I. ISOLATION AND CHARACTERIZATION OF ENZYME-BOUND ACETYL ADENYLATE*

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Acetyl coenzyme A synthetase catalyzes the formation of acetyl coenzyme A (acetyl-CoA), adenosine monophosphate (AMP), and pyrophosphate (PPi) from acetate, adenosine triphosphate (ATP), and reduced coenzyme A. The proposed intermediate in this reaction is acetyl adenylate (1), as shown in partial Reactions 1 and 2:

\[
\text{Enzyme} + \text{acetyl} + \text{ATP} + \text{H}^+ \rightarrow \text{enzyme-acetyl adenylate} + \text{PPi} \tag{1}
\]

\[
\text{Enzyme-acetyl adenylate} + \text{CoA-SH} \rightarrow \text{acetyl-CoA} + \text{AMP} + \text{enzyme} + \text{H}^+ \tag{2}
\]

The acyl adenylates participate in a variety of synthetase reactions, and their recent isolation from reactions containing substrate quantities of enzyme suggests that these compounds act as enzyme-bound intermediates (2-7). However, such evidence does not exclude the possibility, emphasized by Boyer, Mills, and Fromm, that the adenylate occurs only as a free side product that is in equilibrium with an enzyme-bound intermediate (8,9). For this reason an attempt was made with acetyl-CoA synthetase to isolate an enzyme-bound intermediate. This effort was facilitated greatly by the recent preparation of an acetyl-CoA synthetase in relatively homogeneous form (10). The present report presents strong evidence that an enzyme-bound intermediate participates in the acetyl-CoA synthetase reaction and that it is acetyl adenylate. Certain characteristics of its formation in partial Reactions 1 and 2 are also described.

EXPERIMENTAL PROCEDURE

Assay and Preparation of Enzymes

Acetyl-CoA synthetase was assayed by measuring the acetyl-dependent disappearance of coenzyme A according to Mahler, Wakil, and Boek (11) under conditions described by Campagnari and Webster (10). Enzyme-dependent disappearance of acetyl adenylate (deacylase activity) was determined by the hydroxamate reaction of Lipmann and Tuttle (12) according to the technique published by Webser and Campagnari (7).

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Charges are not included for the enzyme and coenzyme A molecules; the charge assigned to ATP does not allow for possible chelation with magnesium.

Protein concentrations were measured by the method of Gornall, Bardawill, and David with the use of crystalline bovine albumin as the standard (13). Units of enzymatic activity are expressed in micromoles of substrate disappearance per minute at 37°; specific activities are expressed as units of activity per mg of protein.

Acetyl-CoA synthetase was purified from bovine heart mitochondria as described by Campagnari and Webster (10). In the analytical centrifuge, nearly all of the protein traveled in a single peak with a sedimentation constant of 4.4 S. The best preparations catalyzed the formation of 13.4 moles of acetyl-CoA per minute per mg of protein.

Acetyl adenylate deacylase (hydrolase) was freed from acetyl-CoA synthetase by heating and was purified 15-fold over a crude supernatant fluid from bovine mitochondria as previously described (10). An ammonium sulfate precipitate of this material was diluted to a protein concentration of 30 mg per ml with 0.02 M KHCO₃-0.0005 M Versene (EDTA), and was placed on a column of Sephadex G-100, 80 x 1.6 cm. When eluted with 0.02 M KHCO₃-0.0005 M Versene, the deacylase appeared after most of the protein had been recovered, and the activity of this enzyme increased as the protein concentration decreased. The pooling of the fractions having the highest specific activities resulted in an additional 23-fold purification. The final preparation catalyzed the disappearance of 14 to 17 micromoles of acetyl adenylate per minute per mg of protein.

