and for a separate experiment at a concentration of 1.60 mg/ml. These values are summarized in Table III.

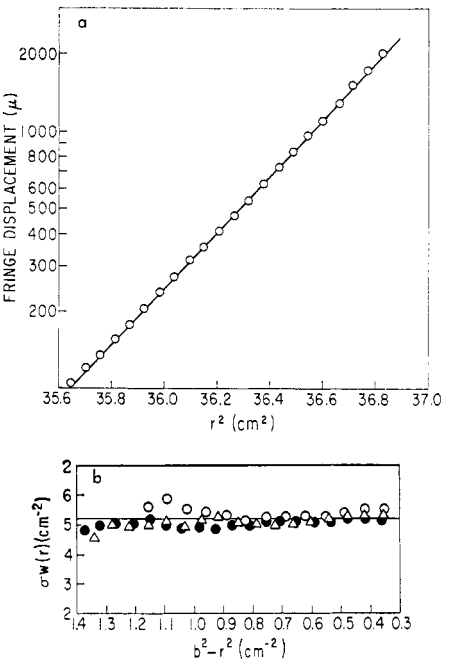

FIGURE 5: Molecular weight determination of chicken liver fatty acid synthetase by the Yphantis method (1964): speed, 10,000 rpm; time, 87 hr after reaching speed. (a) A plot of the natural logarithms of net fringe displacement against the square of radius, r^2, for a protein concentration of 3.68 mg/ml. (b) Direct comparison of the effective reduced weight-average molecular weights observed in the three channels of the equilibrium experiment. The initial protein concentrations were: (●) 3.68 mg/ml, (△) 1.51 mg/ml, and (○) 0.58 mg/ml.

Ultraviolet Light Absorption Spectrum and Extinction Coefficient. Purified chicken liver fatty acid synthetase did not absorb visible light. Its ultraviolet absorption spectrum (Figure 6) has a maximum at 279 mμ. The extinction coefficient at this wavelength was found to be 0.980 and 0.950 for a 0.1% protein solution (w/v on a dry weight basis) in two separate determinations. From these values, a mean value of 0.965 ± 0.015 was calculated.

TABLE III: Molecular Weight of Chicken Liver Fatty Acid Synthetase.

Expt	Initial Concn (mg/ml)	$\bar{M}_w$	$\bar{M}_z$
1	3.68	496,000	506,000
	1.51	487,000	505,000
	0.58	507,000	503,000
2[a]	1.60	510,000	
		Mean 500,000 ± 5,500[b]	

[a] Centrifuged for 80 hr. [b] Standard error.

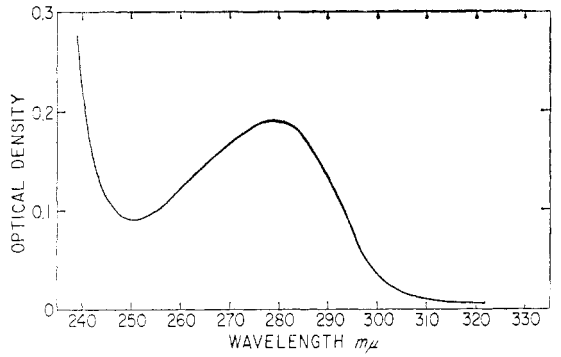

FIGURE 6: Ultraviolet absorption spectrum of chicken liver fatty acid synthetase. Protein concentration, 0.203 mg/ml. The purified enzyme was dialyzed against 0.005 M potassium phosphate buffer (pH 7.0), containing 1 mM EDTA and dithiothreitol. The spectrum was measured using the dialysis medium as blank in a Cary Model 16 spectrophotometer equipped with a wavelength scanning accessory.

Discussion

High fatty acid synthetase activity was found in livers of starved and refed chickens. Crude supernatant fraction prepared from these livers had a specific activity of 4.6, which is 1.5 to 2 times higher than that reported for the pigeon enzyme (Hsu *et al.*, 1965), and as much as three times higher than the normal laying hen (Chang *et al.*, 1967). This enzyme was purified by a method analogous to that described previously for the enzyme from pigeon liver (Hsu *et al.*, 1965), with small but significant alterations. During the last step, reproducible purity in the final preparations was obtained by replacing Sephadex G-100 with G-200.

R_2DG enzyme prepared from Columbian–New Hampshire chickens was resolved into two activity peaks by gradient ion-exchange chromatography. These two activity peaks gave identical fatty acid patterns. Results of sucrose density gradient centrifugation of a combined fraction containing both enzymes gave a simple, symmetrical activity peak comparable with that obtained for the purified enzyme, indicating similarity in molecular size. These above observations led us to the tentative conclusion that two fatty acid isozymes are present in the livers of Columbian–New Hampshire chickens differing only in their negative charges. Further studies on the characterization of these isozymes will be carried out pending on a supply of these experimental chickens.

Purified chicken liver fatty acid synthetase exhibited homogeneity on velocity sedimentation, density gradient centrifugation, and cellulose acetate electrophoresis. It has a final specific activity of 80 units/mg of protein and synthesizes only saturated fatty acids of which palmitic acid is the major product. The sedimentation coefficient of 12.8 S obtained by density gradient centrifugation is in reasonable agreement with the $s^0_{20,b}$ of 11.8 S obtained by velocity sedimentation. These properties are very similar to the enzyme isolated from pigeon liver (Hsu *et al.*, 1965; Yang *et al.*, 1967; Yang *et al.*, 1965), as would be assumed on the basis of their close relationship in evolution.

