Conformational Changes, Inactivation, and Dissociation of Pigeon Liver Fatty Acid Synthetase Complex

EFFECTS OF IONIC STRENGTH, pH, AND TEMPERATURE*

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SUMMARY

The pigeon liver fatty acid synthetase complex (\(s_{2n,m} = 14.0\) S) is inactivated and dissociated into half-molecular weight subunits (\(s_{2n,m} = 0.0\) S) in the presence of low ionic strength buffers. The rates of inactivation and dissociation of the complex are dependent upon the ionic strength, pH, and temperature of the medium. At low ionic strengths (0.01 or less) and mildly alkaline pH (8.35), the rates of inactivation and dissociation of the complex into subunits are coincident and the rate of inactivation is independent of protein concentration over a 62-fold range. At higher ionic strengths (above 0.02) and in the presence of 2-mercaptoethanol, active complex, inactive enzyme complex, and inactive subunits are found. Increasing the pH above neutrality at constant ionic strength increases the rate of inactivation as well as dissociation of the complex. The inactivation rate is also increased as the pH is lowered below 7.0, but the rate of dissociation of the complex is quite slow; the enzyme thus exists mainly as inactive complex. Temperature also has a marked effect on the rate of inactivation and dissociation of the complex. The rate of inactivation and dissociation in Tris-glycine buffer, pH 8.35 (\(\mu = 0.008\)) is nearly 10-fold greater at 0°C than at room temperature. The lowest rate of inactivation is found at 18-25°C. Above 25°C the rate of dissociation of the complex increases as the temperature rises.

The fatty acid synthetase complex (\(s_{2n,m} = 14.0\) S) appears to undergo a transition to an enzymatically active intermediate at low ionic strength. The "active intermediate" is then further converted to either an inactive enzyme complex or to inactive subunits (\(s_{2n,m} = 9.0\) S).

There is no requirement for the oxidation of \(-\text{SH}\) groups of the enzyme prior to its dissociation to subunits. However, the subunits obtained in the presence of dithiothreitol (conditions under which there is no loss of \(-\text{SH}\) groups of the enzyme) do not transfer the acetyl group from the 4'-phosphopantetheine to the cysteine site. Hence, these subunits have no activity for fatty acid synthesis.

The results presented in this paper suggest that hydrophobic forces and a diminution of electrostatic repulsions are factors which contribute to the stability of the complex in high ionic strength buffers at neutral pH. A lowering of the temperature weakens the hydrophobic interactions and a decrease in the ionic strength increases the electrostatic repulsion between the subunits. These cumulative effects lead ultimately to dissociation of the complex to two subunits.

Enzyme complexes are a class of protein aggregates in which the functional unit of the complex arises through the specific organization of nonidentical protein entities. One of the simpler of these is perhaps hemoglobin, and one of the more complex (isolated as a soluble component of the cell) is fatty acid synthetase. The dissociation of enzyme complexes, the isolation of their diverse subunits, and the reconstitution of the subunits to the enzymatically active complex have been the subject of intense recent interest. \(\alpha\)-Keto acid dehydrogenase complexes have been purified from pigeon breast muscle (1, 2), pig heart muscle (3, 4) and Escherichia coli (5). An account of the dissociation of these complexes, their separation into three individual functional, nonidentical subunits, and the reassembly of these subunits into an active enzyme system has been given by Reed and Cox (6). The existence of tryptophan synthetase of Neurospora crassa (7) and E. coli (8) and of anthranilate synthetase and phosphoribosyl transferase (the first two reactions in the synthesis of tryptophan) of E. coli (9) as heteropolymeric proteins has also been demonstrated.

In contrast to the relatively simple heteropolymeric structures mentioned above, homogenous preparations of the fatty acid synthetase complex of avian (10) and mammalian (11) species, consist of at least seven different functional catalytic units. These fatty acid synthetase complexes have not as yet been separated into individual enzymes, whereas those obtained from bacteria (12) and plants (13) have been separated into their component enzymes. Yang et al. (14) demonstrated the presence of
a to 8 polypeptide units by disc gel electrophoresis of the irre-
versibly inactivated pigeon liver fatty acid synthetase complex,
and Butterworth et al. (15) showed that the native complex can
be dissociated into two subunits of identical or nearly identical
molecular weights. It was also demonstrated in these studies
(14, 15) that "ageing" of the complex in the presence of 2-mer-
captoethanol results in a progressive loss of fatty acid synthetase
activity. Ultracentrifugation analyses of the enzymatically in-
active species showed that both complex and half-molecular
weight subunits were present. Inactivation and dissociation of
the fatty acid synthetase complex were attributed to the loss of
essential —SH groups of the enzyme either through auto-oxida-
tion or by disulfide exchange with the disulfide formed from 2-
mercaptoethanol. Based on these observations, Butterworth
et al. (15) were able to demonstrate the dissociation of the pigeon
liver enzyme complex with carboxymethyl disulfide and potas-
sium maleate. It was suggested, therefore, that the mainte-
nance of —SH groups of the pigeon liver enzyme is essential for
the integrity of the complex even though treatment with carbox-
ymethyl disulfide resulted in a total loss of fatty acid synthetase
activity but only a 50 to 70% dissociation to subunits. Com-
pared to the complex, the subunits obtained by the above treat-
ment showed a decreased capacity to bind acetyl and malonyl
groups from acyl-coenzyme A thioesters (19). These subunits
could be partially reactivated to active enzyme on incubation
with dithiothreitol.

In the present paper we report the results of a study of the mecha-
nism of inactivation and dissociation of the pigeon liver
fatty acid synthetase complex. The process of dissociation has
been studied under controlled conditions of ionic strength, pH,
and temperature. Variations in enzyme activity have been
Correlated with changes in sedimentation characteristics of the
enzyme and with conformational changes in the complex prior
to dissociation. Conditions for the formation of subunits with-
out the loss of —SH groups of the enzyme have also been estab-
lished and assays for the covalent binding of acetyl groups to the
binding sites on the reduced subunits have been carried out. A
preliminary report of a portion of this work has been published
(16).

EXPERIMENTAL PROCEDURE

Materials—DTNB1 and DTT were obtained from Aldrich and
Calbiochem, respectively, and were used without further purifi-
cation. Sodium dodecyl sulfate (Fisher) and 2-amino-2-methyl-
1,3-propanediol (Eastman) were recrystallized from ethanol
prior to use. The sources of additional compounds used in this
investigation were reported previously (17). The remaining reagents
were of analytical grade, and deionized glass distilled
water was used in all experiments.