Materials

Sodium acetate-1-¹⁴C was purchased from the Isotopes Specialties Company, Inc. Acetyl-1-¹⁴C-CoA, purchased from New England Nuclear Corporation, contained significant contaminating radioactivity, which was not separated from the acetyl-¹⁴C-adenylate fraction by chromatography on Dowex 1 (Cl⁻). This contamination was removed by chromatography on DEAE-cellulose in a manner similar to that described below for the isolation of enzymatically synthesized acetyl-CoA. The purified acetyl-¹⁴C-CoA had a specific activity of 1.81 x 10⁶ c.p.m. per micromole. Chemically synthesized nonradioactive acetyl-CoA, purchased from Pabst Laboratories, was purified by chromatography on Whatman No. 1 paper in a descending system with the use of ethyl alcohol-0.5 M ammonium acetate buffer (pH 4.0), 5:2, as the solvent mixture. Under these conditions, the bulk of the ultraviolet light-absorbing material migrated with an Rf of 0.30 to 0.32. The presence of free sulfhydryl groups was indicated only after treatment with hydroxylamine or dilute alkali (14).
Acetyl adenylate was prepared from acetic anhydride and adenosine monophosphate according to the method of Avison (15). Acetyl-1-14C-adenylate was prepared enzymatically as described below from acetate-1-14C, ATP, and substrate quantities of acetyl-CoA synthetase. The protein was precipitated at 4°C by trichloroacetic acid, which was subsequently removed by ether extraction. The final solution containing the radioactive adenylate was brought to pH 6.0 by slow addition of NaOH. This product was used within 15 minutes after its preparation.

Adenosine triphosphate and adenosine monophosphate were products of the Pabst Laboratories. Other chemicals were the commercially available reagent grade.

Methods

For the biosynthesis of acetyl-14C-adenylate from acetate-14C and ATP, the standard reaction mixture contained 29 μmoles of potassium phosphate buffer (pH 7.3) or 72 μmoles of Tris buffer (pH 8.0), 3.6 μmoles of magnesium chloride, 1.25 μmoles of potassium-ATP (pH 8.0), 2.5 μmoles of sodium acetate-1-14C (62,500 to 71,800 c.p.m.), and 1.8 to 7.2 units of acetyl-CoA synthetase per 0.9 ml. The incubation mixture for the reverse partial reaction contained 29 μmoles of potassium phosphate buffer (pH 7.3) or 72 μmoles of Tris buffer (pH 8.0), 0.9 μmole of potassium-AMP (pH 8.0), 2.65 μmoles of acetyl-1-14C-CoA (48,000 c.p.m.) and 1.8 to 7.2 units of acetyl-CoA synthetase per 0.9 ml. The mixtures were incubated for 0.5 minute at 37°C and were then either chilled to 0°C in an ice bath or treated with 0.1 ml of 25% trichloroacetic acid to denature the enzyme.

Enzyme-associated radioactivity was separated from other isotopic compounds by placing an aliquot from a chilled reaction mixture on a Sephadex G-100 column, 1.5 x 20 cm, that was equilibrated with 0.02 M KHCO3-0.0005 M Versene at 4°C. When eluted with the equilibrating solution, acetyl-CoA synthetase activity appeared slightly behind the solvent front. The fractions containing enzyme were assayed for radioactivity in a Nuclear-Chicago liquid scintillation counter.

Acetyl-14C-adenylate was isolated from either the incubation mixtures or the Sephadex fractions by a method previously described in detail (7). Briefly, the enzymatic protein was denatured by trichloroacetic acid and removed by centrifugation at 4°C. Nonradioactive acetyl adenylate was added to the supernatant solution, which was brought to pH 4 and chromatographed on a Dowex 1-X2 column, 1 x 15 cm (chloride, 200 to 400 mesh), at 4°C. The adenylate fraction was eluted with 0.025 M HCl and was estimated by the hydroxamic acid procedure of Lipmann and Tuttle (12). Aliquots from each fraction were extracted with ether to remove free acetic acid-14C and were assayed for radioactivity in a Nuclear-Chicago liquid scintillation counter. The identity of acetyl-14C-adenylate was established further by its electrophoretic mobility and its conversion to acetyl-14C-hydroxamate (7).

For conversion of enzyme-associated radioactivity to acetyl-14C-CoA, the incubation mixtures contained 25 μmoles of Tris buffer (pH 8.0), 37 μmoles of oceonzyme A, and 3000 to 9000 c.p.m. of enzyme-associated radioactivity from a Sephadex column in a volume of 1.7 ml. For the control experiment, the Sephadex fraction was boiled for 1.5 minutes before it was used; this resulted in the inactivation of the enzyme and in the destruction of 10 to 15% of the acetyl-14C-adenylate. After incubation for 1 minute at 37°C, each tube received 0.2 ml of 25% trichloroacetic acid, and the denatured protein was removed by centrifugation. The supernatant solutions were brought to pH 4.0 with μ NaOH and were chromatographed on DEAE columns, 1.5 x 13 cm, equilibrated with water at 4°C. Radioactivity was eluted with 0.003 M HCl-0.03 M LiCl according to a modification of the technique of Moffatt and Khorana (16).

