ACYL ADENYLATES: AN ENZYMATIC MECHANISM OF ACETATE ACTIVATION*

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Several different pathways are now known for the activation of acetate. One of these, found thus far only in certain microorganisms (1, 2), is initiated by the phosphorylation of acetate with ATP1 by acetokinase (3, 4), followed by the transfer of the acetyl group to CoA by the action of phototransacetylase (5-7).

\[
\text{(1)} \quad \text{ATP} + \text{acetate} \rightleftharpoons \text{acetyl phosphate} + \text{ADP}
\]

\[
\text{(2)} \quad \text{Acetyl phosphate} + \text{CoA} \rightleftharpoons \text{acetyl CoA} + \text{phosphate}
\]

In animal tissues (8-11), yeast (12, 13), plants (14), and Rhodospirillum rubrum (15), another pathway of acetate activation has been demonstrated. This involves a reaction of ATP, acetate, and CoA, resulting in a split of ATP with the formation of acetyl CoA, A5P, and PP, and has been termed the aceto-CoA-kinase reaction (12, 16).

\[
\text{(3)} \quad \text{ATP} + \text{acetate} + \text{CoA} \rightleftharpoons \text{acetyl CoA} + \text{A5P} + \text{PP}
\]

Analogous reactions with higher fatty acids have also been reported and characterized (17-19).

In a recent study of the mechanism of Reaction 3, Jones, Lipmann, Hilz, and Lynen (20) reported that a partially purified enzyme preparation from yeast catalyzed an exchange of PP and ATP in the absence of acetate and CoA. They also found that acetate-C14 exchanged with the acetyl group of acetyl CoA in the absence of A5P and PP. To account for these observations, the following mechanism was proposed (20).

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† Scholar in Cancer Research of the American Cancer Society.

1 The following abbreviations have been used: adenosine triphosphate, ATP; adenosine-5'-phosphate, A5P; uridine triphosphate, UTP; inosine triphosphate, ITP; guanosine triphosphate, GTP; cytidine triphosphate, CTP; coenzyme A, CoA; inorganic pyrophosphate, PP; di- and triphosphopyridine nucleotide, DPN and TPN; trichloroacetic acid, TCA; tris(hydroxymethyl)aminomethane, Tris; flavin adenine dinucleotide, FAD; uridine diphosphoglucose, UDPG; nicotinamide mononucleotide, NMN; flavin mononucleotide, FMN; uridine-5'-phosphate, U5P.
MECHANISM OF ACETATE ACTIVATION

(4) \( ATP + \text{enzyme} \rightleftharpoons \text{enzyme} - A5P + PP \)

(5) \( \text{Enzyme} - A5P + \text{CoA} \rightleftharpoons \text{enzyme} - \text{CoA} + A5P \)

(6) \( \text{Enzyme} - \text{CoA} + \text{acetate} \rightleftharpoons \text{acetyl CoA} + \text{enzyme} \)

Since the existence of the postulated enzyme-bound substrates was inferred solely from isotope exchange experiments, we investigated this hypothesis once more to obtain further information on the nature of the intermediates and the mechanism of their formation. With a more highly purified enzyme than that previously employed, it has been found that the exchange of PP and ATP does not occur unless acetate is added. This, together with the absence of any exchange of A5P and ATP in the presence of CoA alone, as predicted by Reactions 4 and 5, indicates that the proposed mechanism is untenable. In the present paper we wish to present evidence in support of another mechanism of acetyl CoA synthesis. This involves a primary reaction of ATP and acetate to form a hitherto undescibed compound, adenyl acetate (shown in the accompanying diagram), and its subsequent reaction with CoA to form acetyl CoA (Reactions 7 and 8).

\[
\begin{align*}
\text{Adenyl acetate} & \quad \text{has been synthesized and demonstrated to react enzymatically in the manner shown below.} \\
\text{(7)} & \quad \text{ATP} + \text{acetate} \rightleftharpoons \text{adenyl acetate} + PP \\
\text{(8)} & \quad \text{Adenyl acetate} + \text{CoA} \rightleftharpoons \text{acetyl CoA} + A5P
\end{align*}
\]

A preliminary communication of this work has been reported elsewhere (21). The synthesis, purification, and characterization of adenyl acetate are described in the following paper (22). The apparently analogous formation of an amino acid acyl adenylate in the reaction of \( \text{r-methionine} \) with ATP by an enzyme from yeast has been briefly mentioned earlier (21) and will be reported in detail in an accompanying paper (23).

**Materials and Methods**

\( \text{P}^{32} \) was prepared by heating \( \text{Na}_2\text{HPO}_4 \) at \( 225^\circ \) for 18 hours (24), and purified by anion exchange chromatography. ATP, labeled with \( \text{P}^{32} \) in the
terminal pyrophosphate group, was prepared either by exchange of \( ^{32}P_4\) with unlabeled ATP by using purified aceto-CoA-kinase (Reaction 7) or from A5P and \( ^{32}P_4\) by rat liver mitochondria in the presence of \( \alpha\)-ketoglutarate. Adenine-C\(^{14}\)-labeled ATP was prepared by first coupling adenine-C\(^{14}\) with 5-phosphoribosyl pyrophosphate (25) and converting the A5P-C\(^{14}\) to ATP-C\(^{14}\) with adenylic kinase (26), phosphopyruvate, and pyruvate phosphokinase (27). The ATP was purified both by adsorption and elution from Norit and by anion exchange chromatography (28). Acetyl CoA was prepared from acetyl phosphate and CoA by purified phosphotransacetylase (5), and partially purified by anion exchange chromatography by using a 2 per cent cross-linked Dowex 1 Cl\(^-\) column and eluting with 0.1 \( \times \) HCl-0.1 \( \times \) KCl. According to the optical density at 260 \( \mathrm{m}\mu\) and acetyl CoA assay (11, 29), it was 50 per cent pure. Sodium acetate-1-C\(^{14}\) was obtained from Tracerlab, Inc., and sodium fluoroacetate was kindly supplied by Dr. R. O. Brady. CoA (75 per cent pure) and CTP were obtained from the Pabst Brewing Company, and DPN, TPN, ITP, UTP, and GTP from the Sigma Chemical Company. Reduced CoA was prepared with potassium borohydride by the method of Jones et al. (13). Hexokinase was prepared by a modification of the method of Brown\(^2\) and contained 5 units per mg. (1 unit forms 1 \( \mu\)mole of glucose-6-phosphate per minute at pH 8.0 and 25\(^\circ\)). Glucose-6-phosphate dehydrogenase was obtained from the Sigma Chemical Company and contained 0.8 unit (30) per mg. Crystalline condensing enzyme (31), recovered from the mother liquor of the first crystallization, was generously given by Dr. S. Ochoa. This preparation, when used in the coupled assay for acetyl CoA (11, 29), contained malic dehydrogenase in adequate excess. The amine-acetylating enzyme of pigeon liver (acetone fraction) was prepared according to Tabor et al. (32). Crystalline inorganic pyrophosphatase (33) was generously supplied by Dr. G. Perlmann and Dr. M. Kunitz. Phosphotransacetylase was prepared by the method of Stadtman (5), and A5P deaminase by Procedure A of Kalckar (34), adenylic kinase according to Colowick and Kalckar (26), and pyruvate phosphokinase by the method of Reisenherz et al. (35).

