The oxidation of pyruvate, catalyzed by the pyruvic oxidase of pigeon breast muscle (2), yields active acetate. Evidence in support of this is (a) the synthesis of acetyl sulfanilamide in the pigeon liver acetylation system (3, 4) and (b) formation of acetyl phosphate in the phosphotransacetylase system (5) from pyruvate. In bacterial preparations, also, pyruvate forms active acetate, as shown by the coupled synthesis of either acetyl phosphate or citrate (6). CoA is essential for the above reactions, and it has been inferred that the active acetate is acetyl CoA. However, acetyl CoA was not isolated from the reaction mixture. Since relatively crude preparations of enzymes, either of pyruvic oxidase or of auxiliary enzymes, were used in these studies, the possibility that the first product of pyruvate oxidation is some other compound which is subsequently converted to acetyl CoA is not excluded.

We have studied the primary oxidative reaction which can now be formulated as follows:

\[
\text{Pyruvate} + \text{DPN}^+ + \text{CoA} \rightarrow \text{acetyl CoA} + \text{DPNH} + \text{CO}_2 + \text{H}^+ \quad (1)
\]

**Results**

Spectrophotometric Demonstration—The purified pyruvic oxidase of pigeon breast muscle catalyzes the reduction of DPN by pyruvate in the presence of CoA (Fig. 1, Curve 1). On adding lactic dehydrogenase, the optical density at 340 nm decreases as a result of reoxidation of DPNH by pyruvate. Owing to the difficulty of removing traces of lactic dehydrogenase from pyruvic oxidase, a different system has been devised for following pyruvate oxidation. Pyruvate is produced by the oxidation of lactate (Reaction 2) catalyzed by lactic dehydrogenase.

\[
\text{Lactate} + \text{DPN}^+ \rightarrow \text{pyruvate} + \text{DPNH} + \text{H}^+ \quad (2)
\]
After equilibrium is reached, as shown by the constant optical density at 340 nm, pyruvic oxidase is added to start the oxidation (Fig. 1, Curves 2 and 3). The over-all reaction is represented by Reaction 3, which is the sum of Reactions 1 and 2. The rate of DPN reduction is high initially but declines rapidly when the available CoA is utilized. Addition of phosphotransacetylase (7) increases the rate markedly due to acetyl phosphate formation (Reaction 4), and the resultant liberation of CoA which can recycle in Reaction 1. As expected (7), addition of arsenate further increases the rate of DPN reduction by promoting acetyl phosphate breakdown to acetate and phosphate. The change in optical density produced by the addition of pyruvic oxidase (corrected for the slow continued DPN reduction, indicating, perhaps, non-enzymatic liberation of CoA from acetyl

\[
\text{Acetyl CoA} + \text{phosphate} \rightleftharpoons \text{acetyl phosphate} + \text{CoA} \quad (4)
\]
CoA), is proportional to CoA added. It must be noted that one-half of the DPN reduced at this stage is involved in the oxidation of lactate to pyruvate. In order to satisfy the requirements of Reaction 3, as much lactate is oxidized as the pyruvate which disappears.

TPN will not replace DPN in pyruvate oxidation, and pantetheine² (synthetic Lactobacillus bulgaricus factor) (8), at levels of $6.7 \times 10^{-3} \text{ M}$ or lower, will not replace CoA. Protogen B³ (9), α-lipoic acid³ (10), and

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Pyruvate</th>
<th>−SH</th>
<th>Active acetate</th>
<th>DPNH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-2.9</td>
<td>-2.5</td>
<td>+2.4</td>
<td>+1.9</td>
</tr>
<tr>
<td>2</td>
<td>-7.3</td>
<td>+6.6</td>
<td>+12.6</td>
<td></td>
</tr>
</tbody>
</table>

Experiment 1—CoA (8 mg. or 1800 units at pH 6.5) and sodium borohydride (20 μM) in 1.0 ml. were heated for 1 minute in boiling water. The mixture was cooled in an ice bath and the excess borohydride decomposed by adding 0.2 ml. of 1 per cent acetic acid. Phosphate (50 μM), MgCl₂ (20 μM), cocarboxylase (40 γ), pyruvate (30 μM), and DPN (3 μM) were added. Final volume 3.0 ml., pH 6.0. The initial concentration of the components listed in Table I was determined and the reaction started by adding 26 units of pyruvic oxidase. After incubation at 37° for 5 minutes in an atmosphere of N₂, the mixture was reanalyzed. For DPNH assay the enzyme was inactivated with methanol. Active acetate is measured by the hydroxamic acid reaction (12).