Apparatus—All spectrophotometric assays were performed
with a Gilford model 220 optical density converter-Zeiss mono-
chromator recording spectrophotometer, equipped with a ther-
mostatted cell compartment. A radiometer pH meter, type
TTT1C, was used to measure buffer pH values; Fisher, pH 5.0,
7.0, and 9.0 buffers were used as standards. High voltage elec-
trophoresis of radioactive peptides was carried out with a Gilson
model D high voltage electrophorator. Radioactivity was lo-
ted by scanning the paper with a Vanguard ultrascanner, model
880.

1 The abbreviations used are: DTNB, 5,5'-dithiobis(2-nitro-
benzoic acid); DTT, dithiothreitol.

METHODS

Preparation of [3-14C]Acetyl-CoA

[2-14C]Acetyl-CoA was prepared according to the procedure of
Simon and Shemin (18), and then purified by the method of
Brodie and Porter (19).

Enzyme Purification and Assay

Pigeon liver fatty acid synthetase was purified according to
the procedure of Hsu et al. (10) with the slight modifications reported
previously (17). Assays for fatty acid synthetase activity were
conducted at the same substrate concentrations as previously
described (17). Unless otherwise specified in the text, enzyme
activity determinations were carried out in 0.2 mM potassium phos-
phate, pH 7.0, containing 3 mM EDTA and 1 mM 2-mercap-
teto ethanol, at 30°.

Inactivation and Dissociation of Enzyme

Variation in Tris-Glycine Concentration—Fatty acid synthe-
tase in 0.2 mM potassium phosphate, pH 7.0, containing 1 mM
EDTA and 10 mM DTT was precipitated by the slow addition
of one-half volume of a solution of saturated ammonium sulfate,
ph 7.0. The precipitated protein was centrifuged and the pellet
was dissolved in a minimum volume of the appropriate Tris-
glycine buffer. The enzyme solution (less than 0.3 ml) was then
desalted on a G-25 Sephadex column (22 × 1 cm) previously
equilibrated with the same Tris-glycine buffer. The desalting
procedure usually took about 10 min. After desalting, aliquots
were removed at various time periods and after appropriate dilu-
tion assayed for enzyme activity.

Variation in Temperature—The ammonium sulfate-precipi-
tated enzyme was dissolved in 35 mM glycine, 1 mM EDTA, and
1 mM 2-mercaptoethanol adjusted to pH 8.35 with 1 M Tris
at the temperature to be used in the experiments. KCl was added
to adjust the ionic strength to that of the sample at 37°. Des-
ataling was performed at 4° for the experiments performed at 0°,
6°, and 12°, and at room temperature (approximately 22°) for
experiments performed at 18° or higher. The enzyme solution
was placed at the desired temperature soon after the desalting
procedure was completed. Initial activities represent the activity
at the time of placement of the enzyme at a particular tempera-
ture.

Variation in Buffer pH—The procedure followed was nearly
the same as that described for variation in Tris-glycine buffer
concentration. However, all solutions were adjusted to the pro-
per pH and the same ionic strength (with KCl) prior to de-
salting.

Variation in Phosphate Buffer Concentration, pH 7.0—The
desalting procedure at the various phosphate concentrations em-
ployed (1, 2, 5, 10, and 100 mM) was similar to that described for
variation in Tris-glycine concentration. However, when 1 and
2 mM phosphate was used, the enzyme was desalted in 5 mM
phosphate and then diluted to yield the final phosphate and
EDTA concentrations.

Titration of Sulphydryl Groups in Complex and in Subunits

The number of —SH groups per mole of the enzyme complex
was determined, in the absence and presence of 0.5% sodium

1 Ionic strength values were calculated with pK, values from
Reference 20.
Cubated with a 10-fold molar excess of [14C]acetyl-CoA (8 x 10^5 dpm per nmole) in ice for a period of 30 s. At the end of this period, the reaction was stopped with the addition of 125 ml of 5% HClO (final pH, approximately 1). The precipitated complex and reduced subunits were washed, digested with pepsin, and the resulting peptides subjected to high voltage paper electrophoresis as previously described (17). The paper was then cut into 1-cm strips, and each was assayed for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer as described previously (17).

Analytical Ultracentrifugation Analysis

All sedimentation studies were performed in a Spinco model E ultracentrifuge. Sedimentation velocity runs employed standard single or double sector cells, schlieren optics, and a rotor speed of 59,780 rpm. Sedimentation velocity experiments were usually performed at 20°. However, when dissociation was carried out at 0° and 6°, the rotor temperature was maintained at 1°. Sedimentation constants were calculated from the plot of log of radial boundary position against time (21). In all cases density and viscosity corrections were employed to calculate \( r_{02}, \) values. Density determinations were made by pycnometry and viscosity measurements were made with an Ostwald viscometer that had a drainage time of 86.2 s for distilled water at 20°.

High speed sedimentation equilibrium studies with the use of interference optics were carried out by the short column method of Yphantis (23) in a standard double sector cell equipped with a Bausch and Lomb windows and a column height of less than 3 mm. Weight average molecular weight \((M_w)\) values were obtained from the slopes of natural logarithms of the corrected fringe displacement \( (y) \) with respect to radial distance \( (r) \) squared according to the relationship,

\[
M_w = \frac{d \ln y}{dr^2} \times \frac{2RT}{(1 - \nu \omega^2)}
\]

where \( R, T, \rho, \) and \( \omega \) are the gas constant, absolute temperature, solvent density, and angular velocity, respectively. A value of 0.744 ml per g was used for the partial specific volume, \( \bar{V} \) (24). The measurement of schlieren and fringe patterns was made with a Gaertner microcomparator (Gaertner Scientific Corp., Chicago, Ill.).

Areas under the sedimentation velocity peaks were obtained by weighing the 20-fold magnified tracings obtained with a Bausch and Lomb microcomparator. For pauci-disperse systems, the apparent area measurements were corrected for media solvent density, and angular velocity, respectively. A value of 0.744 ml per g was used for the partial specific volume, \( \bar{V} \) (24). The measurement of schlieren and fringe patterns was made with a Gaertner microcomparator (Gaertner Scientific Corp., Chicago, Ill.).

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Results

Loss of Enzyme Activity in Presence of Tri-Glycine Buffer—

The inactivation of pigeon liver fatty acid synthetase in Tris-glycine buffer, pH 8.35 (\( \mu = 0.008 \)) is a relatively slow process; about 95% of the original enzyme activity is lost during a 24-hour incubation at 25°. The loss in activity is accompanied by

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Fig. 1. Sedimentation velocity pattern of pigeon liver fatty acid synthetase complex after various time periods at 25°C in 5 mM Tris, 35 mM glycine, 1 mM EDTA, and 1 mM 2-mercaptoethanol, pH 8.35. Times of incubation were 1 hour 42 min (upper left); 4 hours 7 min (upper right); 6 hours 55 min (lower left); and 24 hours (lower right). Centrifugations were performed at 20°C and photographs were taken about 32 min after reaching a speed of 59,780 rpm. Enzyme activities at these time periods were 61, 57.4, 27.9, and <1% of the initial value. The protein concentration in each solution was 5 mg per ml. Further details are described under “Experimental Procedure.”