A solution of 2 \( \times \) hydroxylamine was prepared daily by neutralizing a 4 \( \times \) solution of hydroxylamine hydrochloride with KOH. More concentrated solutions of hydroxylamine were made by adding solid barium hydroxide to a solution of hydroxylamine sulfate until the pH was about 7.2. The barium sulfate was removed by centrifugation and the supernatant fluid was concentrated under reduced pressure. The volume was adjusted so that the concentration of hydroxylamine was 5 \( \times \), based on the initial

\(^2\) We are grateful to Dr. D. H. Brown for giving us this method before its publication.
amount of hydroxylamine sulfate. The solution was kept at 4° for periods of no longer than 2 weeks.

Acetyl CoA was measured by DPN reduction by the citrate-condensing enzyme system containing malic dehydrogenase (11, 29), and ATP was determined with hexokinase, glucose, glucose-6-phosphate dehydrogenase, and TPN (36). Adenyl acetate was determined either by conversion to acetyl CoA, measured as mentioned above, or by conversion of P$_{32}$P$_{32}$ to ATP (see Table VI). Adenyl acetate concentration was also determined as acethydroxamic acid after treatment with hydroxylamine. The sample was incubated for 5 minutes at 37° in 1 ml. of 0.2 M hydroxylamine, and 0.5 ml. of acidified ferric chloride (13) was added and the optical density at 540 mμ measured. Under these conditions 1 μmole of acethydroxamic acid has an optical density of 0.630. This was in good agreement with the values determined enzymatically (22). Protein was measured by the phenol method of Lowry et al. (37).

**Assay of Aceto-CoA-kinase**

**Formation of Acetyl CoA**—The assay procedure was essentially that described by Jones et al. (13). The reaction mixture contained, in 1.0 ml., 0.1 M potassium phosphate, pH 7.5, 0.005 M MgCl$_2$, 0.01 M ATP, 0.05 M potassium fluoride, 0.01 M glutathione, 0.15 mg. of CoA (equivalent to 0.1 μmole), 0.01 M potassium acetate, 0.2 M neutralized hydroxylamine, and the enzyme. The mixture was incubated for 20 minutes at 37°, then 2 ml. of an acidified ferric chloride solution (13) were added. After centrifugation to remove precipitated protein, the optical density at 540 mμ was determined with a Beckman model DU spectrophotometer. In all cases, a control tube in which CoA was omitted was incubated with each amount of enzyme. The formation of 1 μmole of acethydroxamic acid resulted in an increase in optical density of 0.325, and the unit of activity was defined as the amount of enzyme catalyzing the formation of 1 μmole of acethydroxamic acid in 20 minutes under these conditions. As previously shown, the formation of acethydroxamic acid was proportional to enzyme concentration in the range of 0 to 0.5 unit (13).

**Exchange of P$_{32}$P$_{32}$ with ATP**—The standard assay mixture contained, in 1.0 ml., 0.1 M potassium phosphate, pH 7.5, 0.005 M MgCl$_2$, 0.002 M ATP, 0.001 M acetate, 0.05 M fluoride, 0.002 M P$_{32}$P$_{32}$ containing between $10^4$ and $10^5$ c.p.m. per μmole, and the enzyme. When the purified enzyme was used, the fluoride was omitted, since there was no demonstrable inorganic pyrophosphatase activity. The mixture was incubated for 20 minutes at 37° and the reaction stopped by the addition of 0.5 ml. of 7 per cent perchloric acid, followed by 0.2 ml. of a suspension of acid-washed Norit (15 per cent, dry weight). After 3 minutes, the Norit was centrifuged, washed
three times with 3 ml. portions of water, and then suspended in 3 ml. of 50 per cent ethanol containing 0.3 M ammonium hydroxide. An aliquot of the suspension was plated and the \( P^{32} \) content determined with a Geiger-Müller counter. The activity is expressed as the micromoles of \( P^{32}P^{32} \) incorporated into ATP and is calculated by dividing the total \( P^{32} \) activity in ATP by the specific activity of the initial \( P^{32}P^{32} \). 1 unit of enzyme activity is defined as the incorporation of 1 \( \mu \)mole of \( P^{32}P^{32} \) into ATP in 20 minutes under these conditions. Comparison of the values obtained by this procedure with those found by chromatographic separation (28) of ATP showed that they were in good agreement. Thus in one experiment the total counts per minute in ATP determined by the Norit procedure were 6000 (complete), 520 (no acetate), and 500 (no ATP), as compared with the respective values of 6700, 360, and 600, obtained by chromatographic analysis.

The assay was proportional to enzyme concentration in the range of 0 to 0.4 unit. Thus with 0, 0.59, 1.5, 3.0, and 7.3 \( \gamma \) of enzyme protein, 0, 76, 80, 80, 69 units per mg. of enzyme were calculated to be present.

**Results**

Purification of Aceto-CoA-kinase—All the operations were carried out at \( 4^\circ \). Pressed bakers’ yeast (200 gm.), obtained from Fleischmann’s yeast, Standard Brands Incorporated, was mixed to a paste with 200 ml. of cold 0.1 M dipotassium phosphate and treated in a 10 kc. sonic oscillator (Raytheon) for 40 minutes. The mixture was centrifuged in a Servall centrifuge at \( 10,000 \times g \) for 30 minutes, and the turbid supernatant fluid was filtered through glass wool to remove particles of fat. This crude extract (Table I) was stable for at least 2 days when kept at \(-15^\circ\).

To 180 ml. of the crude extract were added, with stirring, 27 ml. of a 2 per cent solution of protamine sulfate, generously supplied by Eli Lilly and Company. After 5 minutes, the solution was centrifuged for 5 minutes at \( 10,000 \times g \) and the precipitate discarded. To the clear supernatant fluid were added 55 ml. of the protamine solution, and after 5 minutes the mixture was centrifuged as above. The supernatant fluid was discarded and the gummy precipitate washed twice with a total volume of 100 ml. of cold water by homogenizing with a motor-driven pestle and then centrifuging. The washing was repeated as described above, 100 ml. of 0.01 M potassium phosphate, pH 7.0, being used. The washings were discarded and the precipitate was dissolved in 125 ml. of 0.25 M potassium phosphate, pH 7.5 (protamine eluate).

