COUPLING OF ACETYL DONOR SYSTEMS WITH CHOLINE ACETYLASE

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Previous studies of the choline acetylation system of brain and of the head ganglion of the squid have utilized acetate, ATP, and CoA to generate "active acetate" (1, 2). Following the isolation of acetyl CoA by Lynen et al. (3), its identity with "active acetate" has been established by the demonstration that the acetylated coenzyme is the acetyl donor in the biological acetylation of sulfanilamide (3), choline,2 and orthophosphate (4), and in the synthesis of citrate (5) and acetoacetate (6).

The synthesis of acetylcholine from acetyl CoA and choline has been confirmed with a partially purified choline acetylase from the head ganglion of the squid. Experiments on the coupling of choline acetylase with various acetyl donor systems, in the presence of catalytic amounts of CoA, are also reported. The acetyl donors used were acetyl phosphate, pyruvate, and citrate in the presence of phosphotransacetylase (4), the pyruvate oxidation system (7, 8), and the condensing enzyme (5), respectively.

Acetyl CoA As Acetyl Donor—An experiment demonstrating the enzymatic synthesis of acetylcholine from acetyl CoA and choline is given in Table I. The formation of acetylcholine is accompanied by the appearance of a stoichiometric amount of sulfhydryl groups. This indicates that the choline acetylase preparation employed catalyzes the following reaction.

\[
\text{CoA} - \text{S} - \text{C} - \text{CH}_3 + (\text{CH}_3)_2\text{N} - \text{CH}_2 - \text{CH}_2\text{OH} \rightleftharpoons \text{CoA} - \text{SH} + (\text{CH}_3)_2\text{N} - \text{CH}_2 - \text{CH}_2\text{OC} - \text{CH}_3
\]

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† Fellow of the Rockefeller Foundation.

2 The following abbreviations are used: ATP, adenosinetriphosphate; CoA or CoASH, coenzyme A; acetyl CoA, acetyl coenzyme A; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

* Unpublished experiments of O. Wieland (quoted by Lynen et al. (3)).
Whether Reaction 1 is catalyzed by a single enzyme remains to be established.

**Acetyl Phosphate As Acetyl Donor**—The choline acetylase preparation

### TABLE I

**Acetyl CoA As Acetyl Donor in Acetylcholine Synthesis**

The sample contained 100 μM of potassium phosphate buffer, pH 7.4, 8 μM of MgCl₂, 20 μM of KCl, 24 μM of choline chloride, 0.68 μM of acetyl CoA* (purity 4.4 per cent), 0.03 μM of tetraethyl pyrophosphate, and choline acetylase fraction with 14 mg. of protein. Final volume 2.0 cc. Incubation, 15 minutes at 25°. Acetylcholine determined by bioassay.

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Sulfhydryl (μM)</th>
<th>Acetylcholine (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.05</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0.55</td>
<td>0.51</td>
</tr>
</tbody>
</table>

* Determined by enzymatic conversion to citrate with excess oxalacetate and condensing enzyme.

### TABLE II

**Acetyl Phosphate As Acetyl Donor in Acetylcholine Synthesis**

The reaction mixture contained 2 μM of MgCl₂, 30 μM of choline chloride, 10 μM of L-cysteine, 40 μM of acetyl phosphate, 20 units of CoA, 0.1 μM of tetraethyl pyrophosphate, 0.10 cc. of fractionated choline acetylase (containing 1.2 mg. of protein), and either 0.30 cc. of *E. coli* extract (Experiment 1) or 0.30 cc. of an ammonium sulfate fraction from *E. coli* extract at 69 per cent saturation (Experiment 2). The total volume was brought to 1.75 cc. with 0.02 M potassium phosphate buffer, pH 7.4. Incubation, 60 minutes. Temperature, 30°. Values in micromoles of acetylcholine determined chemically.

<table>
<thead>
<tr>
<th>System</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete.</td>
<td>19.6</td>
<td>37.0</td>
</tr>
<tr>
<td>No CoA.</td>
<td>6.9</td>
<td>0.18</td>
</tr>
<tr>
<td>&quot; acetyl phosphate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>&quot; choline.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>&quot; <em>E. coli</em>.</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

of squid can be coupled with phosphotransacetylase (4), which catalyzes Reaction 2, to effect the synthesis of acetylcholine from acetyl phosphate and choline. A typical experiment, with *Escherichia coli* extract as a source of phosphotransacetylase, is shown in Table II. Acetylcholine synthesis in this system requires CoA, acetyl phosphate, choline, and both enzyme preparations. The CoA requirement is rendered complete by
ammonium sulfate fractionation of the *E. coli* extract and dialysis, procedures which remove CoA present in the initial extract.

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

*Pyruvate As Acetyl Donor*—The coupling of pyruvate oxidation with the acetylation of choline is due to the formation and further reaction of acetyl CoA and has been obtained experimentally through Reactions 3 to 6.