The close relationship between the appearance of the slower moving component and the loss in enzyme activity for fatty acid synthesis in the presence of 2-mercaptoethanol is shown in Fig. 2. The percentage of loss of enzyme activity closely parallels the percentage of conversion of 14 S component to half-molecular weight subunits up to about 70% enzyme inactivation.

Fig. 2. Relationship between loss of fatty acid synthetase activity and the appearance of 9 S components in Tris-glycine buffer, pH 8.35, containing 1 mM EDTA. Losses in enzyme activity in the presence of 1 mM 2-mercaptoethanol (○) and 5 mM DTT (●) were determined from assays performed at 25°C. Protein concentrations of the incubation mixtures were 4 to 5 mg per ml. The quantity of complex remaining in the presence of 2-mercaptoethanol (△) and DTT (●) at each time period was determined from the areas under the protein peak obtained on ultracentrifugation. In a separate experiment, fatty acid synthetase was inactivated in the Tris-glycine-EDTA system in the presence of 1 mM 2-mercaptoethanol (○) and 1 mM DTT (●). Assays for enzyme activity were then made at 25°C. The protein concentration of each incubation mixture was approximately 1.7 mg per ml.

The close relationship between the appearance of the slower moving component and the loss in enzyme activity for fatty acid synthesis in the presence of 2-mercaptopetanol is shown in Fig. 2. The percentage of loss of enzyme activity closely parallels the percentage of conversion of 14 S component to half-molecular weight subunits up to about 70% enzyme inactivation.

An earlier report from this laboratory showed that the inactivation and dissociation of the fatty acid synthetase in low ionic strength Tris-glycine buffer was accompanied by the loss of enzyme sulfhydryl groups (16). Hence, it was of interest to see if inactivation and dissociation of the enzyme complex also occurred in the presence of DTT. Fig. 2 shows that the rate of dissociation of the enzyme was similar in the presence of 5 mM DTT to that obtained with 2-mercaptoethanol. However, a slower rate of loss of enzyme activity was observed in the presence of DTT and the enzyme activity was considerably higher (Fig. 2) than predicted from the amount of complex found by sedimentation analysis, even after corrections for the Johnston-Ogston effect were taken into account. The schlieren pattern obtained after incubation for 24 hours in the presence of DTT was nearly identical with that obtained for the enzyme incubated with 2-mercaptoethanol (Fig. 1). When enzyme activity measurements were carried out in 0.2 M phosphate on the 9 S species obtained after a 24-hour incubation in the presence of Tris-glycine and 5 mM DTT, enzyme activity was approximately 20% of that originally present. In addition, there was a considerable increase in the rate of NADPH utilization during the assay period. Similar results were obtained when DTT was separated from the subunits on a Sephadex G-25 column prior to assay in Tris-glycine buffer, pH 8.35 (the same buffer composition as was used to effect dissociation of the complex). Further experiments revealed that the reduced subunits were rapidly converted to the active complex not only when the ionic strength is increased with 0.2 M phosphate but also when NADPH is present at extremely low concentrations. Finally, it was found in the late phases of this study that reactivation of the enzyme inactivated and dissociated in the presence of DTT could be prevented by assaying for enzyme activity at tempera-

3 R. Muesing, S. Kumar, and J. W. Porter, unpublished results.
TABLE I

<table>
<thead>
<tr>
<th>Initial protein concentration (mg/ml)</th>
<th>( \bar{M}_w \times 10^{13} )</th>
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</thead>
<tbody>
<tr>
<td>0.67</td>
<td>2.40</td>
</tr>
<tr>
<td>1.00</td>
<td>2.26</td>
</tr>
<tr>
<td>1.17</td>
<td>2.28</td>
</tr>
<tr>
<td>1.35</td>
<td>2.35</td>
</tr>
<tr>
<td>2.29</td>
<td>2.22</td>
</tr>
</tbody>
</table>

Subunits were obtained as described under "Experimental Procedure." The solvent was Tris-glycine buffer containing 1 mM EDTA and either 1 mM 2-mercaptoethanol or 1 mM DTT. Centrifugations were performed at 20°C and 13,410 rpm.

A value of 0.744 ml per g was used for the partial specific volume.

![Graph of In y against r^2](image1)

**Fig. 3.** Representative plots of In y (y = fringe displacement in microns) plotted against the square of the distance from the axis of rotation for the subunits of fatty acid synthetase complex in Tris-glycine, pH 8.35, containing 1 mM EDTA and 1 mM DTT (\( \mu = 0.008 \)). Fringe patterns were obtained 20 hours (upper curve, outer ordinate) and 45 hours (lower curve, inner ordinate) after attaining a speed of 13,419 rpm. The initial protein concentration was 1.17 mg per ml. Structures near 0°. Under these conditions dissociation in the presence of 2-mercaptoethanol and DTT gave identical rates of inactivation (Fig. 2).

**Determination of Molecular Weight of Subunits—**The high speed equilibrium sedimentation analysis method was used in an attempt to establish the molecular weight of the 9 S component obtained in Tris-glycine buffer (\( \mu = 0.008 \)). Centrifugations were performed at several initial protein concentrations between 0.67 to 2.29 mg per ml. Plots of the natural log of the fringe displacement (y) versus the radial distance squared (r^2) are shown for the subunits at an initial concentration of 1.17 mg of protein per ml (Fig. 3). The fringe patterns were obtained after 20 hours (upper curve) and 45 hours (lower curve). The plots obtained for the two time periods were linear and parallel. The weight average molecular weights (\( \bar{M}_w \)) of the subunits at various initial protein concentrations are given in Table I. No significant variation in \( \bar{M}_w \) was found for changes in the initial protein concentration. The average value obtained from the data of Table I at these concentrations is 2.29 \( \times 10^4 \pm 0.08 \times 10^3 \), which is approximately one-half of that reported for the complex (14). The value of the partial specific volume (0.744 ml per g) used in the calculations is the same as that used for the complex (14).