To the protamine eluate (125 ml.) were added 97 ml. of a solution of ammonium sulfate, saturated at \( 4^\circ \). After 10 minutes, the mixture was centrifuged for 5 minutes at \( 10,000 \times g \) and the precipitate was dissolved in 65 ml. of cold water (Fraction AS-1). To this solution was added alumina
Cγ gel (38) (1.1 mg., dry weight, of gel per mg. of protein) and after 5 minutes the gel was centrifuged and washed with a total of 100 ml. of water, then twice with a total of 100 ml. of 0.05 M potassium phosphate, pH 6.9. The enzyme was then eluted with two 40 ml. portions of 0.1 M potassium phosphate, pH 7.5 (Cγ gel eluate).

To the Cγ gel eluate (80 ml.) were added 21 gm. of ammonium sulfate and, after 5 minutes, the precipitate was separated by centrifugation and dissolved in 14 ml. of 0.5 M potassium phosphate, pH 7.6 (Fraction AS-2).

In several trials the purification achieved in the AS-2 fraction in relation to the crude extract ranged from 35- to 60-fold, and the yield was between 25 and 35 per cent. Fraction AS-2 has been repeatedly frozen and thawed several times a week for periods of up to 4 months without appreciable loss of activity.

* Assay, formation of acetyl CoA, measured by formation of acethydroxamic acid as described in “Methods.”

P$_{32}$P$_{32}$ Exchange with ATP—It had been reported previously (20) that aceto-CoA-kinase catalyzed an exchange of P$_{32}$P$_{32}$ and ATP in the absence of acetate and CoA. Since such a reaction could have occurred by other previously reported reactions (36, 39, 40) involving endogenous substrates, and therefore could be unrelated to the formation of acetyl CoA, the exchange reaction was again examined. It was found that, in addition to ATP, P$_{32}$P$_{32}$, and Mg$^{++}$, acetate was required for the exchange reaction with the most purified fraction of aceto-CoA-kinase (Table II). There was no appreciable exchange when either ATP, Mg$^{++}$, acetate, or the enzyme was omitted. The requirement for acetate became increasingly apparent with purification; in one experiment the ratio of activities in the presence and absence of acetate was 4 in the crude extract, 60 in Fraction AS-1, and 143 in Fraction AS-2.

The rate of exchange as a function of acetate concentration is shown in Table III. Since the rôle of acetate is catalytic, small amounts of acetate...
effect an appreciable amount of exchange of $P^{32}P^{32}$ with ATP, the extent of which is dependent on the time of incubation. It therefore seems likely that the previously reported exchange reaction (20) was due, at least in part, to the presence of acetate and perhaps to other compounds unrelated to acetyl CoA formation. The inhibition of acetate at a concentration of $3 \times 10^{-3}$ M and higher has not been observed with acetyl CoA formation as

**TABLE II**

*Requirements for $P^{32}P^{32}$ Exchange with ATP*

<table>
<thead>
<tr>
<th>Components</th>
<th>$P^{32}P^{32}$ incorporated into ATP ($\mu$ mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>0.55</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>0.01</td>
</tr>
<tr>
<td>&quot; Mg$^{+4}$</td>
<td>0.01</td>
</tr>
<tr>
<td>&quot; acetate</td>
<td>0.02</td>
</tr>
<tr>
<td>&quot; enzyme</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Usual assay procedure for PP-ATP exchange with 0.002 M acetate and 7.5 $\gamma$ of enzyme (Fraction AS-2); specific activity 92.

**TABLE III**

*Effect of Acetate Concentration on $P^{32}P^{32}$-ATP Exchange*

<table>
<thead>
<tr>
<th>Concentration</th>
<th>$P^{32}P^{32}$ incorporated into ATP ($\mu$ mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X 10^4$ M</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>0.02</td>
</tr>
<tr>
<td>0.4</td>
<td>0.15</td>
</tr>
<tr>
<td>2.0</td>
<td>0.39</td>
</tr>
<tr>
<td>10.0</td>
<td>0.57</td>
</tr>
<tr>
<td>30.0</td>
<td>0.43</td>
</tr>
<tr>
<td>40.0</td>
<td>0.37</td>
</tr>
</tbody>
</table>

The conditions are the same as for the experiment described in Table II.

a measure of the aceto-CoA-kinase reaction. The specificity of the reaction for acetate (see below) and the effectiveness of small amounts in the exchange reaction suggest the use of this reaction for measuring acetate in the range of $10^{-4}$ to $10^{-6}$ M.

**Time-Course and Extent of Exchange**—The exchange of $P^{32}P^{32}$ and ATP approaches the calculated value for complete equilibration of the pyrophosphoryl group of ATP and the $P^{32}P^{32}$ (Fig. 1). ATP prepared in this way contains essentially equal amounts of $P^{32}$ in the two terminal phosphate groups (41). Since the enzyme preparation did not contain any adenylic kinase activity as measured with ATP and A5P in the presence of pyruvate
phosphokinase and lactic dehydrogenase (42), the reaction appears to represent an exchange of the two pyrophosphate moieties.

**Specificity of Nucleotide and Fatty Acid Components**—ATP was the only nucleoside triphosphate which was active in the exchange reaction. UTP, CTP, ITP, and GTP did not replace ATP. Formate, butyrate, caproate, and octanoate at concentrations of 1 to 2 × 10⁻³ M did not replace acetate. Propionate, the only other fatty acid activated by the aceto-CoA-kinase (16), also catalyzed the exchange reaction. At 5 × 10⁻³ M, the rate was about 30 per cent that found with 1 × 10⁻³ M acetate. With fluoroacetate at levels of 1 × 10⁻³ M and 5 × 10⁻³ M, there was about 2 and 5 per cent the exchange found with 1 × 10⁻³ M acetate. Whether this is a property of fluoroacetate or due to contamination by acetate is uncertain. There was no significant inhibition of the exchange when equimolar amounts of acetate and fluoroacetate were present. Brady (43) has reported that neither yeast nor liver aceto-CoA-kinase converts fluoroacetate to fluoroacetyl CoA but that kidney preparations do.