For identification purposes the appropriate radioactive fractions from a DEAE column were desalted with Dowex 50-H+, concentrated by evaporation, and spotted on Whatman No. 1 paper along with nonradioactive acetyl-CoA (repurified from the commercial preparation). Descending chromatography was performed with ethyl alcohol-0.5 M ammonium acetate buffer (pH 4.0), 5:2, as the solvent system. The location of radioactive compounds, ascertained by radioautography, was compared with that of the ultraviolet light absorption of authentic acetyl-CoA. Conversion to acetoxyhydroxamic acid and identification of this derivative by paper chromatography was accomplished as previously outlined (7).

For experiments concerning the stability of free and enzyme-associated acetyl-14C-adenylate in the presence of acetyl adenylate deacetylase, the reaction tubes contained 40 μmoles of potassium phosphate buffer, pH 7.0, and 0.017 to 2.0 units of deacetylase at 37°C. The reactions were started by the addition of 0.05 to 0.32 μmole of acetyl-1-14C-adenylate either in the enzyme-associated form (fraction from a Sephadex column) or the free form to make a final volume of 1.6 ml. They were terminated by the addition of 0.1 ml of 25% trichloroacetic acid at 0, 0.5, 1.0, and 1.5 minutes. The denatured protein was removed by centrifugation and acetyl-14C-adenylate was isolated from the supernatant solutions by chromatography on Dowex 1 (Cl) as noted above.

RESULTS

Enzymatic synthesis of acetyl adenylate from acetate and ATP was observed under equilibrium conditions with substrate quantities of highly purified acetyl-CoA synthetase. The first partial reaction was completely dependent on enzyme, ATP, and a magnesium salt (Table I). Radioactive acetyl adenylate was isolated also when substrate quantities of synthetase were reacted, instead, with acetyl-14C-CoA and AMP (Table II). The reverse of the second partial reaction was completely dependent on AMP and active enzyme; pyrophosphate, 0.1M, decreased the yield of the adenylate significantly, as would be expected. Equilibrium for both partial reactions was achieved.

TABLE I

<table>
<thead>
<tr>
<th>System</th>
<th>Acetyl-14C-adenylate c.p.m.</th>
<th>mmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>4620</td>
<td>0.198</td>
</tr>
<tr>
<td>Complete system minus MgCl</td>
<td>31</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Complete system minus ATP</td>
<td>41</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Boiled enzyme</td>
<td>28</td>
<td>&lt;0.002</td>
</tr>
</tbody>
</table>
Enzymatic formation of acetyl adenylate from acetyl coenzyme A and adenosine monophosphate

Triate buffer (pH 8.0), 72 moles, and acetyl-CoA synthetase (specific activity, 10), 2.5 units, were employed for each 0.9 ml of incubation mixture. Otherwise, mixtures were made up and incubated, trichloroacetic acid was added, and acetyl-14C-adenylate was isolated by chromatography over Dowex 1 (Cl-) as described under "Experimental Procedure." Quantities of product (acetyl-14C-adenylate) are expressed per ml of incubation mixture.

<table>
<thead>
<tr>
<th>System</th>
<th>Acetyl-14C-adenylate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>2400</td>
</tr>
<tr>
<td>Complete system plus MgCl2</td>
<td>4800</td>
</tr>
<tr>
<td>Complete system plus PPi (1 mmol)</td>
<td>2960</td>
</tr>
<tr>
<td>Complete system minus AMP</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Boiled enzyme</td>
<td>42</td>
</tr>
</tbody>
</table>

TABLE II

3. Chromatography on Sephadex G-100 of a complete reaction mixture containing acetate-14C, ATP, and substrate quantities of acetyl-CoA synthetase. The incubation mixture contained 1.25 mmoles of potassium-ATP, pH 8.0, in addition to the components described for Fig. 2. The mixture, 0.9 ml, was incubated at 37° and an 0.85-ml aliquot was subsequently chromatographed on Sephadex at 4° as described in "Methods." One hundred four per cent of the added radioactivity and 94% of the enzymatic activity were recovered.