**Effect of Ionic Strength on Inactivation and Dissociation of Complex—**The rate of loss of fatty acid synthetase activity is dependent upon the concentration of glycine and Tris in incubation mixtures. Semilogarithmic plots of the rate of loss of enzyme activity as a function of varying buffer concentrations are shown in Fig. 4. The rate of loss of enzyme activity is an apparent first order process up to a 90% inactivation of the enzyme, and the rate depends upon the concentration of buffer. The rate is highest with 10 mM glycine and it decreases as the glycine concentration increases.
centration is increased; the time for 50% inactivation at 10, 35, 200, and 500 mM glycine are 2 hours 55 min, 3 hours 30 min, 5 hours 20 min, and 6 hours 10 min, respectively. In 35 mM glycine containing 0.2 M KCl (uppermost line) the rate of loss of activity is slowest. This rate is similar to that found in the presence of 0.2 M phosphate, pH 8.35. The rate of loss of enzyme activity then appears to be closely dependent upon ionic strength and the data suggest that electrostatic interactions within the complex result in enzyme inactivation.

The existence of intramolecular rearrangements leading to dissociation of the complex is indicated by the finding that the relative rates of inactivation of the complex in 35 mM glycine were identical over a 60-fold variation in protein concentration (0.1, 1.35, and 6.15 mg of protein per ml). These results rule out the existence of an intermolecular interaction, prior to inactivation and dissociation of the enzyme complex, as the rate-limiting step.

Further studies were carried out to determine the relationship between the extent of enzyme inactivation and the appearance of low molecular weight species at various glycine concentrations. While the rates of inactivation and dissociation of the complex corresponded closely in 35 mM glycine (1 mM 2-mercaptoethanol) as shown previously (Fig. 2), the proportion of complex present at higher buffer concentrations was considerably greater than that expected from the loss in enzyme activity (Fig. 5). In 200 mM glycine ($\mu = 0.014$) approximately 5% of the original enzyme activity remained after 24 hours whereas 49% of the protein was present as the 14 S complex. The difference between enzyme activity and percentage of complex is accentuated even further at higher glycine concentrations. After 24 hours, at a glycine concentration of 500 mM ($\mu = 0.024$), approximately 85% of the enzyme was present as the 14 S component whereas only 10% of the initial enzyme activity was retained (Fig. 5). At the higher concentrations of glycine an equilibrium appeared to be established between inactive halves and inactive complex. We have also found that the enzymatically inactive complex obtained in the presence of 500 mM glycine can be completely converted to 9 S subunits when it is dialyzed against the 35 mM glycine buffer.

Effect of Other Anions and Cations on Inactivation of Fatty Acid Synthetase Complex—Further studies were carried out on the inactivation and dissociation of the enzyme complex in the presence of anions other than glycinate. In these experiments, the ionic strength was kept constant ($\mu = 0.036$) and in all cases apparent first order kinetics were observed up to at least 80% inactivation of the enzyme. Clear differences between the effects of glycine and other anions on the rates of inactivation and the extent of dissociation of the complex were obtained (Table II). Thus, in Tris-glycine, pH 8.35, only 26.6% of the original enzyme activity was present after 22 hours, even though 80% of the species existed as the complex. In contrast, in Tris-acetate 8.574 of the enzyme activity was retained after 23 hours and approximately 27.5% of the enzyme existed as a complex. Qualitatively similar results were obtained with Tris-sulfate and Tris-chloride buffers (Table II). The time for 50% inactivation in Tris-acetate buffer is 4.8 hours, whereas in Tris-glycine it is 7.1 hours. The slower rate of inactivation and the drastic reduction in the rate of dissociation of the enzyme complex is peculiar to glycine. In Tris-acetate and other buffers given in Table II, the extent of dissociation is also less than that indicated by the rate of inactivation, but the differences are not as great as with glycine buffers.

The effect of varying the cation was not extensively studied. Substitution of 2-amino-2-methyl-1,3-propanediol for Tris.

**Table II**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Half-life</th>
<th>Time</th>
<th>Complex</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-glycine</td>
<td>7.1</td>
<td>22</td>
<td>80.5</td>
<td>26.6</td>
</tr>
<tr>
<td>Tris-acetate</td>
<td>4.8</td>
<td>23</td>
<td>27.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Tris-sulfate</td>
<td>3.0</td>
<td>24</td>
<td>29.3</td>
<td>8.9</td>
</tr>
<tr>
<td>Tris-phosphate</td>
<td>4.4</td>
<td>26</td>
<td>38.5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Tris-chloride</td>
<td>4.3</td>
<td>26</td>
<td>38.5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>2-Methyl-2-amino-1,3-propanediol chloride</td>
<td>6.6</td>
<td>4.5</td>
<td>70.4</td>
<td>62.0</td>
</tr>
</tbody>
</table>

a The ionic strength of each buffer was adjusted to 0.036 by the addition of NaCl. The pH of each incubation mixture was 8.35.

b Tris, 55 mM, was used when the anion was varied, and HCl, 17.8 mM, was used when the cation was varied. Each buffer contained 1 mM EDTA and 1 mM 2-mercaptoethanol.

c Time interval between the desalting procedure and the attainment of full centrifugation speed.

d Percentage of initial value.

**Fig. 5.** Semilogarithmic plots of the rate of enzyme inactivation of pigeon liver fatty acid synthetase complex in 200 mM (A) and 500 mM (●) glycine and the percentage of complex remaining (●, 200 mM glycine; ○, 500 mM glycine) at various time intervals. The pH of the glycine buffers was adjusted to 8.35 with 1 M Tris. Each buffer contained 1 mM EDTA and 1 mM 2-mercaptoethanol. The percentage of complex was determined from schlieren patterns obtained at the indicated times.
(Table II) resulted in a significantly lowered rate of inactivation; however, the difference between the extent of inactivation and dissociation is not as dramatic as found with Tris-glycine at the same strength.

Effect of pH on Inactivation and Dissociation of Fatty Acid Synthetase Complex—The maximum stability of the fatty acid synthetase complex at neutral pH was previously recognized (24). High pH values (>10) and low ionic strengths were shown to produce subunit species with values of 2, 6.0, 8.5, 9.5, and 10.5 S. No qualitative or quantitative correlation between the pH of the medium, its ionic strength, and the nature of the subunit species was contributed by KCl. On the alkaline side of neutrality the inactivation rate increases as the pH is increased from 7.5 to 9.5 (Fig. 6A). The times taken for a 50% loss of enzymatic activity are 8 hours, 3 hours 8 min, 1 hour 6 min, and 28 min, respectively, at pH 8.0, 8.5, 9.0, and 9.5. At pH 9.0 and 9.5, the process of inactivation deviates slightly from a linear semilogarithmic relationship. At pH values lower than 6.5 (Fig. 6B), a sharp decline in enzyme activity occurs with a small decrease in pH; at pH 5.5, 90% of the original enzyme activity is lost in about 35 min (Fig. 6B). The loss of fatty acid synthetase activity at the lower pH may be attributed in part to aggregation and precipitation of the protein as it nears its pI value of 5.6, since a distinct turbidity develops as activity is lost in pH 5.5 buffer. When the enzyme is incubated at pH 5.5 to 6.0 and then assayed at pH 7.0, there is an appreciable increase in the rate of fatty acid synthesis during the assay. This was particularly noticeable after about 50% of the activity had been lost. For this reason only the initial slopes were used for the activity determinations of the samples at pH 5.5 and 6.0. Hence, the percentage of activity values given in Fig. 6B may be somewhat higher than the actual values. The inactivation cannot, however, be ascribed completely to readily reversible aggregation. After the enzyme had been incubated at pH 6.0 for 8 hours, only 50% of the original activity was recovered when phosphate was added to a final concentration of 0.2 M, pH 7.0.