**Formation of Acethydroxamic Acid from ATP and Acetate**—The observation that acetate was required for the PP-ATP exchange suggested that the initial step in acetyl CoA synthesis was a reversible enzymatic interaction of ATP and acetate with the formation of adenyl acetate and PP (Reac-

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**Fig. 1.** Time-course and extent of PP-ATP exchange. Reaction mixture: 1.0 ml. containing 0.10 IU potassium phosphate buffer, pH 7.5; 0.005 M MgCl₂; 0.0016 M ATP; 0.001 M potassium acetate; 0.002 M P₃P³ containing 5 × 10⁴ c.p.m.; 4 γ of enzyme, specific activity 96. Temperature 37°.
tion 7). This hypothesis was tested with hydroxylamine to trap the acetyl group of adenyl acetate as acethydroxamic acid. With large amounts of enzyme, ATP, and acetate, and high concentrations of hydroxylamine (2.5 M), there was a net formation of acethydroxamic acid (Table IV), which increased with time and was dependent on the presence of ATP, acetate, and the enzyme. When a smaller amount of hydroxylamine was used, e.g. 1 M, there was only 16 per cent as much acethydroxamate formed. The rate of acethydroxamic acid formation was also dependent on the amount of enzyme; thus, with 33, 82, 164, and 330 γ of enzyme, there was a forma-

**Table IV**

*Formation of Acethydrozamic Acid from ATP and Acetate in Absence of CoA*

<table>
<thead>
<tr>
<th>Components</th>
<th>30 min.</th>
<th>60 min.</th>
<th>120 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmole</td>
<td>μmole</td>
<td>μmole</td>
</tr>
<tr>
<td>Complete........</td>
<td>0.52</td>
<td>0.89</td>
<td>1.60</td>
</tr>
<tr>
<td>Minus acetate</td>
<td>0.22</td>
<td>0.20</td>
<td>0.22</td>
</tr>
<tr>
<td>&quot; enzyme.......</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>&quot; ATP.........</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

All the values are expressed in micromoles of acethydroxamic acid and the change (Δ) was calculated from the difference between the values obtained in the presence and the absence of acetate. Reaction mixture: 1.0 ml. contained 0.1 M potassium phosphate, pH 7.5; 0.005 M MgCl₂; 0.01 M ATP; 0.01 M acetate; 2.5 M hydroxylamine; and 330 γ of enzyme (specific activity 96); temperature 37°. The reaction stopped by the addition of 0.2 ml. of acid (2 N TCA, 6.6 N HCl), followed by the addition of 0.5 ml. of ferric chloride reagent (12).

Acethydroxamic acid formation under these conditions was accompanied by the liberation of an equivalent amount of A₅P and PP (Table V). In another experiment, with 412 γ of enzyme, A₅P formation was measured at the end of the incubation with A₅P deaminase. In the presence of acetate, 1.1 μmoles of acethydroxamic acid and 1.3 μmoles of A₅P were formed, and, in the absence of acetate, 0.1 μmole of acethydroxamic acid and 0.1 μmole of A₅P were found.

The above results are in agreement with the postulated formation of adenyl acetate as an intermediate, an interpretation, however, dependent on the assumption that small amounts of CoA were not present. In order to determine whether CoA was present, a sensitive assay for CoA was developed which involved the use of high concentrations of hydroxylamine (2.5 M) in the usual acetyl CoA assay system. By developing the color in a
volume of only 1.5 ml., as little as 0.0005 µmole of CoA could be detected. Heat-inactivated samples of the enzyme (330 γ) tested in place of CoA in the above assay gave no detectable amounts of acethydroxamic acid in either the presence or the absence of glutathione. As a control, CoA added to the enzyme solution before or after heating was quantitatively recovered. Moreover, when a sample of heated enzyme (330 γ) was added to the reaction mixture (Table IV), there was no increase in the amount of acethydroxamic acid over that found in the presence of 330 γ of unheated enzyme alone, whereas the addition of 0.0005 µmole of CoA doubled the formation of acethydroxamic acid. These experiments indicate that the acethydroxamic acid formed in the absence of added CoA is not due to endogenous traces of CoA. This conclusion is further supported by the observations reported in the section pertaining to A5P exchange with ATP in which both acetate and added CoA were required for the exchange. These experiments, therefore, are consistent with the formulation of adenyl acetate as an intermediate in acetyl CoA synthesis.

**Utilization of Adenyl Acetate for ATP Synthesis**—From the experiments discussed thus far, it was considered likely that the intermediate formed from ATP and acetate was the phosphoacetyl derivative of A5P. This compound was synthesized first from the silver salt of A5P and acetyl chloride by a modification of the method of Lipmann and Tuttle (45), but better yields and purer material were obtained by direct acetylation of A5P with acetic anhydride by the method of Avison (46). These are presented in detail in Paper II (22).

### Table V

<table>
<thead>
<tr>
<th>Components</th>
<th>Acethydroxamate (µmole)</th>
<th>Δ</th>
<th>PP* (µmole)</th>
<th>Δ</th>
<th>A5P† (µmole)</th>
<th>Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>0.74</td>
<td>0.71</td>
<td>1.11</td>
<td>0.76</td>
<td>1.12</td>
<td>0.73</td>
</tr>
<tr>
<td>Minus acetate</td>
<td>0.09</td>
<td>0.06</td>
<td>0.45</td>
<td>0.10</td>
<td>0.46</td>
<td>0.07</td>
</tr>
<tr>
<td>&quot; enzyme</td>
<td>0.03</td>
<td>0.35</td>
<td></td>
<td></td>
<td>0.39</td>
<td></td>
</tr>
</tbody>
</table>

* Calculated from the original specific activity of ATP.
† Calculated from the optical density at 260 mμ, with an extinction coefficient of 16 × 10^4 cm.^−1 m.−1 (44). Reaction mixture: 1.0 ml. contained 0.1 M Tris, pH 7.4, 0.005 M MgCl₂; 0.0077 M P³²-labeled ATP containing 3.31 × 10⁶ c.p.m. per µmole; 0.01 M acetate; 2.5 M hydroxylamine; 330 γ of enzyme (specific activity 125). Incubated 120 minutes at 37°. Heated aliquots of the incubation mixture were chromatographed on Dowex 1 Cl⁻ columns to separate A5P and PP (24). The amount of acethydroxamic acid formed was determined colorimetrically in a parallel experiment, as described in Table IV.
Synthetic adenyl acetate was converted to ATP in the presence of PP, Mg++, and aceto-CoA-kinase (Table VI). This was shown with $^{32}$P$^{32}$ and

**Table VI**

<table>
<thead>
<tr>
<th>Components</th>
<th>Time (min)</th>
<th>PP incorporated into ATP (μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete...........</td>
<td>20</td>
<td>0.23</td>
</tr>
<tr>
<td>&quot;</td>
<td>45</td>
<td>0.28</td>
</tr>
<tr>
<td>Minus enzyme........</td>
<td>20</td>
<td>0.01</td>
</tr>
<tr>
<td>Complete*...........</td>
<td>20</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* The adenyl acetate was previously incubated in 0.1 M KOH for 5 minutes at room temperature and then neutralized to pH 7.0. Reaction mixture: The volume of 1.0 ml. contained 0.1 M potassium phosphate, pH 7.5; 0.005 M MgCl$_2$; 0.002 M PP$^{32}$; 0.28 μmole of adenyl acetate (measured by hydroxamic acid assay); and 7.2 γ of enzyme (specific activity 72).

