THE ACETATE ACTIVATING ENZYME SYSTEM OF HEART MUSCLE

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Nachmansohn and Machado (1) discovered a soluble enzyme system in extracts of brain tissue which catalyzed the formation of active acetate in the presence of ATP and CoA. Active acetate was detected by the acetylation of choline. Later Lipmann (2, 3) reported an enzyme system in extracts of pigeon liver which catalyzed the CoA and ATP-dependent acetylation of sulfanilamide by acetate. The identification of active acetate as acetyl CoA by Lynen et al. (4) opened the way for a systematic study of the acetate activation system.

The present investigation2 deals with the properties of a system prepared from heart muscle of pig or rabbit which catalyzes the over-all basic reaction,

\[\text{ATP + CoA + acetate \to AMP + acetyl CoA + PP}\]

The assay of the enzyme system is based on trapping acetyl CoA either as acethydroxamic acid (Equation 2) or as citrate (Equations 3 and 4) in the presence of malic dehydrogenase and the condensing enzyme of Ochoa, Stern, and Schneider (6).

\[\text{Acetyl CoA + hydroxylamine \to acethydroxamic acid + CoA}\]

\[\text{Acetyl CoA + oxalacetate \to citrate + CoA}\]

\[\text{Malate + DPN^+ \to oxalacetate + DPNH + H^+}\]

Lipmann and Tuttle (7) were the first to use hydroxylamine as a trapping reagent for "activated carboxyl" groups. Stern et al. (8) have introduced the coupling possibilities of the reactions shown in Equations 3 and 4. Lipmann et al. (9) have made a preliminary announcement of an enzyme

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1 The following abbreviations will be used: adenosine triphosphate, ATP; adenosine diphosphate, ADP; adenosine-5-phosphate, AMP; diphosphopyridine nucleotide, DPN; coenzyme A, CoA; acetate activation enzyme, AAE; and inorganic pyrophosphate, PP.

2 A preliminary account of this work has been given (5).
from yeast which catalyzes the activation of acetate according to Equation 1.

**Materials and Methods**

*Preparation of Enzymes*—The acetate activation enzyme (AAE) system was prepared in crude form from pig heart muscle as follows: The bicarbonate (0.02 M) extract of an “acetone” powder of fresh, minced muscle prepared as described previously (10) was serially precipitated with ammonium sulfate at neutral pH, first at 21 gm. per 100 ml. and then at 42 gm. per 100 ml. The latter was refractionated with ammonium sulfate stepwise into three fractions, the middle one of which (precipitated between 28 and 35 gm. of ammonium sulfate per 100 ml.) showed the highest specific activity. All manipulations were carried out at 0°. The precipitates were taken up in the minimal volume of 0.02 M potassium bicarbonate and the solutions were stored at −10°. The enzyme was stable for an indefinite period under these conditions. When the activity of the preparation per mg. of protein is relatively high (>3 μM of acetate activated per 15 minutes per mg. at 38°), it is not necessary to remove salts from the solution before testing.

When essentially the same procedure was applied to a mitochondrial suspension of rabbit heart as starting material (11), the final preparation was found to be about 3 times more active per mg. of dry weight than the corresponding preparation from the whole homogenate of pig heart.

A preparation containing both malic dehydrogenase and condensing enzyme free from the AAE was made from pig heart muscle by a modification (10) of the procedure of Ochoa et al. (6).

We are indebted to Dr. S. A. Kuby for a sample of highly purified ATP-creatine transphosphorylase prepared from rabbit skeletal muscle.

*Assay Procedures*—The details of, or references for, some of the analytical procedures have been given in a previous communication (10). Inorganic pyrophosphate was determined as orthophosphate after hydrolysis by yeast pyrophosphatase (12, 13). Creatine phosphate was determined as inorganic phosphate in the Fiske-Subbarow procedure (14) and differentiated from inorganic phosphate by the Lowry-Lopez method (15). Sulfhydryl was estimated by the method of Grunert and Phillips (16).