Loss in enzyme activity and changes in sedimentation behavior of the enzyme complex incubated at various pH values are reported in Table III. At alkaline pH values the dissociation of the enzyme is nearly equal to that expected on the basis of loss in enzyme activity. On the other hand, at pH values less than neutrality, differences between the extent of inactivation and the proportion of the 9 S component were drastically increased. Thus, at pH 6.0, 85% of the enzyme remained as complex, whereas only 15% of the original activity remained.

**Table III**

<table>
<thead>
<tr>
<th>pH</th>
<th>Half-life (hr)</th>
<th>Time (hr)</th>
<th>Complex (%)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.5</td>
<td>0.47</td>
<td>2.3</td>
<td>14.3</td>
<td>7</td>
</tr>
<tr>
<td>8.5</td>
<td>3.1</td>
<td>2.9</td>
<td>60.3</td>
<td>51</td>
</tr>
<tr>
<td>6.0</td>
<td>0.9</td>
<td>2.8</td>
<td>84.6</td>
<td>15</td>
</tr>
</tbody>
</table>

* The buffers employed were 2-methyl-2-amino-1,3-propanediol, pH 9.5, Tris, pH 8.5, and histidine, pH 6.0, all at a concentration of 22 mM. The pH was adjusted with 0.1 N HCl, and KCl was added to adjust the ionic strength to 0.023.

† Time interval between the desalting procedure and the attainment of full speed.

‡ Values are given as percentage of initial activity.
It was also noted that the total area under the schlieren peaks was approximately 60% of that measured for the sample at pH 7.0 at the same protein concentration. This suggests that a large portion of the enzyme is present as higher aggregates which quickly sediment to the bottom of the cell during the initial phase of centrifugation. No peaks were observed which would indicate the presence of dimers or tetramers.

**Effect of Temperature on Rate of Inactivation and Dissociation of Fatty Acid Synthetase Complex**—Polymeric structures stabilized by hydrophobic interactions tend to dissociate more readily as the temperature is lowered below room temperature. Polymeric enzymes such as argininosuccinase (26), pyruvic carboxylase (27), and tryptophanase apoenzyme (28), have been shown to exhibit the property of cold lability. The results of studies on the rate of inactivation of the fatty acid synthetase complex at various temperatures in low ionic strength Tris-glycine buffer are shown in Fig. 7. The rate of inactivation is highest at 0° and then slows as the temperature is raised. At 0° to 25° the inactivation rate is the lowest, and then it increases as the temperature is raised to 30° and then 37°. The times for the loss of half of the original activity were calculated to be 20 min at 0°, 75 min at 6°, 170 min at 12°, 240 min at 18° and 25°, 140 min at 30° and 45 min at 37°. These results conformed with the relative stability characteristics of hydrophobic interactions at 0° and at ambient temperature. The increased inactivation rate above 25° (Fig. 7) cannot, however, be explained on the basis of weakened hydrophobic interactions. The explanation for this result may lie in the disruption of hydrogen bonds and in the strengthening of electrostatic repulsive forces at higher temperatures.

The loss of enzyme activity of the complex at low or high temperatures could result from either the formation of inactive complex or inactive subunits. To differentiate between these possibilities, sedimentation velocity patterns were observed as the enzyme was inactivated at each temperature. After 3 hours of incubation at 0° only a single boundary was observed in the ultracentrifuge. When corrections were made for density and viscosity the calculated sedimentation coefficient was found to be 7.7 S. No other higher or lower molecular weight materials were observed. Thus, at 0° the rate of inactivation is a direct measure of the dissociation of the 14 S component into 9 S subunits. The results obtained at other temperatures lead to similar conclusions.

We also observed that there is considerable activation of the rate of fatty acid synthesis in the assay medium when the enzyme is inactivated at a low temperature (0° and 6°) and then assayed at 30° in 0.2 M potassium phosphate, pH 7.0. A portion of the subunits appear to be in a state which can be easily activated through reassociation to form the active complex. The trigger for this activation phenomenon may be supplied by the sudden change in temperature and ionic strength. For this reason the computation of the initial rates of fatty acid synthesis for the enzyme at 0° and 6° may be in slight error. The corrected initial rates, however, can only be lower than the values reported here and thus would result in steeper slopes of the plots of log of percentage of initial activity versus time (Fig. 7).

As a result of the findings of the above study it was of interest to ascertain the cold sensitivity of the fatty acid synthetase complex at neutral pH and high ionic strength. When the fully active fatty acid synthetase in 0.2 M phosphate buffer, pH 7.0, containing 5 mM DTT was placed at 0°, enzyme activity was rapidly lost with a corresponding loss of complex. Assays were performed at 2°, analytical centrifugation at 1°, and transfers were made under conditions that minimized the possibility of reassociation and reactivation due to warming of the sample. After 3 hours 36 min incubation, approximately 50% of the enzyme activity remained and 43% complex was present. After 24 hours 96% of the initial activity was lost, while approximately 15% of the enzyme existed as complex. These observations are similar to those recently reported for the cold lability of lactating rat mammary gland fatty acid synthetase (29).