![Fig. 2. Correspondence of ATP formation and adenyl acetate disappearance. Reaction mixture: 1.0 ml. containing 0.1 M potassium phosphate buffer, pH 7.5; 0.005 M MgCl$_2$; 0.0032 M adenyl acetate; 0.002 M PP$^{32}$; and 7.2 γ of enzyme (specific activity 72). Temperature 37°. ATP determined by Norit assay and adenyl acetate by hydroxamic acid assay.](http://www.jbc.org/)

by measuring $^{32}$P in ATP after adsorption and elution from Norit. According to these data, ATP was formed in an amount equivalent to the amount of adenyl acetate added. Treatment of adenyl acetate with dilute alkali, which rapidly hydrolyzed adenyl acetate to ADP and acetate (22), destroyed this activity. The formation of ATP (Fig. 2) occurs concomitantly with a
disappearance of adenyl acetate as measured by hydroxylamine-labile acetyl groups. An over-all balance of the formation of ATP from adenyl acetate and PP has already been presented (21).

The enzymatic conversion of adenyl acetate to ATP has also been verified spectrophotometrically by being coupled to the phosphorylation of glucose with hexokinase. Incubation of adenyl acetate, PP, Mg++, and aceto-CoA-kinase in the presence of an excess of hexokinase, glucose, glucose-6-phosphate dehydrogenase, and TPN resulted in a reduction of TPN which was proportional to the amount of adenyl acetate added (Table VII). The

\[
\text{Table VII} \\
\text{Formation of ATP from Adenyl Acetate and PP Measured Spectrophotometrically with Hexokinase and Glucose-6-phosphate Dehydrogenase}
\]

<table>
<thead>
<tr>
<th>Adenyl acetate added* (µmole)</th>
<th>TPNH formed (µmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>0.024</td>
<td>0.025, 0.025, 0.027</td>
</tr>
<tr>
<td>0.037</td>
<td>0.039, 0.040, 0.038</td>
</tr>
<tr>
<td>0.054</td>
<td>0.056</td>
</tr>
<tr>
<td>0.084</td>
<td>0.085</td>
</tr>
</tbody>
</table>

* The adenyl acetate concentration was determined by acethydroxamic acid formation and by conversion to acetyl CoA as measured with the malic dehydrogenase-citrate-condensing enzymes (10, 29).

† An extinction coefficient of 6.22 × 10³ cm⁻¹µM⁻¹ (47) was used. Reaction mixture contained, in 1.0 ml., 0.05 M Tris, pH 7.4; 0.001 M MgCl₂; 0.025 M glucose; 0.0002 M TPN; 0.002 M PP; 25 γ of hexokinase; and 250 γ of glucose-6-phosphate dehydrogenase. The reaction was started by the addition of 6.6 γ of aceto-CoA-kinase (specific activity 125).

**Table VII**

The reactions involved are as follows:

\[
\begin{align*}
\text{(9)} & \quad \text{Adenyl acetate} + \text{PP} \xrightarrow{\text{aceto-CoA kinase}} \text{ATP} + \text{acetate} \\
\text{(10)} & \quad \text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP} \\
\text{(11)} & \quad \text{Glucose-6-phosphate} + \text{TPN} \xrightarrow{\text{glucose-6-phosphate dehydrogenase}} \text{6-phosphogluconate} + \text{TPNH} + \text{H}^+ 
\end{align*}
\]

By the spectrophotometric assay, the effect of adenyl acetate concentration on the initial rate of ATP formation (TPN reduction) was determined. The maximal rate was obtained with 2 × 10⁻⁴ M and the half maximal rate at 5 × 10⁻⁵ M. In a similar experiment with adenyl acetate in excess and
the PP concentration varied, half maximal rate was found between 1 and $5 \times 10^{-6}$ M.

$Mg^{++}$ Requirement—The conversion of adenylic acetate to ATP required Mg$^{++}$. By measuring the conversion of $^{32}$P$^{32}$ to ATP, a maximal rate was found with $1.5 \times 10^{-5}$ M Mg$^{++}$, a half maximal rate with $2.5 \times 10^{-4}$ M, while with no Mg$^{++}$ the rate was 2 per cent of maximum. According to the PP-ATP exchange assay, the maximal rate of exchange occurred with $5 \times 10^{-3}$ M Mg$^{++}$. The discrepancy of the observed optimal concentration of Mg$^{++}$ by the two methods of measurement may be due to the presence of relatively larger amounts of ATP in the latter assay. Higher concentrations of Mg$^{++}$ were inhibitory; with $1.5 \times 10^{-2}$ M and $2.5 \times 10^{-2}$ M, the inhibition was 15 and 33 per cent, respectively. At these higher concentrations there was a visible precipitate (presumably some complex of Mg$^{++}$ and one or more of the phosphate components).

Conversion of Adenylic Acetate to Acetyl CoA—It was previously reported that CoA inhibits the exchange of PP with ATP by aceto-CoA-kinase (20). This has been confirmed by using the purified enzyme activated with acetate. Moreover, the conversion of adenylic acetate to ATP is markedly inhibited by CoA (Table VIII). This inhibition is due to a competition for the substrate, adenylic acetate, by CoA and PP, as demonstrated by the rapid conversion of adenylic acetate to acetyl CoA. Acetyl CoA formation from adenylic acetate and CoA was measured spectrophotometrically at 340 m$\mu$ by coupling with the malic dehydrogenase-citrate-condensing enzymes (11, 29), as shown in Fig. 3. It can be seen that DPN reduction is dependent on the presence of adenylic acetate, CoA, and aceto-CoA-kinase. The increase in optical density at 340 m$\mu$ in Fig. 3 (0.789) is equivalent to the formation of 0.127 $\mu$mole of acetyl CoA, which is in agreement with the 0.126 $\mu$mole of adenylic acetate added.

### Table VIII

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Components</th>
<th>$\mu$mole incorporated into ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Complete</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>+ 2 $\mu$moles CoA</td>
<td>0.14</td>
</tr>
<tr>
<td>2</td>
<td>Complete</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>+ 2 $\mu$moles CoA</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Experiment 1. A volume of 1.0 ml. contained 0.1 M potassium phosphate, pH 7.5; 0.005 M MgCl$_2$; 0.002 M ATP; 0.002 M $^{32}$P$^{32}$; 0.001 M acetate; 0.01 M glutathione; 7.2 $\gamma$ of enzyme (specific activity 96). Incubated at 37° for 20 minutes. Experiment 2. Same as above, except with 0.0003 M adenylic acetate instead of ATP.
With the malic dehydrogenase-citrate-condensing enzyme assay, the effect of adenyl acetate concentration on the initial rate of acetyl CoA formation was studied. A maximal rate was obtained with $2 \times 10^{-4}$ M and a half maximal rate at $5 \times 10^{-5}$ M.