Hydroxamic acid formation was followed by the method of Lipmann and Tuttle (17). The incubated enzyme mixture (2 ml.) was cooled to 0° and mixed with 1 ml. of hydrochloric acid (1 volume of acid to 3 volumes of water) and 1 ml. of a ferric chloride solution (5 per cent in 0.1 N hydrochloric acid). The suspension was clarified by centrifugation. The absorption of the supernatant fluid at 540 με was then measured against a blank containing all components except acetate. An optical density reading of 0.16 corresponds to 0.25 μM of acethydroxamic acid per ml.
Coenzymes—The sources of some of the coenzymes have been given previously (10). Acetyl CoA was prepared by interaction of thiolacetic acid and CoA by the method of Wilson (18).

Neutralization of Reagents—The AAE system is sensitive to sodium ions. Therefore, all reagents which were added to the enzyme mixture had to be sodium-free. The practice of neutralizing all acidic reagents with potassium hydroxide was routinely followed. Dipotassium ATP (Pabst) was used instead of the usual disodium salt. Hydroxylamine with a low salt content was prepared as follows: Hydroxylamine hydrochloride (5.6 gm.), dissolved in 20 ml. of absolute methyl alcohol, was titrated with potassium hydroxide (about 6 gm.) dissolved in 20 ml. of methyl alcohol to about pH 7.2. In testing the pH of the solution, the aliquot must be diluted at least 10-fold before measurement. The precipitate of potassium chloride was removed by filtration. The solution was then concentrated by distillation in vacuo down to about 4 ml. and then diluted with water to 12 ml. The amount of hydroxylamine present in the solution was determined by titration of an aliquot with standard acid, and, after neutralization with potassium hydroxide (3 N) to pH 8, the solution was diluted to a final molarity of 2.5.

Results

Acethydroxamic Assay System—Table I summarizes a component study of the system. Fig. 1 shows the dependence of the rate of reaction on the concentration of CoA, glutathione, magnesium ions, ATP, acetate, and hydroxylamine, respectively. Cysteine is as effective as glutathione. In another communication, Von Korff will deal with the strong inhibitory effect of sodium ions on the reaction rate.

A balance study of the acethydroxamic assay system is summarized in Table II. Calcium ions were included in the complete system to inhibit pyrophosphatase activity. PP was also identified by isolation as the cadmium salt according to the procedure of Cohen and Kolthoff (19). The cadmium salt contained neither an adenine moiety (absence of absorption at 260 μm) nor inorganic phosphate. The PP content, determined by yeast pyrophosphatase (13), accounted for 83 per cent of the phosphate hydrolyzable by 0.1 N acid at 100° in 10 minutes.

Somewhat more ATP disappears than can be accounted for as AMP, but the sum of ADP and AMP formed tallies closely with the amount of ATP disappearing. The formation of ADP is referable to the action of myokinase whose presence in the AAE preparation of pig heart was demonstrated by the procedure of Kielley and Kielley (20). Both the value for ATP disappearing and that for AMP formed have been corrected by adding to each half the amount of ADP formed.

The interaction of ATP with acetate and catalytic amounts of CoA to
form AMP and PP proceeds in the absence of added hydroxylamine provided calcium ions are present and the enzyme preparation contains acetyl CoA deacylase (21). For this purpose a relatively crude enzyme preparation has to be used. Under these conditions acetyl CoA is broken down to

**Table I**

Component Study of AAE System with Hydroxylamine As Acyl Acceptor

<table>
<thead>
<tr>
<th>Component</th>
<th>Hydroxamic acid formed (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>7.00</td>
</tr>
<tr>
<td>No ATP</td>
<td>0.28</td>
</tr>
<tr>
<td>&quot; CoA</td>
<td>0.26</td>
</tr>
<tr>
<td>&quot; magnesium ions</td>
<td>0.68</td>
</tr>
<tr>
<td>&quot; acetate</td>
<td>0.78</td>
</tr>
<tr>
<td>&quot; glutathione</td>
<td>1.92</td>
</tr>
</tbody>
</table>

**Fig. 1.** Activity as a function of the concentration of the various components of the hydroxylamine assay system. The standard test system, unless otherwise indicated, was set up as in the legend to Table I. The blank in each series was the system minus the component under test.

acetate and CoA, and PP is removed from solution by precipitation with calcium. The equilibrium is thus shifted completely in favor of the breakdown of ATP.

Formation of Acetyl CoA—When the enzyme is allowed to act upon acetate, ATP, and stoichiometric amounts of CoA in the absence of hydroxylamine, an extensive conversion of CoA to acetyl CoA can be demonstrated.
The actual yield is determined by the amount of acetyl CoA deacylase present in the preparation.