**Effect of Low Ionic Strength Phosphate Buffer on Stability of Fatty Acid Synthetase Complex at pH 7.0**—It has been suggested that the attainment of the proper orientation of the individual enzyme of the fatty acid synthetase complex for optimum enzyme activity requires phosphate ions (30, 31). It was desirable, therefore, to determine the stability of the complex at pH 7.0 in the presence of different concentrations of phosphate buffer. The rates of enzyme inactivation at these phosphate concentrations were found to differ markedly (Fig. 8). After 1 hour 50% of the original enzyme activity was lost in 1 mM phosphate, whereas in 10 mM phosphate, no appreciable change in activity occurred. A characteristic feature of the inactivation rate profile in 1 to 5 mM phosphate buffer was the nonlinearity of the plot of the log of the percentage of the initial activity against time. At higher phosphate concentrations the rates of enzyme inactivation were not followed for sufficient...
phosphate ions, at low concentrations, are not capable of controlling the orientation of the component enzymes and behave in a manner which is similar to that observed with other buffers. It is apparent from these results that the enzyme exists mainly as 9 S subunits, whereas 4 S component(s) in the presence of low ionic strength buffer, pH 8.35, and 2-mercaptoethanol. This dissociation was accompanied by the loss of 5 to 6 —SH groups per mole of the complex and an almost complete loss of activity for the synthesis of fatty acids. The enzyme activity loss was attributed to the modification or nonavailability of the —SH group of the cysteine site of the complex (17). In the results discussed below, we have clarified two main questions associated with the inactivation and dissociation process. First, is it possible to effect dissociation of the complex without any apparent oxidation of —SH groups, and secondly, do the 9 S subunits thus obtained possess fatty acid synthetase activity?

**Sulfhydryl Oxidation and Dissociation**—The change in the number of —SH groups per mole of the complex was determined for enzyme dissociated under various conditions. The active fatty acid synthetase complex in 0.2 mM phosphate and 5 mM DTNB contains 39 to 51 free —SH groups. However, when the —SH determinations are made in the presence of 0.5% sodium dodecyl sulfate, 62 to 63 groups are found. The time course for the reaction of —SH groups of the enzyme with DTNB at pH 7.5 is shown in Fig. 9A. When the enzyme was dissociated in the presence of Tris-glycine buffer containing 1 mM 2-mercaptoethanol at pH 8.35 and 25°, titrations for —SH groups gave results (Fig. 9B) which were identical with those we previously reported (16). In the subunits obtained under the above conditions, 44 to 45 free —SH groups per mole of the complex were titrated with DTNB. The same subunits had 57 to 58 free —SH groups per mole of the complex when titrated in the presence of 0.5% sodium dodecyl sulfate. The rates of enzyme inactivation were nearly identical. However, when the 9 S components were tested for their —SH content, significantly different results were obtained (Fig. 9B); a total of 34 to 35 and 46 to 47 —SH groups per mole of the complex were titrated in the absence and presence of 0.5% sodium dodecyl sulfate. The difference in the number of free —SH groups per mole of enzyme under the two sets of conditions where the rates of enzyme inactivation were nearly identical can only be attributed to a difference in the concentration of 2-mercaptoethanol disulfide. The rate or extent of oxidation of the —SH groups does not, therefore, control the rate of inactivation of the complex. If this were so, inactivation would be faster in 5 mM than in 1 mM 2-mercaptoethanol. The results of analysis for —SH groups of the subunit species obtained in the presence of Tris-glycine buffer, 1 mM 2-mercaptoethanol in melting ice (0-4°) at pH 8.35, support the above conclusion. At 0° the oxidation of 2-mercaptoethanol is expected to be slower and the concentration of disulfide available for the exchange reaction would then be less than at room temperature. If the inactivation and dissociation were dependent only on the oxidation of —SH groups, then this process would be minimized at 0°. We found, however, that the rate of loss of fatty acid synthetase activity and of dissociation is faster at 0° than at 25° (Fig. 7). Thus, after 6 to 7 hours the complex is completely dissociated at 0°, whereas 24 to 28 hours are required at 25°. The titration of —SH groups of the subunits obtained at 0° showed the presence of 48 to 49 and 61 to 62 —SH groups per mole of enzyme, respectively, in the absence and in the presence of 0.5% sodium dodecyl sulfate (Fig. 9B). These

**Relationship between Oxidation of Sulfhydryl Groups and Inactivation and Dissociation of Complex**

Recently Kumar, Dorsey, and Porter (16) showed that the pigeon liver fatty acid synthetase complex is completely dis-
FIG. 9. Titration of the —SH groups in the fatty acid synthetase complex and its subunits in the presence and absence of 0.5% sodium dodecyl sulfate. Reactions were performed at 30° in 0.1 M Tris-HCl, pH 7.5, containing 2 mM DTNB and protein at a concentration of approximately 0.5 mg per ml. A, untreated complex (C, ●); subunits obtained in Tris-glycine buffer containing 5 mM DTT (D, ▲); and subunits obtained in degassed 1 mM potassium phosphate, pH 7.0, containing 1 mM EDTA and 1 mM 2-mercaptoethanol (□, ■). B, enzyme dissociated in Tris-glycine containing 1 mM EDTA and 1 mM 2-mercaptoethanol at 25° (O, ●); enzyme dissociated in Tris-glycine containing 1 mM EDTA and 5 mM 2-mercaptoethanol at 25° (Δ, ▲); and enzyme dissociated in Tris-glycine containing 1 mM EDTA and 1 mM 2-mercaptoethanol at 0-4° (□, ■). The closed symbols are for the titrations done in the presence of 0.5% sodium dodecyl sulfate. The optical density increase due to the thiophenolate anion was measured at 412 nm. Details of the controls performed are given under "Experimental Procedure." An extinction coefficient of 1.36 × 10^4 M^-1 cm^-1 was utilized to calculate the number of —SH groups per mole of complex.

numbers are within 2 of the numbers of —SH groups per mole of enzyme titrated in the fully active 14 S species (17, 32). From these results, it is clear that the dissociation of the complex and oxidation of its —SH groups are processes which are quite unrelated.

We have confirmed the above conclusion with another set of experiments. The enzyme was dissociated in Tris-glycine buffer containing 5 mM DTT instead of 1 mM 2-mercaptoethanol at pH 8.35 and 25°C. DTT has been shown to be an extremely efficient reducing agent (33). This reagent derives its effectiveness from its low oxidation-reduction potential (−0.33 volts at pH 7.0) and its irreversible oxidation to a cyclic disulfide. It can thus keep monothiols in a completely reduced state. After 36 hours in Tris-glycine buffer containing 5 mM DTT, the 14 S complex was converted completely to 9 S subunits as determined by analysis of sedimentation velocity patterns. A portion of the enzyme was also dialyzed at room temperature against degassed 1 mM phosphate buffer, pH 7.0, containing 1 mM 2-mercaptoethanol. The Tris-glycine, DTT-treated enzyme, and the enzyme dialyzed against 1 mM phosphate had very little activity for fatty acid synthesis. The inactive species obtained above were compared with enzymatically active complex for the rate and extent of reaction of their —SH groups with DTNB in the absence and presence of 0.5% sodium dodecyl sulfate (Fig. 9A). The total number of —SH groups titrated per mole of the enzyme complex remained the same for dissociated and undissociated enzyme. In addition, the rates of reaction with DTNB were the same for dissociated and undissociated enzyme. These results clearly demonstrate that it is possible to obtain inactive subunits without any apparent oxidation of —SH groups. It should be mentioned, however, that the method is not sufficiently sensitive to exclude completely the possibility of the loss of one or possibly two —SH groups per mole of enzyme.