![Graph](http://www.jbc.org/)  
**Fig. 3.** Formation of acetyl CoA from adenyl acetate and CoA. Reaction mixture: 1.0 ml. containing 0.1 M potassium phosphate, pH 7.5; 0.005 M MgCl$_2$; 0.00015 M CoA; 0.005 M glutathione; 0.0005 M DPN; 0.005 M l-malate; 40 μg of crystalline citrate-condensing enzyme; 18 μg of aceto-CoA-kinase (specific activity 72); and 0.126 μmole of adenyl acetate (assayed by conversion to ATP). Adenyl acetate was added initially to Curves 1, 2, and 3, and, at 6 minutes, to Curve 4. CoA was added to 3 at 23 minutes. The experiments were carried out at room temperature in cuvettes with a 1 cm. light path.

The acetyl CoA formed from adenyl acetate and CoA was further identified by its ability to acetylate p-nitroaniline with a partially purified amine-acetylase from pigeon liver (32); (Fig. 4). The over-all stoichiometry of the reaction could not be determined under these conditions, probably due to the non-enzymatic acetylation of thioglycolate by acetyl CoA (32).

*Mg$^{++}$ Requirement for Conversion of Adenyl Acetate to Acetyl CoA*—In contrast to the ATP-PP exchange and the conversion of adenyl acetate to ATP reactions, which have an absolute requirement for Mg$^{++}$, acetyl CoA
formation occurs maximally in the absence of added Mg++. Adenyl acetate and CoA were incubated with the enzyme, and various concentrations of Mg++ and the acetyl CoA were measured as previously described. The same amount of acetyl CoA was formed in the absence of added Mg++ as in the presence of up to $5 \times 10^{-2}$ M Mg++. 

Attempts to Demonstrate Enzymatic Synthesis of Adenyl Acetate—The reactions of adenyl acetate demonstrated in the previous sections are in agreement with those predicted by the hypothesis shown in Reactions 7 and 8.

![Graph](http://www.jbc.org) **Fig. 4.** Acetylation of p-nitroaniline with adenyl acetate and CoA. Reaction mixture: A volume of 1.0 ml. contained 0.1 M potassium phosphate, pH 7.8; 0.005 M MgCl₂; 0.00013 M p-nitroaniline; 0.005 M ethylenediaminetetraacetate; 0.005 M thioglycolate; 0.00015 M CoA; 0.003 M adenyl acetate; 18 γ of aceto-CoA-kinase (specific activity 72); 0.05 ml. of acetylating enzyme (acetone fraction, 1060 units per ml. (32)). Adenyl acetate was added at zero time. The experiments were carried out at room temperature in cuvettes with a 1 cm. light path.

Attempts to achieve the net enzymatic synthesis and isolation of adenyl acetate from ATP and acetate have been unsuccessful to date. By using $^{32}$P-labeled ATP and an amount of crystalline inorganic pyrophosphatase (33) sufficient to hydrolyze 150 μmoles of PP per minute (200 γ), there was no significant increase in the $^{32}$P of the non-nucleotide phosphate fraction above a blank, with no acetate, under conditions in which a liberation of 0.005 μmole of PP could have been detected.

In an attempt to trap adenyl acetate by an experiment in isotope dilution, adenine-$^{14}$C-labeled ATP, adenyl acetate, Mg++, and the enzyme were incubated in the presence and the absence of acetate. After 30 minutes, the mixture was adjusted to pH 10 to hydrolyze the adenyl acetate to A₅P (22),
and then chromatographed on an anion exchange column (28). The results (Table IX) show that there was no significant incorporation of the A5P moiety of ATP into the adenyl acetate pool. Similar results were obtained in the presence of 100 γ of inorganic pyrophosphatase.

A rational explanation for the above failure of isotope equilibration between ATP-C14 and adenyl acetate became apparent when it was found that adenyl acetate inhibited the utilization of ATP. ATP, labeled with P32 in the terminal pyrophosphate group, was incubated with acetate, reduced CoA, inorganic pyrophosphatase, and aceto-CoA-kinase in the presence and the absence of adenyl acetate. After incubation for various periods of time, aliquots were assayed for acetyl CoA and the PP liberated was determined in the supernatant fluid after adsorption of the ATP-P32 with Norit. In

<table>
<thead>
<tr>
<th>Components</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.p.m. per μmole</td>
</tr>
<tr>
<td>Complete</td>
<td>117</td>
</tr>
<tr>
<td>Minus acetate</td>
<td>125</td>
</tr>
<tr>
<td>&quot; enzyme</td>
<td>91</td>
</tr>
</tbody>
</table>

A volume of 1.0 ml. contained 0.10 M potassium phosphate, pH 7.5; 0.005 M MgCl2; 0.002 M ATP containing 2.5 × 104 c.p.m. per μmole; 0.002 M acetate, 0.0055 M adenyl acetate, and 36 γ of enzyme (specific activity 72). Incubated 30 minutes at 37°.

the absence of adenyl acetate, P32 liberation and acetyl CoA formation were equal throughout the incubation (Fig. 5). In the presence of adenyl acetate, however, there were a marked decrease in the P32P32 formed and an increase in the rate of acetyl CoA formation. With smaller amounts of adenyl acetate, the inhibition of P32P32 formation was not as marked, and as the adenyl acetate was utilized for acetyl CoA formation, the rate of P32P32 formation increased. These data suggest that, in the presence of adenyl acetate and ATP, there was a preferential use of the adenyl acetate. This conclusion was supported by competitive inhibition experiments with PP formation from ATP as a measure of ATP utilization. With ratios of ATP to adenyl acetate of 1.7, 0.56, and 0.42, the inhibition was 68, 85, and 92 per cent, respectively. In measuring the conversion of adenyl acetate to ATP, a ratio of 8 of ATP to adenyl acetate produced only a 25 per cent inhibition. From this it appears that the affinity of adenyl acetate for the enzyme is greater than that of ATP. This finding, therefore, makes trapping experiments with labeled ATP or acetate experimentally difficult.
Exchange of $\text{A}5\text{P}-\text{C}^{14}$ and ATP—According to the hypothesis proposed by Jones et al. (20), aceto-CoA-kinase should catalyze an exchange of $\text{A}5\text{P}$ with the corresponding moiety of ATP in the presence of CoA alone (see Reactions 4, 5, and 6). By the hypothesis proposed in Reactions 7 and 8, the exchange requires both CoA and acetate. Adenine-$\text{C}^{14}$-labeled $\text{A}5\text{P}$ was incubated with unlabeled ATP and the enzyme in the presence or absence of acetate and CoA, and then $\text{A}5\text{P}$ and ATP were separated by anion exchange chromatography (28) and the radioactivity determined (Table X).