In a typical experiment 331 μM of CoA reduced by sodium amalgam were incubated with 720 μM of ATP, 840 μM of acetate (labeled in the carboxyl group with C¹⁴), 240 mg. of AAE and the other reagents except for glutathione at the usual concentrations brought to scale (X 60). 200 μM of potassium borohydride were added to keep CoA in its reduced form. After 35 minutes at 38°, the mixture was acidified with perchloric acid to pH 2 and clarified by centrifugation. The isolation procedure has essentially been described in a previous communication by Littlefield and

### Table II

**Balance Study of ATP-Acetate Reaction with Hydroxylamine As Acyl Acceptor**

The complete system contained magnesium chloride (24 μM), calcium chloride (60 μM), glycine buffer of pH 9 (1200 μM), glutathione (120 μM), ATP (46.5 μM), CoA (1.1 μM), hydroxylamine (3000 μM), acetate (120 μM), and AAE (26 mg.) in a final volume of 12.15 ml. The mixture was incubated for 30 minutes at 38°. The results are expressed in micromoles.

<table>
<thead>
<tr>
<th></th>
<th>ATP corrected for ADP formation</th>
<th>AMP corrected for ADP formation</th>
<th>Inorganic pyrophosphate</th>
<th>Inorganic phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ</td>
<td>-30.5</td>
<td>+27.3</td>
<td>+29.4</td>
<td>+30.8</td>
</tr>
<tr>
<td>Theory based on ATP disappearance</td>
<td>+28.4</td>
<td>+28.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ATP, ADP, and AMP were determined by the column technique of Cohn and Carter (25).

* Corrected for the blank without added acetate.

Sanadi (22). The hydroxamic acid assay showed that 248 μM of an acyl CoA had been recovered, representing a yield of 75 per cent based on the amount of CoA added. The purity of the crude product in terms of acetyl CoA was 56 per cent. 80 per cent of the adenine present, as determined by the absorption of the product at 260 mμ, could be accounted for in the form of acetyl CoA.

Acetyl CoA was identified as citrate after condensation with oxalacetate in the presence of the condensing enzyme. The yield of citrate was about 80 per cent of theory, based on the hydroxamic acid assay. Citrate was estimated colorimetrically by the procedure of Natelson et al. (23) and shown to be radioactive after extraction into ether and then chromatographing on paper. We have used the ATP-acetate system preparatively for the isolation of relatively large amounts of acetyl CoA.

**Reversibility of Reaction**—When acetyl CoA is incubated with the AAE
system in the presence of PP and AMP, a decline of the hydroxamic re-
action is observed, with an equivalent liberation of sulfhydryl (Table III). 
There is no such decline when either AMP or PP is omitted. The negative 
result in absence of added AMP provides evidence that AMP is not a con-
taminant of our standard CoA preparation (24), which for this experiment

**TABLE III**

*Reversal of Acetate-ATP Reaction*

The incubation mixture contained magnesium chloride (8 μM), glycine buffer of 
pH 9 (200 μM), acetyl CoA (3.3 μM), and AAE (1.6 mg.) in a final volume of 2.15 ml. 
AMP (43 μM) or potassium pyrophosphate (30 μM) was added as indicated. Incuba-
tion for 30 minutes at 38°.

<table>
<thead>
<tr>
<th></th>
<th>Acetyl CoA</th>
<th>Sulfhydryl*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>3.3 μM</td>
<td>0 μg</td>
</tr>
<tr>
<td>Incubated without additions</td>
<td>3.0 μM</td>
<td>0 μg</td>
</tr>
<tr>
<td>&quot; with AMP</td>
<td>3.0 μM</td>
<td>0.25 μg</td>
</tr>
<tr>
<td>&quot; pyrophosphate</td>
<td>3.0 μM</td>
<td></td>
</tr>
<tr>
<td>&quot; AMP and pyrophosphate</td>
<td>1.2 μM</td>
<td>1.9 μg</td>
</tr>
</tbody>
</table>

* Based on glutathione as standard.