Are 9.0 S Subunits Active for Fatty Acid Synthesis?—Like the active complex, the subunits obtained in the presence of Tris-glycine and DTT showed 50 to 51 —SH groups in the absence, and 63 to 64 groups in the presence of 0.5% sodium dodecyl sulfate. These subunits can be reassociated to the active complex by increasing the ionic strength in the absence of DTT, showing thereby that the essential —SH groups of the subunits exist in a reduced state.

The problem concerning the presence or absence of fatty acid synthetase activity in 9 S species was approached in another manner. It is well documented (34) that fatty acid synthesis is initiated by the binding of an acetyl group from acetyl-CoA to the hydroxyl site of the complex followed by successive transfers of this group to the 4′-phosphopantetheine and cysteine—SH sites. The malonyl group is similarly transferred from the hydroxyl site to the 4′-phosphopantetheine—SH site. Condensation between the acetyl group bound to the cysteine—SH site and the malonyl group bound to the 4′-phosphopantetheine—SH site then results in the formation of enzyme-bound acetoacetate. Transfer of the acetyl group to the cysteine site and the subsequent condensation reaction are two critical steps in fatty acid synthesis (17). The binding of the 14C-acetyl group of radioactive acetyl-CoA to the 14 S enzymatically active complex and the 9 S subunits at pH 8.35 (μ = 0.008) and in the presence of 5 mM DTT was thus compared. High voltage electrophoresis of a peptic digest of the fatty acid synthetase complex...
(Fig. 10A) showed the presence of three radioactive peptides (A, B, and C) which corresponded to the peptide residues containing the 4'-phosphopantetheine, cysteine, and hydroxyl sites, respectively. The peptide peptides of the 9 S subunits, Fig. 10B, exhibit binding of radioactive acetyl groups to the hydroxyl (B1) and 4'-phosphopantetheine (A) sites. However, very little radioactivity was bound to the cysteine—SH site (B). The radioactivity obtained in the B region, Fig. 10B, was not much above background and it was about 2% of the total radioactivity found in the B region of Fig. 10A. A quantitative comparison of the results is valid since the protein and acetyl-CoA concentrations were identical in each case. It was also found that the total radioactivity covalently bound per mg of protein is essentially the same for both the active complex and the reduced subunits. Under the experimental conditions used, 1.31 moles of acetyl groups were bound per mole of the complex compared to 1.10 for the subunits. A breakdown of the percentages showed that 19%, 58.8%, and 22.2% of the total radioactivity were bound to the hydroxyl, 4'-phosphopantetheine, and cysteine sites of the complex, respectively. The comparative values for the peptic digest from the subunits were 29.5%, 68%, and 2.5%. It is evident that over 90% of the radioactivity bound to the cysteine—SH site of the active complex is about evenly distributed between the hydroxyl and 4'-phosphopantetheine sites of the dissociated subunits. These results demonstrate that under identical experimental conditions the acetyl group is not transferred to the cysteine—SH site of the subunits to any appreciable extent. In the absence of such a transfer condensation between covalently bound acetyl and malonyl groups does not proceed and hence fatty acid synthesis is eliminated.

**Conformational States of Enzyme Species at Different Ionic Strengths**

When the sedimentation coefficient of the complex in low ionic strength buffer at pH 8.35 was measured from sedimentation velocity data, the value obtained was significantly lower than that for enzyme in 0.2 M phosphate, pH 8.35. This difference could be attributed to the incomplete suppression of primary charge effects in low ionic strength buffer (35). Assuming the salt effect to be applicable in this system, extrapolation of the values to zero protein concentration should have given an s20,w value identical with that obtained at higher ionic strength. The variations in the s20,w values with protein concentration for the subunits and the complex in Tris-glycine, pH 8.35 (µ = 0.008) and for the complex in 0.2 M phosphate.
pH 8.35, are shown in Fig. 11 as plots of 1/s hat against protein concentration as suggested by Fessler and Ogston (36). The values of s_{hat} calculated from the results given in Fig. 11A for the complex in low and high ionic strength buffers are 13.5 and 14.0 S, respectively. The small difference (3 to 4%) in s_{hat} may be attributed to the use of identical values of partial specific volume at high and low ionic strengths and to the slight dissociation of the complex at low ionic strength during the period of centrifugation. Further experimentation is needed to clarify this point. However, we have previously shown (37) that the sedimentation coefficient of the complex at low ionic strength changes in the presence of small concentrations of NADPH.

The calculation of s_{hat} of the half-molecular weight subunits gives a value of 9.0 S (Fig. 11B). The points fall on the same line whether the subunits are obtained in the presence of Tris-glycine and 2-mercaptoethanol or Tris-glycine and DTT. These results indicate that the gross conformation of the subunits is unchanged whether the —SH groups are maintained in a fully reduced state or not. Some of the sedimentation coefficient values obtained for the subunits when measured in the presence of undissociated complex are slightly lower than the values obtained in the absence of the complex (Fig. 11B). The differences, however, are within the limits of experimental error. The s_{hat} value of 9.0 S is in agreement with the value predicted for subunits having half the molecular weight of the active fatty acid synthetase complex.

**DISCUSSION**

The present study has demonstrated that electrostatic and hydrophobic interactions and possibly hydrogen bonding are involved in the maintenance of the integrity and enzyme activity of the fatty acid synthetase complex. The rates of inactivation and dissociation of the complex into half-molecular weight subunits are highly dependent on the ionic strength, pH, and temperature of the medium. Furthermore, the inactivation and dissociation of the complex may be affected without oxidation of the sulphydryl groups of the enzyme. Hence, the oxidation of these groups is not essential for the dissociation of the complex.

The stabilization of the fatty acid synthetase complex as a result of electrostatic attractions between oppositely charged groups of several polypeptide chains appears unlikely. Such salt linkages are weakened by high concentrations of electrolytes because of the stabilizing ionic atmosphere created by ionic species surrounding the charged groups (38, 39). Alternatively, attractive forces between oppositely charged groups are strengthened as the ionic strength is lowered. In contrast, the fatty acid synthetase complex is quite stable in high ionic strength buffer and unstable at low ionic strength.