![Graph](http://www.jbc.org)

**Fig. 5. Effect of adenyl acetate on utilization of ATP for acetyl CoA synthesis.** Reaction mixture: 1.0 ml., containing 0.05 M Tris, pH 7.5; 0.005 M MgCl$_2$; 0.005 M acetate; 0.001 M ATP$^3$P$^2$ (ARPP$^3$P$^2$) containing 43,500 c.p.m. per µmole; 0.0007 M reduced CoA; 100 µ of crystalline inorganic pyrophosphatase; 3.6 µ of enzyme (specific activity 72). Temperature 37°.

In the complete system during the time the exchange occurred, there was 0.32 µmole of acetyl CoA formed, as measured by acethydroxamic acid. These data demonstrate that there was an exchange of $\text{A}5\text{P}$ and ATP only under conditions in which complete reversal of the reaction occurs; namely, when both acetate and CoA were present, and are in agreement with the mechanism proposed in Reactions 7 and 8. Moreover, this experiment supports the earlier conclusion that trace quantities of CoA are not present in the enzyme preparation.

Exchange of Acetate-$\text{C}^{14}$ with Acetyl CoA—Jones et al. (20) have reported that aceto-CoA-kinase catalyzed an exchange of acetate-$\text{C}^{14}$ with the acetyl moiety of acetyl CoA in the absence of $\text{A}5\text{P}$, PP, and Mg$^{++}$. Since this observation was inconsistent with the mechanism proposed here, the ex-
change experiment was reexamined with the purified enzyme (Table XI). In the absence of both A5P and PP, and with PP alone, there was only a small amount of exchange detectable. When A5P alone was added, the incorporation of acetate-C\textsuperscript{14} into acetyl CoA was about 30 per cent of the

**Table X**

*Exchange of A5P-C\textsuperscript{14} and ATP by Aceto-CoA-kinase*

<table>
<thead>
<tr>
<th>Components</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.p.m. per μmole</td>
</tr>
<tr>
<td>Complete</td>
<td>69,500</td>
</tr>
<tr>
<td>Minus acetate</td>
<td>113,000</td>
</tr>
<tr>
<td>&quot; CoA</td>
<td>118,000</td>
</tr>
<tr>
<td>&quot; acetate and CoA</td>
<td>114,000</td>
</tr>
</tbody>
</table>

A volume of 1.0 ml. contained 0.1 M potassium phosphate, pH 7.5; 0.005 M MgCl\textsubscript{2}; 0.0024 M ATP; 0.0069 M C\textsuperscript{14}-A5P, specific activity 1 × 10\textsuperscript{4} c.p.m. per μmole; 0.0005 M reduced CoA; 0.003 M acetate; 8.3 γ of enzyme (specific activity 125). Temperature 37°; incubation, 30 minutes.

**Table XI**

*Exchange of Acetate-C\textsuperscript{14} and Acetyl CoA by Aceto-CoA-kinase*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Additions</th>
<th>Specific activity of acetyl CoA</th>
<th>Per cent exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>1,400</td>
<td>0.38</td>
</tr>
<tr>
<td>2</td>
<td>A5P</td>
<td>106,000</td>
<td>29.2</td>
</tr>
<tr>
<td>3</td>
<td>PP</td>
<td>1,950</td>
<td>0.54</td>
</tr>
<tr>
<td>4</td>
<td>A5P + PP</td>
<td>111,000</td>
<td>30.5</td>
</tr>
<tr>
<td>5</td>
<td>&quot; + PP\textsuperscript{ase}</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>6</td>
<td>&quot; + fluoride + PP</td>
<td>102,500</td>
<td>28.2</td>
</tr>
</tbody>
</table>

A volume of 0.27 ml. contained 0.15 M Tris, pH 7.4; 0.01 M MgCl\textsubscript{2}; 0.001 M acetate-C\textsuperscript{14}; 6.7 × 10\textsuperscript{4} c.p.m. per μmole; 0.00084 M acetyl CoA; 5.4 γ of enzyme (specific activity 128); and, where indicated, 0.0011 M A5P; 0.0019 M PP (Experiment 3); 0.007 M PP (Experiment 6); 50 γ of inorganic pyrophosphatase; 0.003 M potassium fluoride. Temperature 37°; incubation 30 minutes. Acetyl CoA was separated from acetate-C\textsuperscript{14} by adsorption on Norit and elution with 50 per cent ethanol containing 0.005 M KOH. Aliquots were counted and the acetyl CoA content determined as acethydroxamic acid.
calculated maximum under these conditions, and this was not increased by adding PP. Since the exchange in the presence of A5P alone also required Mg++, it appeared likely that this was due to small amounts of PP in the reaction mixture. Addition of inorganic pyrophosphatase in the presence of A5P completely blocked the incorporation of acetate-C\(^{14}\) into acetyl CoA. When, in addition to the inorganic pyrophosphatase, sufficient fluoride to inhibit the pyrophosphatase and PP were added, the exchange was restored. These data show, therefore, that both A5P and PP are required for the acetate-acetyl CoA exchange and are in agreement with the mechanism postulated in Reactions 7 and 8. These experiments also suggest that the acetyl moiety of adenyl acetate does not exchange with free acetate. This has been confirmed by incubating adenyl acetate and acetate-C\(^{14}\) with the enzyme and reisolating the adenyl acetate. There was no detectable incorporation of acetate-C\(^{14}\) into the recovered adenyl acetate.

**DISCUSSION**

The formation of adenyl acetate as an intermediate in acetyl CoA formation by yeast aceto-CoA-kinase is supported by the following observations. (1) The exchange of PP and ATP requires acetate; (2) synthetic adenyl acetate is readily converted to ATP in the presence of PP and to acetyl-CoA with CoA; (3) the CoA-independent accumulation of acethydroxamic acid, PP, and A5P in the presence of hydroxylamine; (4) the requirement of acetate and CoA for the exchange of A5P with ATP (5); and the requirement of A5P and PP for the exchange of acetate with acetyl CoA. Further support for the proposed mechanism has been provided by the recent O\(^{18}\) exchange studies of Boyer et al. (48). It was found that O\(^{18}\)-carboxyl-labeled acetate gave rise to excess O\(^{18}\) in the phosphate group of A5P. These results would be predicted from Reactions 7 and 8 and are discussed below.

The failure to accumulate enzymatically formed adenyl acetate has been puzzling. Whether this is a reflection of a low dissociation of adenyl acetate from the enzyme is not clear. The apparent dissociation constant \((K_a)\) of adenyl acetate is about \(5 \times 10^{-5}\) M, but whether this is the same as the dissociation constant of enzymatically formed adenyl acetate is not known. Further work is required to elucidate this point.