Labile enzymes can often be stabilized by the addition of glycerol. In the presence of this compound, stabilization of mammary glucose 6-phosphate dehydrogenase and human placental 17β-hydroxysteroid dehydrogenase were achieved (Langer and Engel, 1958; Levy, 1963). In the present study chicken liver fatty acid synthetase was completely stabilized by 20% glycerol. Previously, many studies on the properties of the pigeon enzyme were handicapped by its instability. This remarkable stability exhibited by the chicken enzyme overcomes this difficulty and provides an excellent enzyme source for further studies on the avian enzyme, particularly those requiring a knowledge of the unaltered, native complex. Suitability of this enzyme as an alternate source is further enhanced by the ready availability of chickens of known pedigree, sex, and age in quantity and by its high initial enzyme content and high yield of purified enzyme.

Light absorption characteristics of the chicken enzyme are similar to the enzyme from pigeon liver (Hsu *et al.*, 1965). Lack of absorption in the visible range is compatible with the absence of flavin in the former. The extinction coefficient at its maximum ultraviolet absorption peak (279 mμ) was found to be 0.965 $\pm$ 0.015 for a 0.1% protein on a dry weight basis. This value is useful for a direct and quick estimation of the concentration of the purified enzyme and provides better precision than other colorimetric methods (1–2% *vs.* 5% error for the Biuret method).

Two separate equilibrium sedimentation experiments performed on the purified enzyme yielded weight-average molecular weights in excellent agreement with each other. Further analysis of the sedimentation data is consistent with a homogeneous preparation whose molecular weight is not concentration dependent. The latter observation allowed us to average the molecular weights, and a mean value of 500,000 $\pm$ 5,500 was calculated. This value is nearly identical with an average (498,000) of two values (540,000 and 456,000 from Hsu *et al.*, 1965, and Yang *et al.*, 1967, respectively) previously calculated for the pigeon liver enzyme, indicating their similarity in molecular size.

Acknowledgment

The authors wish to thank Drs. Fisher and Grimminger of the Animal Nutrition Department at Rutgers University for their generous supply of Columbian–New Hampshire chickens.

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Reduced Triphosphopyridine Nucleotide Binding Sites of the Fatty Acid Synthetase of Chicken Liver*

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ABSTRACT: Purified chicken liver fatty acid synthetase (a multienzyme complex) forms a tight binary complex with reduced triphosphopyridine nucleotide. This binding is accompanied by enhancement of nucleotide fluorescence, a small red shift of excitation maximum from 345 to 353 mμ, and a blue shift of emission maximum from 460 to 447 mμ. Stoichiometric titration of the enzyme with reduced triphosphopyridine nucleotide yields a binding weight of 119,700 (±2,400) g of protein/mole of reduced triphosphopyridine nucleotide, and a dissociation constant of 0.29 (±0.016) μM. From a molecular weight of 500,000, four reduced triphosphopyridine nucleotide binding sites were calculated for the native enzyme. Reduced diphosphopyridine nucleotide does not exhibit significant binding. Acetylcoenzyme A appears to be bound at a site different from the reduced triphosphopyridine nucleotide binding site. Potassium maleate caused dissociation of the enzyme complex into subunits approximately equal in size. Inactivation of the native enzyme by dialysis against phosphate buffer at low concentration and dissociation by treatment with maleate resulted in the loss of up to two reduced triphosphopyridine nucleotide sites. The latter treatment also produced significantly lower fluorescence yield and a tenfold increase in dissociation constant of the binary ligand complex.

Fatty acid synthetase exists as a multienzyme complex in yeast and higher animals (Lynen, 1967a,b; Hsu *et al.*, 1965; Burton *et al.*, 1968; Smith and Abraham, 1969). It contains all of the enzyme components required for the synthesis of long-chain fatty acids from acetyl-CoA, malonyl-CoA, and TPNH. Confirmation of individual enzyme activities within this complex has been achieved through the use of model substrates (Lynen, 1961, 1967b) and characterization of acyl intermediates (Lynen, 1961, 1967b; Brodie *et al.*, 1964; Goldman and Vagelos, 1962). However, resolution of these compounds in active form has not been achieved.

Recent studies on the substrate binding sites of the yeast, pigeon, and rat liver complexes (Lynen, 1961, 1967b; Jacob *et al.*, 1968; Burton *et al.*, 1968) indicated that acetyl and malonyl moieties from respective coenzyme A thioesters are bound covalently to sulfhydryl groups on the enzyme molecule, with 4′-phosphopantetheine and cysteine playing significant roles in the binding process. Additional information suggests that the hydroxyl group of serine may also be involved in the initial phase of binding (Lynen, 1967b). TPNH, the third substrate for the overall synthetic reaction, is a coenzyme for β-keto acyl-acyl carrier protein reductase and enoyl-acyl carrier protein reductase, both components of the multienzyme (Majerus and Vagelos, 1967; Lynen, 1967b). These enzymes carry out reduction of the respective acyl intermediates with concomitant oxidation of TPNH in a manner analogous to other dehydrogenases. At the present, information on TPNH binding sites on these enzyme complexes is not available.

Fluorescence titration has been used as an effective tool in a study of binding sites for other pyridine nucleotide linked dehydrogenases (Udenfriend, 1962; Jarabak and Sack, 1969; Hsu and Lardy, 1967). Formation of the enzyme-reduced pyridine nucleotide binary complex usually results in significant enhancement of nucleotide fluorescence which can be used for the stoichiometric titration of nucleotide binding sites. These titration studies yield useful information on the number and nature of binding sites on these enzymes and provide insight to their mechanism of action. An elegant theoretical treatment of ligand binding to proteins was described by Weber (Weber and Anderson, 1965; Anderson and Weber, 1965; Weber, 1965).

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