FIG. 2. Chromatography on Sephadex G-100 of a control reaction mixture containing acetate-14C and AMP, and substrate quantities of acetyl-CoA synthetase. The reaction mixtures contained 72 moles of Tris buffer (pH 8.0), 0.9 mmole of potassium-AMP, 2.55 mmoles of acetyl-14C-CoA (48,000 c.p.m.), and 2.25 units of enzyme (0.25 mg) in a volume of 0.9 ml. They were incubated at 37° for the periods shown, and the mixture was denatured with 0.1 ml of 25% trichloroacetic acid; acetyl-14C-adenylate was isolated from an aliquot of the trichloroacetic acid supernatant as described in "Methods." Quantities of product are expressed per ml of incubation mixture.

One hundred one per cent of the added radioactivity and 95% of the enzymatic activity were recovered. Within 0.2 minute at 37°, so that a 0.5-minute period of incubation was selected for routine studies (see Fig. 1 for the reverse of the second partial reaction). The addition of a magnesium salt did not affect the yield of adeylate in the reverse of the second partial reaction (Table II), although complete magnesium dependence was shown with the same preparation of enzyme for the formation of acetyl adenylate from ATP and acetyl in the first partial reaction. When acetate-14C, MgCl2, and substrate quantities of enzyme were incubated together and subsequently chromatographed on Sephadex G-100, the enzyme was eluted shortly after the solvent front and was separated practically completely from all radioactivity (Fig. 2). When ATP was included in the above reaction mixture, a peak of radioactivity that coincided with the activity of the enzyme appeared near the solvent front (Fig. 3). This peak was not noted if the enzyme was boiled or if the magnesium salt was omitted from the incubation mixture. A similar peak was observed when the enzyme reacted instead with acetyl-14C-CoA and AMP. In this case, AMP and active enzyme, but not MgCl2, were required.

When the enzymatically active protein from a Sephadex fraction was precipitated with trichloroacetic acid, radioactivity was released that, when chromatographed on Dowex 1 (Cl), behaved identically with chemically synthesized acetyl adenylate; about 80 to 94% of the total counts were recovered in this fraction. For further identification, this radioactive fraction was concentrated and subjected to paper electrophoresis with subsequent radioautography as outlined previously (7). Under these conditions, the darkened spot on the exposed film corresponded in position to the place where ultraviolet absorption of chemically synthesized acetyl adenylate occurred. In another type of experiment, the radioactive fraction was incubated in the presence of 0.1 M hydroxylamine, pH 6.8, for 10 minutes at 37°. Nonradioactive acetyl adenylate, 20 mmoles, was then added, and the solution was chromatographed on Dowex 1 (Cl) at 4°. No radioactivity was found in the ether-extracted acetyl adenylate fractions. This result is consistent with the formation of acet-14C-hydroxamate.

The conversion of enzyme-associated radioactivity recovered from a Sephadex column to acetyl-14C-CoA was tested by incu-
hating the former with refined coenzyme A and chromatograph-
ing the radioactive products on DEAE-cellulose (see "Methods").
A peak of radioactivity, not seen in the control, was eluted after
approximately 185 to 200 ml of 0.003 M HCl-0.03 M LiCl had
passed through the DEAE column. This peak contained 86
to 94\% of the radioactivity initially added, and it was separate
from acetate and acetyl adenylate. Fractions in this major
peak were combined, desalted, concentrated, and chromato-
graphed on Whatman No. 1 paper in the presence and absence
of repurified commercial acetyl-CoA. Virtually all of the radio-
activity in the dried chromatogram migrated with an \( R_f \)
of 0.30 to 0.32, and in the former case it appeared in the ultraviolet-
absorbing spot attributed to authentic acetyl-CoA.