**TABLE IV**

*Reversal of Acetate-ATP Reaction with Creatine As Phosphate Acceptor*

The reaction mixture contained magnesium chloride (6 μM), AMP (5 μM), potas-
sium pyrophosphate (10.7 μM), acetyl CoA (5 μM), glycine buffer of pH 9 (150 μM), 
AAE (0.8 mg.), ATP-creatine transphosphorylase (2.6 mg.), and solid creatine (10 
mg.) in a final volume of 1.45 ml. Incubated at 38° for 1 hour. The results are 
expressed in micromoles and have been corrected for blanks without either PP or 
acetyl CoA.

<table>
<thead>
<tr>
<th></th>
<th>Hydroxamic acid</th>
<th>Inorganic phosphate</th>
<th>Creatine phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ</td>
<td>-2.3</td>
<td>+0.25</td>
<td>+5.0</td>
</tr>
</tbody>
</table>

had been converted to acetyl CoA by treatment with thiolacetate (18). 
If the reversal reaction is formulated as in Equation 5,

\[(5) \quad \text{Acetyl CoA} + \text{AMP} + \text{PP} \rightarrow \text{ATP} + \text{acetate} + \text{CoA}\]

ATP would be expected to be formed when acetyl CoA disappears in the 
presence of AMP and PP. The experiment described in Table IV demon-
strates that ATP formed in the reversal reaction can be converted to 
creatine phosphate and AMP in the presence of the ATP-creatine trans-
phosphorylase and myokinase systems according to Equations 6 and 7.
If we allow for the recurrent operation of Equation 7, the balance equation would be

\[
\text{Acetyl CoA} + \text{PP} + 2 \text{ creatine} \rightarrow \text{acetate} + 2 \text{ creatine phosphate} + \text{CoA}
\]

Creatine phosphate was determined as the difference between apparent inorganic (Fiske-Subbarow) phosphate and true inorganic (Lowry-Lopez) phosphate after correction for the slow breakdown of PP in the former method. Myokinase was present in the crude preparation of the AAE system used in the balance study. ATP-creatine transphosphorylase was added as a supplement.

**Specificity of System**—The AAE system of pig heart muscle is active only with acetate and propionate, the latter showing about half the activity of the former. Ethyl acetate, thiolacetate, butyrate, acetylglucine, acetooacetate, and succinate were inactive.

**Citrate Assay System**—Although the hydroxamic acid assay system has the virtue of simplicity, it has one serious drawback in studies directed at the purification of the enzyme. As purification proceeds, the rate of reaction with hydroxylamine no longer serves as a reliable measure of enzymatic activity (see Fig. 2). This circumstance has compelled us to devise a more satisfactory assay system which is based on the reactions of Equations 3 and 4. The rate of DPN reduction in the presence of an excess of malic dehydrogenase and condensing enzyme is a reliable index of the concentration of the AAE system (see Fig. 3). The component and balance studies for the second assay system are shown in Tables V and VI. The assay of ATP and AMP by the Cohn-Carter column procedure (25) is interfered with by DPN and DPNH. But qualitatively the column procedure confirmed the disappearance of ATP and the appearance of an equivalent amount of AMP.

**Number of Enzymes in AAE System**—The reaction described by Equation 1 is probably a composite one consisting of two separate reactions which can be provisionally represented as follows:

\[
(1, a) \quad \text{ATP} + \text{CoA} \rightarrow [\text{ATP-CoA}]
\]

\[
(1, b) \quad [\text{ATP-CoA}] + \text{acetate} \rightarrow \text{AMP} + \text{PP} + \text{acetyl CoA}
\]

The compound enclosed in brackets was previously visualized as CoA pyrophosphate (5, 9). To date our experiments do not point to the accumulation of any significant amount of such an intermediate.

In so far as our studies on the purification of the AAE system permit a conclusion, there appears to be no evidence that more than one enzyme is
involved or that the system can be resolved into more than one enzymatic component.

Although evidence for a limited interaction of ATP with CoA has been obtained by following the disappearance of sulfhydryl, a reversal in the

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**Fig. 2.** Specific activity of different fractions of AAE as measured by two assay systems. The conditions for the hydroxylamine and oxalacetate assay procedures are as in the legends to Tables I and V, respectively. The comparison was made on different fractions obtained by fractionating the AAE seriatim with saturated ammonium sulfate. The numbers on the ordinate represent the range of saturation in per cent in which the precipitation of the fraction was carried out. Specific activity is defined as the micromoles of acetate metabolized per mg. of enzyme per 15 minutes at 38°.