In considering the mechanism of the aceto-CoA-kinase reaction and its analogy with the formation and utilization of acetyl phosphate, certain similarities are apparent. Koshland (49), in a discussion of the mechanism of group transfer reactions, pointed out that many of the well known enzymatic group transfers may be considered as single displacement or substitution reactions. With the phosphorylation of acetate to illustrate this,
the reaction may be formulated as follows:

\[
\text{CH}_3\text{O} - \text{O} \quad + \quad \text{O}^- \quad \text{P}^+ \quad \text{P}^+ \quad \text{P}^+ \quad \text{O}^- \quad \text{O}^- \quad \text{O}^- \quad \text{adenosine} \rightarrow
\]

\[
\text{CH}_3\text{C} - \text{O} \quad \text{P}^+ \quad \text{O}^- \quad \text{P}^+ \quad \text{O}^- \quad \text{O}^- \quad \text{O}^- \quad \text{adenosine} \rightarrow
\]

\[
\text{CH}_3\text{C} - \text{O} \quad \text{P}^+ \quad \text{O}^- \quad + \quad \text{O}^- \quad \text{P}^+ \quad \text{O}^- \quad \text{O}^- \quad \text{adenosine}
\]

First, there is a nucleophilic attack on the terminal P atom by the unshared pair of electrons of the acyl oxygen atom, forming an activated complex which breaks down to ADP and acetyl phosphate. In the case of the aceto-CoA-kinase reaction, however, the nucleophilic attack by acetate may be considered to occur in a similar way, but on the adenyl acid phosphorus atom liberating PP and forming a derivative of acetyl phosphate; namely, adenyl acetate. The formation of acetyl CoA by phosphotransacetylase and by aceto-CoA-kinase also appears to be analogous when considered in this way. Thus a nucleophilic attack by CoA on the acyl carbon atom displaces, in one case phosphate, and in the other, A5P (Reaction 13).

\[
\text{CH}_3\text{O} \quad \text{O}^- \quad \text{CoA} \quad \text{S:} \quad + \quad \text{C} - \text{O} \quad \text{P}^+ \quad \text{O}^- \quad \text{adenosine} \rightarrow \text{CoA} \quad \text{S:} \quad \cdots \quad \text{C} - \text{O} \quad \text{P}^+ \quad \text{O}^- \quad \text{adenosine} \rightarrow
\]

Analogous reactions which lead to the liberation of PP are well known. In the enzymatic formation of DPN (36), FAD (39), and UDPG (40), one may consider the nucleophilic displacing unit to be the phosphate-oxygen atom of NMN, FMN, and glucose-1-phosphate, and the acceptor, the A5P moiety of ATP, or the U5P moiety of UTP.

Although it is clear that the formation of acetyl CoA via acetyl phosphate is catalyzed by two separate enzymes, all attempts to show that the aceto-
CoA-kinase reaction involves more than one enzyme have been negative. Thus far our own studies with the yeast enzyme have given no indication of a separation of the activities shown in Reactions 7 and 8 during a purification of the enzyme of about 50-fold, although further studies would be required to establish this point rigorously. Similar results have been obtained with the acetyl CoA- and butyryl CoA-forming systems of animal tissue (11, 19).

There is now plentiful evidence that the formation of acyl adenylate compounds may be of considerable significance in other acyl group activations. Thus, in the butyrate-activating system (19), the utilization of synthetic adenyl butyrate for ATP and butyryl CoA formation has been demonstrated. Furthermore, Jencks (50) has reported a CoA-independent formation of octanoyl hydroxamic acid and PP by a pig liver enzyme, and it appears possible that this represents an initial formation of adenyl octanoate which is subsequently split by hydroxylamine.

During the course of this work (21) an enzyme was purified (23) from yeast which catalyzes a PP-ATP exchange requiring specifically l-methionine, and which in the presence of hydroxylamine carries out the following reaction.

\[
(14) \text{ATP} + \text{l-methionine} + \text{hydroxylamine} \rightarrow \text{l-methionine hydroxamate} + \text{A}5\text{P} + \text{PP}
\]

Hoagland et al. (51) have also presented evidence for the formation of amino acid hydroxamates and PP from ATP, and some fifteen amino acids by a soluble protein fraction of liver. A similar system which requires amino acids for an exchange of PP and ATP has been demonstrated in extracts of a number of microorganisms (52). Maas (53), in his studies of pantothenic acid synthesis by an enzyme from Escherichia coli, has shown that pantoic acid is required for the exchange of PP with ATP. Because of this and other evidence, Maas has suggested (53) the formation of a pantoyl adenylate compound which is subsequently cleaved by β-alanine, forming pantothentic acid. It is interesting to note that the nitrogen atom of amino groups contains an unshared pair of electrons and is capable of initiating a nucleophilic displacement of the A5P in the manner proposed for the sulfur atom of CoA. Evidence has also been presented for the formation of adenyl sulfate from ATP and sulfate in the esterification of nitrophenol by liver enzymes (54) and for the formation of adenyl carbonate in the carboxylation of β-hydroxyisovaleryl CoA (55).

It therefore seems possible that acyl adenylate formation may represent a general mechanism for the activation of fatty acids, amino acids, and a variety of compounds containing an acyl group.

\[1\] Private communication from Dr. H. Beinert and Dr. C. N. Lee Peng.
MECHANISM OF ACETATE ACTIVATION

SUMMARY

Studies of the enzymatic mechanism of acetyl CoA synthesis from ATP, acetate, and CoA have shown that the reaction occurs in two steps.

\[
\begin{align*}
\text{ATP} + \text{acetate} & \rightleftharpoons \text{adenyl acetate} + \text{PP} \\
\text{Adenyl-acetate} + \text{CoA} & \rightleftharpoons \text{acetyl CoA} + \text{A}_5\text{P} \\
\text{ATP} + \text{acetate} + \text{CoA} & \rightleftharpoons \text{acetyl CoA} + \text{A}_5\text{P} + \text{PP}
\end{align*}
\]

In support of this formulation are the following observations: (1) The exchange of P\text{32}\text{P}_{32} with ATP requires acetate; (2) acethydroxamic acid is formed from ATP, acetate, and hydroxylamine in the absence of added CoA; (3) synthetic adenyl acetate is converted to ATP in the presence of PP and to acetyl CoA in the presence of CoA; (4) the exchange of A\text{5P-C}_{4} with ATP is dependent on the presence of acetate and CoA; and (5) the exchange of acetate-C\text{14} with acetyl CoA requires both A\text{5P} and PP.

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ACYL ADENYLATES: AN ENZYMATIC MECHANISM OF ACETATE ACTIVATION

Paul Berg and With the technical assistance of Georgia Newton


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