The lowered rate of inactivation of the fatty acid synthetase with increasing ionic strength can be attributed to a dampening of electrostatic repulsions by the ionic medium. Insight into the nature of the ionic interactions of the subunits is provided by the study on the effect of pH on the rates of inactivation and dissociation of the enzyme. At pH values higher than pH, the fatty acid synthetase complex would be more negatively charged and charge repulsions would be increased. This effect would be expected to bring about an increase in the rate of dissociation of the complex. The experiments on the effect of pH, at constant ionic strength, on the rate of enzyme inactivation show that the rate of inactivation, as well as the extent of dissociation, is increased as the pH is increased above 7.0. This inactivation and dissociation at low ionic strength and mildly alkaline pH may be a consequence of the mutual electrostatic repulsion between the two similarly charged halves or between similarly charged regions of the halves which are in close proximity. It cannot be discounted, however, that the initial interactions cause rearrangements within the two subunits which in turn result in secondary interactions prior to dissociation of the complex.

The concept of electrostatic repulsion as the only cause of inactivation and dissociation of the enzyme complex is not supported by the results of studies on the rate of enzyme inactivation as a function of temperature. The rate of inactivation and dissociation is increased by a factor of about 10 when the temperature is lowered from 25° to 0° (Fig. 7). Electrostatic interactions are not altered extensively under these conditions (40). On the other hand, hydrophobic interactions are highly dependent on temperature and are decreased (in terms of interaction between nonpolar regions) as the temperature is lowered. Furthermore, such interactions are strengthened by high ionic strengths but destabilized by low ionic strengths (38, 39). Thus the instability of the fatty acid synthetase complex in low ionic strength Tris-glycine and other buffers can be attributed in large part to a weakening of hydrophobic interactions which maintain the tertiary structure within the component subunits or hold the subunits together.

Additional evidence for a significant contribution of hydrophobic interactions in maintaining the structural stability of the fatty acid synthetase complex is found in the cold lability of the enzyme in 0.2 M phosphate buffer, pH 7.0, containing DTT. This pH and buffer concentration are highly favorable for the maintenance of the integrity of the active complex. However, at 0° activity is rapidly lost and nearly complete dissociation occurs in 24 hours. These results are similar to those reported by Smith and Abraham (39) for the purified lactating rat mammary gland fatty acid synthetase.

Mechanism I incorporates the results reported in this paper. The active enzyme complex (14.0 S) is converted to an enzymatically active intermediate species, Reaction 1, in low ionic strength Tris-glycine buffer at mildly alkaline pH. The active intermediate enzyme species exists in a conformationally altered state as judged from its relative instability and its increased tendency for —SH group oxidation. While it is clear that the 14.0 S complex undergoes a conformational transition at low ionic strength, it is, however, not quite certain whether this change is accompanied by a gross conformational change similar to the one seen on the addition of a low concentration of NADPH to the fatty acid synthetase complex at low ionic strength. The formation of enzymatically inactive complex, Reaction 3, occurs in the presence of 2-mercaptoethanol and is
favored by elevated ionic strength and pH values lower than neutrality. In Reactions 2 and 4, oxidized subunits are obtained in the presence of 2-mercaptoethanol, whereas only reduced subunits are found in the presence of DTT. The formation of oxidised subunits is favored by an increase in temperature and pH. Dissociation of the complex is increased with an increase in pH and a decrease in ionic strength and temperature.

The above changes can be explained as follows. An increase in pH (7.0 to 9.0) and a decrease in ionic strength results in an increased charge repulsion among components of the complex. The enzyme is then converted to an active "intermediate" complex which in turn undergoes sulphydryl oxidation to yield an enzymatically inactive complex or it dissociates to inactive subunits. Lowering of the temperature weakens hydrophobic interactions and results in dissociation of the complex without oxidation of sulphydryl groups of the enzyme. At higher temperatures (37°C) the electrostatic repulsions at low ionic strength, as well as the weakening of hydrogen bonds, compensate for stabilizing nonpolar association and result in dissociation of the enzyme. At ambient temperatures (20-25°C) both hydrophobic interactions and electrostatic influences impart relatively greater stability to the enzyme complex.

It is perhaps worth noting at this point that the alkylating agents previously found successful (15) in dissociating the enzyme complex (e.g. carboxymethyl disulfide, potassium mulate) introduce net negative charges onto the enzyme. The present results suggest that the dissociation effected by these agents may be due to an electrostatic repulsion which is sufficient to overcome the hydrophobic stabilization within the complex.

At present we are unable to explain why there is a greater conversion of enzyme to inactive complex in glycine than in the other buffers. It is obvious that in buffers like Tris-acetate or Tris-sulfate, the formation of inactive complex is not favored as much as in Tris-glycine (see Table III). This discrepancy may be related to a difference between glycinate and other anions.

Present studies have clearly established that the complex can be dissociated into subunits without oxidation of its -SH groups. We have also found that these subunits can be reconstituted into an enzymatically active complex in the absence of DTT. If there were any loss of -SH groups, DTT, in addition to high ionic strength, would have been required for reassociation of the active complex as was shown previously by Kumar, Dorsey, and Porter (16).

When the fatty acid synthesizing capacity of the reduced subunits was measured at 30°C appreciable residual activity was obtained when assays were performed in Tris-glycine or phosphate buffers even in the absence of DTT in the assay medium. Initial experiments suggested that in Tris-glycine buffer this apparent activity is a result of the partial reassociation of the subunits in the presence of NADPH in the assay medium. Later we found that the reassociation could be prevented by performing the assay near 0°C.

To further confirm the loss of catalytic activity of fatty acid synthesis, we took the following alternative approach. The pigeon liver fatty acid synthetase complex is known to contain only 1 mole of the prosthetic group, 4'-phosphopantetheine, per mole of the enzyme complex (41) and this group has been shown to be the covalent binding site for the elongation of the carbon chain (42). In addition, hydroxyl and cysteinyl-SH groups have been shown to be covalent binding sites for acyl groups. The binding of acetyl groups of [14C]acetyl-CoA to the three binding sites of the fatty acid synthetase complex, and the absence of binding of acetyl groups to the cysteine site of reduced subunits (Fig. 10) clearly demonstrates the absence of a transfer of the acetyl group to the cysteine site in the reduced subunits. It is quite clear that the initial condensation reaction cannot occur in the absence of binding of the acetyl group to the cysteine-SH site and that further elongation of the carbon chain is impossible. The subunits are thus incapable of fatty acid synthesis.

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Conformational Changes, Inactivation, and Dissociation of Pigeon Liver Fatty Acid Synthetase Complex: EFFECTS OF IONIC STRENGTH, pH, AND TEMPERATURE
Suriender Kumar, Richard A. Muesing and John W. Porter


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