(3) \[ \text{Pyruvate} + \text{DPN}^+ + \text{CoA} \rightarrow \text{acetyl CoA} + \text{DPNH} + \text{H}^+ + \text{CO}_2 \]

(4) \[ \text{Acetyl CoA} + \text{choline} \rightarrow \text{acetylcholine} + \text{CoA} \]

(5) \[ \text{Pyruvate} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{DPN}^+ \]

(6) Net reaction, 2 pyruvate + choline \[ \underset{\text{(DPN, CoA)}}{\longrightarrow} \]

\[ \text{acetylcholine} + \text{lactate} + \text{CO}_2 \]

Reaction 3 is catalyzed by the pyruvate oxidation system (7, 8), Reaction 4 by choline acetylase, and Reaction 5 by lactic dehydrogenase. Table III illustrates typical experiments with the pyruvate dismutation system in which either partially purified enzyme Fractions A and B from *E. coli* (7) or ammonium sulfate fractions from pig heart (8) are employed. In the presence of choline and choline acetylase, pyruvate oxidation occurs to the extent that choline is acetylated. As was shown previously (7, 8), little or no pyruvate is oxidized in the absence of an acetyl acceptor system; the choline acetylase system is as effective in this respect as the phosphotransacetylase or the condensing enzyme system. The CoA dependence is also illustrated by the experiments of Table III.

*Citrate As Acetyl Donor*—It has previously been shown that, in the presence of condensing enzyme and CoA, citric acid can act as an acetyl donor to several acetyl acceptor enzyme systems including choline acetylase (5). However, the yields of acetylcholine were small (approximately 0.1 μM). As already pointed out (5), the enzymatic conversion of CoA and citrate to acetyl CoA and oxalacetate (Reaction 7) is favored by removal of the latter through reduction to L-malate in the presence of DPNH and malic dehydrogenase. In the present experiments with partially purified choline acetylase, the glucose-glucose dehydrogenase system (9) was used to regenerate DPNH. As shown in Table IV, much greater yields of acetylcholine have been obtained under these conditions.

(7) \[ \text{Citrate}^- + \text{CoA} + \text{H}^+ \rightleftharpoons \text{acetyl CoA} + \text{oxalacetate}^- + \text{H}_2\text{O} \]

**Methods**

Acetylcholine was determined either by bioassay (10) or chemically. To determine acetylcholine chemically in the presence of acetyl phosphate, the latter was hydrolyzed by bringing the reaction mixture to pH 4.5 with
TABLE III
Pyruvate As Acetyl Donor in Acetylcholine Synthesis

The basic reaction mixture contained 200 \( \mu \text{M} \) of potassium phosphate buffer, pH 7.4, 2.5 \( \mu \text{M} \) of MnCl\(_2\), 4 \( \mu \text{M} \) of MgCl\(_2\), 12.6 \( \mu \text{M} \) of L-cysteine, 0.15 \( \mu \text{M} \) of DPN, 0.2 \( \mu \text{M} \) of diphosphothiamine, 20 units of CoA, 50 \( \mu \text{M} \) of potassium pyruvate, 0.05 mg. of lactic dehydrogenase, and purified E. coli Fractions A and B (Experiments 1, 2, and 3) or a pig heart fraction (Experiments 4 and 5). To this were added either 100 \( \mu \text{M} \) of choline chloride, 0.34 \( \mu \text{M} \) of tetraethyl pyrophosphate, and choline acetylase with 7.6 to 11.3 mg. of protein (experiments "a") or 10 units of phosphotransacetylase and, if not already present, 100 \( \mu \text{M} \) of potassium phosphate buffer, pH 7.4 (experiments "b"). In Experiments 1 and 2, 290 \( \mu \text{M} \) of tris(hydroxymethyl)aminomethane buffer, pH 7.4, were substituted for the phosphate buffer, and all the enzymes were dialyzed practically free of orthophosphate. Final volume 3.0 cc. (2.4 cc. in Experiment 5a). Gas, nitrogen; incubation, 60 minutes at 25-28°C. Values in micromoles.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>E. coli or heart protein</th>
<th>System</th>
<th>Change on incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pyruvate</td>
</tr>
<tr>
<td>1a</td>
<td>A, 13.9 B, 6.9</td>
<td>Complete</td>
<td>-9.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No choline acetylase</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Complete</td>
<td>0</td>
</tr>
<tr>
<td>1b</td>
<td>A, 7.6 B, 6.5</td>
<td>Complete</td>
<td>+3.2</td>
</tr>
<tr>
<td>2a</td>
<td>A, 16.1 B, 12.2</td>
<td>Complete</td>
<td>-25.6</td>
</tr>
<tr>
<td>2b</td>
<td>23.0</td>
<td>Complete</td>
<td>-30.0</td>
</tr>
<tr>
<td>4a</td>
<td>34.2</td>
<td>Complete</td>
<td>-30.3</td>
</tr>
</tbody>
</table>