The binding of acetyl-\(^{14}\)C-adenylate to the synthetase enzyme
was investigated by comparing the stability of enzyme-associated
acetyl-\(^{14}\)C-adenylate from a Sephadex column to that of free
acetyl-\(^{14}\)C-adenylate. Incubation experiments were carried
out at 37\°C both in the presence and in the absence of a highly
purified preparation of acetyl adenylate deacetylase. When
deacetylase was omitted from the incubation mixture, acetyl
adenylate deteriorated only 8\% or less in 0.5 minute, whether or
not acetyl-CoA synthetase was present. Addition of as little
as 0.017 unit of deacetylase caused a rapid breakdown of free
acetyl-\(^{14}\)C-adenylate but produced no increase in the adenylate
breakdown over the control values when enzyme associated
acetyl-\(^{14}\)C-adenylate was used as the substrate (Fig. 4). Break-
down still occurred if the deacetylase was preincubated with
synthetase before addition of free acetyl-\(^{14}\)C-adenylate; this finding
indicates that, under these conditions, there was no appreciable
inhibition of the deacylase by acetyl-CoA synthetase. In
another experiment, bovine serum albumin did not protect free
acetyl adenylate from the action of deacetylase. Variations in
the initial concentrations of acetyl-\(^{14}\)C-adenylate over a 6-fold
range in different experiments did not cause marked differences
in the percentage of acetyl adenylate broken down by the
deacetylase in the first 0.5-minute period. Only when the amounts
of deacetylase were increased 120-fold did appreciable breakdown
of the enzyme-associated acetyl-\(^{14}\)C-adenylate take place.

In studying the formation of acetyl adenylate from ATP,
acetate, and substrate quantities of enzyme, it was discovered
that the concentration of acetyl adenylate, under equilibrium
conditions, increases asymptotically with increasing concentra-
tions of acetate (7). It appeared that this fact could be used
to investigate the binding of acetyl adenylate to the enzyme. The
maximal number of millimicromoles of acetyl adenylate formed
per unit of enzyme employed could be determined by plotting the
reciprocal of the concentration of product (the adenylate) pres-
cent, under equilibrium conditions, as a function of the reciprocal
of the substrate (acetate) concentration. This quantity could
then be assumed to equal the number of millimicromoles of en-
zyme present. A maximal molecular weight for the enzyme could
then be calculated, since the number of units added and the
specific activity of the homogeneous enzyme were known.
Theoretically, this value should agree with the molecular weight
obtained by physical measurements, if the assumptions of one
active site per enzyme molecule and a 1:1 stoichiometry between
enzyme and maximal adenylate are justified. When three such
experiments were carried out under optimal conditions of pH
and with highly purified fresh acetyl-CoA synthetase, a molecular
weight of 83,000 to 85,000 was found (Fig. 5). This figure is
within the range of molecular weights predictable from the

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{The stability of free and enzyme-associated acetyl-\(^{14}\)C-
adenylate in the presence of acetyl adenylate deacetylase. Each
reaction tube contained 40 \( \mu \)moles of potassium phosphate buffer,
\( pH \) 7.4, and 0.017 unit (0.37 \( \mu \)g) of acetyl adenylate deacetylase
in 0.8 ml at 37\°C. The first reaction was started by the addition of
1800 c.p.m. (0.32 \( \mu \)mole) of free acetyl-\(^{14}\)C-adenylate, and the
second was initiated by adding 3040 c.p.m. (0.12 \( \mu \)mole) of
enzyme-associated acetyl-\(^{14}\)C-adenylate from a Sephadex column.
After the incubation periods shown, the reactions were terminated
with trichloroacetic acid, and acetyl-\(^{14}\)C-adenylate was isolated
by chromatography on Dowex 1 (Cl\(^-\)) as described in "Methods."
The products obtained after the incubation periods shown are
expressed as a percentage of the acetyl-\(^{14}\)C-adenylate recovered
after incubation at zero degrees (zero time).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{The concentration of acetyl adenylate at equilibrium
as a function of acetate concentration in the acetyl CoA syn-
thetase reaction. The reaction mixtures contained 20 \( \mu \)moles
of potassium phosphate buffer (\( pH \) 7.3), 3.6 \( \mu \)moles of magnesium
chloride, 1.25 \( \mu \)moles of potassium-ATP (\( pH \) 8.0), increasing
amounts of sodium acetate-\(^{14}\)C (25,000 c.p.m. per \( \mu \)mole) and
2.02 units of enzyme (0.18 mg) in a volume of 0.9 ml. Mixtures
were incubated for 1 minute at 37\°C, the enzyme was denatured
by 0.1 ml of 25\% trichloroacetic acid, and acetyl-\(^{14}\)C-adenylate
was isolated by chromatography on Dowex 1 (Cl\(^-\)) as stated in
"Methods." Concentrations of substrate (acetate) and product
(acetyl adenylate) at equilibrium are expressed in millimicromoles
per ml of incubation mixture. The units for the reciprocal plot
are expressed in molarity\(^{-1}\). The concentration for \( 1/P_{max} \) is
5 \( \times 10^{4} \) \( \text{M}^{-1} \), and \( P_{max} = 2 \times 10^{4} \) \( \mu \)mole acetyl adenylate.
The 2.25 \( \mu \)moles of enzyme used per ml of incubation mixture is equivalent
to 0.169 \( \mu \)g per liter of homogeneous enzyme (specific activity,
13.4). Then the molecular weight for the enzyme is (0.169 g per
liter)/(2.0 \( \times 10^{4} \) mole per liter), or 84,500 g per mole, if there is
a 1:1 stoichiometry between maximal adenylate and enzyme
concentrations in the incubation mixture.