**Fig. 3.** Activity as a function of the concentration of AAE. Details as in the legend to Table V.

**Fig. 4.** Spectrophotometric demonstration of acetyl CoA formation by the AAE system. The complete system contained ATP (0.2 µM), reduced CoA (0.1 µM), magnesium chloride (6 µM), acetate (30 µM), glycine buffer of pH 9 (300 µM), and AAE (135 γ). Final volume 3.0 ml. The optical path in the cuvette was 1 cm. Allowance has been made for the increase in density due to acetate. The corrected increase in optical density at 230 µm corresponds to the conversion of 48 per cent of available CoA to acetyl CoA.
way of an interaction of acetyl CoA with PP in the absence of AMP could not be obtained (see Table III). Such a reversal would have been evi-

**Table V**

*Component Study of AAE System with Oxalacetate As Acyl Acceptor*

The complete system contained ATP (5 μM), DPN (4 μM), CoA (0.13 μM), magnesium chloride (4 μM), tria(hydroxymethyl)aminomethane buffer of pH 9.0 (100 μM), acetate (10 μM), supplementary enzyme fraction containing malic dehydrogenase and condensing enzyme (0.5 mg.), L-malate (5 μM), glutathione (10 μM), and AAE (0.11 mg.) in a final volume of 1 ml. The reaction was stopped at the end of 15 minutes by the addition of 5 ml. of 0.5 M phosphate buffer of pH 7.4.* The experiment was carried out at 38°.

<table>
<thead>
<tr>
<th>Component Study of AAE System with Oxalacetate As Acyl Acceptor</th>
<th>DPNH formed [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>1.48</td>
</tr>
<tr>
<td>No ATP</td>
<td>0.09</td>
</tr>
<tr>
<td>' CoA</td>
<td>0.18</td>
</tr>
<tr>
<td>' magnesium ions</td>
<td>0.20</td>
</tr>
<tr>
<td>' acetate</td>
<td>0.16</td>
</tr>
<tr>
<td>' malic dehydrogenase and condensing enzyme</td>
<td>0.58</td>
</tr>
<tr>
<td>' malate</td>
<td>0.09</td>
</tr>
<tr>
<td>' glutathione</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* The reaction does not proceed in the presence of 0.4 M phosphate buffer.

**Table VI**

*Balance Study of ATP-Acetate Reaction with Oxalacetate As Acyl Acceptor*

The system was set up as in the legend to Table V but on a scale 14 times as great. Calcium chloride was also added in a concentration of 22 μM per ml. The experiment was carried out at 38° for 60 minutes. The results are expressed in micromoles.

<table>
<thead>
<tr>
<th>DPNH</th>
<th>Citrate</th>
<th>Inorganic pyrophosphate</th>
<th>Inorganic phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ</td>
<td>+36.4</td>
<td>+33.5 (37.4)*</td>
<td>+37.0</td>
</tr>
<tr>
<td>Theory based on DPNH formation</td>
<td>+36.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Corrected for the equilibrium between isocitric acid, citric acid, and cis-aconitic acid which is catalyzed by aconitase present as an impurity in the AAE system.

dence for pyrophosphoryl CoA as an intermediate. The nature of the intermediate formed by interaction of CoA and ATP still remains to be determined.

**Spectrophotometric Assay of AAE System**—Stadtman (26) has recently shown that the activity of the transacetylase system of Clostridium kluy-
Acetate activating system of heart muscle can be followed spectrophotometrically at 230 μμ when the enzyme is incubated with a mixture of acetyl phosphate and CoA. Thiol esters as a class show a band with a maximum at about 230 μμ. The activity of the AAE system can also be followed spectrophotometrically at 230 μμ when the enzyme is allowed to act upon ATP, acetate, and CoA (Fig. 4). The high absorption of ATP and enzyme at 230 μμ imposes a practical limit to the amount of chemical change which can be measured conveniently under conditions of routine spectrophotometry.

**Summary**

An enzyme system has been prepared from pig and rabbit heart which catalyzes the reversible conversion of acetate and CoA to acetyl CoA and the simultaneous conversion of ATP to AMP and inorganic pyrophosphate. The assay, properties, and kinetics of the enzyme system are described.

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**Bibliography**

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