* Values in parentheses determined by bioassay.
† When oxalacetate (30 \( \mu \text{M} \)) and condensing enzyme (76 \( \gamma \)) were used as acetyl acceptor system, 4.3 \( \mu \text{M} \) of citrate were formed.
1.0 N HCl and boiling it for 5 minutes. Water was added to restore the original volume and acetylcholine determined colorimetrically by the hydroxylamine method (11). Pyruvate, lactate, and acetyl phosphate were determined as previously described (7). Sulfhydryl was determined according to the procedure of Grunert and Phillips (12) on an aliquot of the solution after deproteinization with metaphosphoric acid. L-Cysteine was employed as standard. We are indebted to Dr. F. Lynen for a sample of acetyl CoA isolated from yeast. Unless otherwise specified, the CoA used was a commercial liver preparation (Armour) containing 6 units per

### Table IV

**Citric Acid As Acetyl Donor in Acetylcholine Synthesis**

Each cc. of reaction mixture contained 50 \( \mu \text{M} \) of potassium phosphate buffer, pH 7.4, 4 \( \mu \text{M} \) of MgCl₂, 20 \( \mu \text{M} \) of L-cysteine, 0.19 \( \mu \text{M} \) of DPN, 20 \( \mu \text{M} \) of choline chloride, 100 \( \mu \text{M} \) of potassium citrate, 50 \( \mu \text{M} \) of glucose, and 0.03 \( \mu \text{M} \) of tetraethyl pyrophosphate, plus 8 \( \gamma \) of malic dehydrogenase, 600 units of glucose dehydrogenase, 12.5 units of CoA (170 units per mg.), and choline acetylase with 2.3 mg. of protein (Experiment 1) or 16 \( \gamma \) of malic dehydrogenase, 1000 units of glucose dehydrogenase, 7.5 units of CoA (20 units per mg.), and choline acetylase with 5.7 mg. of protein (Experiment 2). Final volume, 4.0 cc. (Experiment 1), 2.0 cc. (Experiment 2). Temperature 25°. The dehydrogenases and choline acetylase were free of condensing enzyme. Values in micromoles of acetylcholine determined by bioassay.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Added condensing enzyme (mg.)</th>
<th>Time</th>
<th>0 min.</th>
<th>30 min.</th>
<th>60 min.</th>
<th>90 min.</th>
<th>120 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td></td>
<td>0</td>
<td>0.72</td>
<td>1.35</td>
<td>1.92</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.42</td>
<td></td>
<td>0</td>
<td>0.72</td>
<td>1.35</td>
<td>1.92</td>
<td>2.88 (2.40)*</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6.52 (4.70)</td>
</tr>
</tbody>
</table>

* Values in parentheses determined chemically.

We are indebted to Dr. F. Lipmann for a preparation containing 170 units per mg.

Crystalline condensing enzyme and highly purified malic dehydrogenase were those previously used (5). Purified glucose dehydrogenase from ox liver was kindly supplied by Dr. H. J. Strecker. The pyruvate oxidation fractions of *E. coli* and the lactic dehydrogenase were prepared as previously (7). For the preparation of the pyruvate oxidation system from heart, fresh pig heart was extracted and fractionated with ammonium sulfate (8). The precipitate obtained at 50 per cent saturation was dissolved in 0.02 M potassium phosphate buffer, pH 7.0, and used after dialysis against the same buffer for 15 hours at 3-4°.

An acetone powder of squid head ganglion was extracted in the manner
previously described (2). The extract was fractionated with ammonium sulfate, and the precipitate between 23 and 46 per cent saturation dissolved in 0.02 M potassium phosphate buffer, pH 7.4, and used after dialysis against the same buffer for 3 hours at 3-4°. The protein content of the choline acetylase preparations was determined colorimetrically by the method of Gornall et al. (13).

E. coli 4157 extract as a source of phosphotransacetylase was prepared and extracted according to Ochoa et al. (14). The extract was also fractionated with ammonium sulfate and the precipitate at 60 per cent saturation dissolved in 0.02 M potassium phosphate buffer, pH 7.4, and dialyzed against the same buffer for 24 hours at 3-4°.

Acetyl phosphate was prepared according to Stadtman and Lipmann (15).

SUMMARY

1. A partially purified choline acetylase from squid head ganglion synthesizes acetylcholine from acetyl coenzyme A and choline with the appearance of a stoichiometric amount of sulfhydryl groups.

2. With choline acetylase, choline, and catalytic amounts of coenzyme A, acetyl phosphate, pyruvate, or citrate acts as an acetyl donor for acetylcholine synthesis in the presence of phosphotransacetylase, the pyruvate oxidation system, or condensing enzyme, respectively.

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