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sedimentation constant of 4.4 S, which was obtained for the purified enzyme (10).

From the above type of experiment, the concentration of acetate at which the enzyme is half saturated with acetyl adenylate can be determined directly. In eight experiments under the conditions employed (high concentrations of ATP and MgCl₂ and relatively low concentrations of enzyme), this figure ranged from 0.6 to 2.6 × 10⁻⁴ M, and the average value was 1.5 × 10⁻⁴ M.

DISCUSSION

Recently, the isolation of acetyl-[¹⁴C]-adenylate from a trichloroacetic acid supernatant of a reaction mixture containing acetate-[¹⁴C], ATP, and substrate quantities of acetyl-CoA synthetase was accomplished (7). The enzyme preparation used for these experiments was not highly purified and contained a contaminating enzyme (acetyl adenylate deacylase) that catalyzed the destruction of chemically synthesized acetyl adenylate (7, 17). The preparation by Campagnari and Webster of acetyl-CoA synthetase in essentially homogeneous form and free of the deacylase has permitted experiments reported herein (10).

Three types of evidence indicating that acetyl adenylate occurs as an enzyme-bound intermediate in the acetyl-CoA synthetase reaction are presented. Most significant is that chromatography on Sephadex G-100 of a reaction mixture containing acetate-[¹⁴C], ATP, MgCl₂, and large amounts of synthetase yielded fractions containing enzymatic activity and radioactivity in constant proportion (Figs. 2 and 3). The same result was noted when substrate quantities of enzyme were incubated, instead, with acetyl-[¹⁴C]-CoA and AMP. In both instances, the major radioactive compound isolated from the Sephadex fractions was acetyl-[¹⁴C]-adenylate. The acetyl-[¹⁴C]-adenylate appeared to be bound to the enzyme, not only because there was a constant ratio between radioactivity and enzyme in these fractions, but also because it was shown that after heat denaturation of the enzyme, the protein separated from acetyl-[¹⁴C]-adenylate when both were chromatographed together on a column of Sephadex G-100. Furthermore, the enzyme-associated radioactivity was converted to acetyl-[¹⁴C]-CoA by incubation with reduced coenzyme A, and denaturation of the enzyme prevented this conversion.

Another indication that adenylate is bound to enzyme was the finding that the radioactive adenylate in the Sephadex fractions was protected from the action of acetyl adenylate deacylase by being associated with acetyl-CoA synthetase (Fig. 4). This result probably is specific for the synthetase protein since it was not noted when bovine serum albumin was substituted.

A more indirect line of evidence that suggested enzymatic binding of acetyl adenylate was derived by determining the maximum molar concentration of acetyl adenylate equivalent to a given concentration of homogeneous enzyme in grams per liter (Fig. 5). Then, a molecular weight was calculated for the enzyme with the assumptions of one active site per enzyme molecule and a 1:1 stoichiometry between enzyme and maximal adenylate. The molecular weight estimated by this method turned out to be consistent with the sedimentation characteristics of the purified synthetase (10).

When, under equilibrium conditions, the effect of increasing acetate levels on the concentration of acetyl adenylate was studied, it was noted that the enzyme was half saturated with adenylate at acetate concentrations of about 0.6 to 2.8 × 10⁻⁴ M. This is of interest in that the Kₐ of 7.9 × 10⁻⁴ M for acetate in the over-all reaction appears to be appreciably higher (10).

A possible role for magnesium in the transfer of acetate to the acceptor CoA has been debated (18-20), but it has not been studied directly because enzyme preparations employed in previous studies have exhibited only a partial requirement for added magnesium (7). Since our highly purified enzyme showed complete magnesium dependence for the biosynthesis of acetyl adenylate from acetate and ATP (the first partial reaction), the effect of magnesium could be evaluated in the reverse of the second partial reaction (formation of acetyl adenylate from acetyl-CoA and AMP). Not only did the formation of acetyl adenylate occur without a magnesium salt in the second partial reaction, but the yield under equilibrium conditions was not increased by adding this divalent cation. Thus, it appears unlikely that magnesium is essential either for the binding of acetyl adenylate to the enzyme or for its formation from acetyl-CoA. These findings are consistent with the formation of an ATP-magnesium chelate in the first partial reaction, but they in no way exclude an effect of magnesium on the rate of acetyl-CoA formation from enzyme-bound acetyl adenylate.

The study of partial reactions in the acetyl-CoA synthetase system has been used to investigate the mechanism of this general type of reaction. The results indicate that much can be learned from this technique. Thus, enzyme-associated acetyl adenylate has been isolated and shown to act as an intermediate in the formation of acetyl-CoA, and the presence of a magnesium salt was not required for the transfer of the acetate moiety to acetyl-CoA. Acetyl adenylate deacylase destroyed free acetyl adenylate under conditions in which it had no effect on synthetase-associated acetyl adenylate. This finding strongly suggests that acetyl adenylate occurs principally in the enzyme-bound form. The 1:1 stoichiometry between acetyl-CoA synthetase and the maximal yield of acetyl adenylate calculated for equilibrium conditions in the first partial reaction provides further support for the enzymatic binding of acetyl adenylate. The future investigation of these partial reactions should provide information concerning the role of monovalent cations in the acetyl-CoA synthetase system (10, 21). Studies with highly purified acetyl-CoA synthetase should also yield data concerning the specificity of the CoA-ester in the formation of enzyme-bound acetyl adenylates.

SUMMARY AND CONCLUSIONS

1. The formation of an enzyme-bound intermediate in the acetyl coenzyme A synthetase reaction has been investigated. When acetyl-[¹⁴C], adenosine triphosphate, MgCl₂, and substrate quantities of a highly purified enzyme were incubated together and the mixture was chromatographed on Sephadex G-100, a peak of radioactivity that coincided with the enzymatic activity appeared. The same peak was observed when the enzyme was incubated instead with acetyl-[¹⁴C]-coenzyme A and adenosine monophosphate. Most of the isotope in the Sephadex fractions containing acetyl coenzyme A synthetase was recovered as acetyl adenylate. Incubation of this enzyme-associated radioactivity with reduced coenzyme A yielded acetyl-[¹⁴C]-coenzyme A when the enzyme was active. Adenosine triphosphate, magnesium salt, and active enzyme were required for the biosynthesis of acetyl adenylate from acetate; active enzyme and adenosine...
monophosphate, but not magnesium salt, were necessary for the formation of acetyl adenylate from acetyl coenzyme A.

2. Free acetyl adenylate was more susceptible to the action of acetyl adenylate deacylase than was synthetase-associated acetyl adenylate. Albumin did not protect free acetyl adenylate from the action of deacylase nor did the synthetase inhibit the deacylase; this result suggests that the protective effect of the synthetase protein was specific.

3. The maximal concentration of acetyl adenylate for a given amount of synthetase was obtained from experiments in which the yield of adenylate at equilibrium was plotted as a function of the acetate concentration. From this experiment, a molecular weight was obtained for the enzyme that was consistent with its sedimentation characteristics by assuming one active site per enzyme molecule and a 1:1 stoichiometry between acetyl adenylate and enzyme.

4. The above findings support the existence of acetyl adenylate as an enzyme-bound intermediate in the acetyl coenzyme A synthetase reaction.

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Studies of the Acetyl Coenzyme A Synthetase Reaction: I. ISOLATION AND CHARACTERIZATION OF ENZYME-BOUND ACETYL ADENYLATE
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