Experiment 2—CoA (17 mg. or 6000 units) was reduced with 30 μM of borohydride in 1.5 ml. as in Experiment 1. DL-Lactate (100 μM), MgCl₂ (20 μM), cocarboxylase (40 γ), DPN (15 μM), lactic dehydrogenase and pyrophosphate (100 μM) were added in a total volume of 3.2 ml., pH 8.5. The oxidation was started by adding 9 units of pyruvic oxidase. Incubated 70 minutes at 37° in N₂. At 20 minutes and 40 minutes, 9 units of pyruvic oxidase were again added since it is inactivated under these conditions.

A boiled extract of α-ketoglutaric oxidase containing protogen presumably in coenzyme form (11) were also inactive.

Stoichiometry—The data on the stoichiometry of the reaction are presented in Table I. Lynen et al. (13) have found that the reactive group of CoA in acetyl CoA formation is the sulfhydryl. Our results also show that for every mole of active acetate formed (shown by the reaction with hydroxylamine) 1 mole of −SH disappears. The results shown in Experiment 1 are consistent with the requirements of Reaction 1, while the data of Experiment 2 satisfy the requirements of Reaction 3. The slightly

² Kindly supplied by Dr. F. M. Strong.
³ We are indebted to Dr. T. Jukes for the protogen and to Dr. L. J. Reed for the α-lipoic acid.
lower DPNH in Experiment 1 may be due to its reoxidation catalyzed by contaminating traces of lactic dehydrogenase.

Isolation of Acetyl CoA—The remainder of the reaction mixture of Experiment 2, Table I, was acidified with hydrochloric acid to pH 3.0, and nearly saturated with ammonium sulfate (50 gm. per 100 ml.). The solution was extracted five times with equal volumes of a phenol-benzyl alcohol mixture (3:1). Each time the mixture was centrifuged to separate the layers. The clear phenol-benzyl alcohol solution was dried for 30 minutes over anhydrous Na₂SO₄, 2 volumes of chloroform were added, and the mixture extracted three times with 0.2 volume of water. The combined aqueous solution was extracted five times with chloroform to remove phenol and then lyophilized. The residue weighed 10 mg. and contained

<table>
<thead>
<tr>
<th>Table II</th>
<th>Synthesis of Citrate from Isolated Active Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active acetate added (hydroxamic acid)</td>
<td>Citrate formed</td>
</tr>
<tr>
<td>µM</td>
<td>µM</td>
</tr>
<tr>
<td>0.20</td>
<td>0.18</td>
</tr>
<tr>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.20 (No condensing enzyme)</td>
<td>0</td>
</tr>
</tbody>
</table>

The complete system contained 25 µM of potassium phosphate buffer, pH 7.4, 4 µM of MgCl₂, 10 µM of cysteine, 5 µM of oxalacetate, active acetate, and condensing enzyme. Final volume 1.0 ml., incubated 15 minutes at 25°. Other details as in Stern et al. (14).

0.34 µM of active acetate (hydroxamic acid reaction) per mg. (purity approximately 28 per cent, assuming the molecular weight of CoA to be 767). The active acetate was identified as acetyl CoA by citrate formation in the presence of condensing enzyme (14). The citrate formed was equivalent to the potential hydroxamic acid (Table II).

DISCUSSION

By the use of highly purified pyruvic oxidase and stoichiometric amounts of CoA, it has been possible to study the oxidation of pyruvate to acetyl CoA uncomplicated by secondary dismutation and condensing reactions as in previous studies (4–6, 15). From these results, it may be concluded that DPN and CoA are the prosthetic groups of pyruvic oxidase. In addition previous work from this laboratory (2, 3) has shown that Mg++ and cocarboxylase are also required for pyruvate oxidation. The function of protogen (thioctic acid) present in large amounts in pyruvic oxidase (2) is not known. The mechanism of pyruvate oxidation involving the four known cofactors may be postulated as shown in the accompanying dia-
The compounds within brackets may be complexes with enzyme. The initial decarboxylation resulting in the formation of an acetaldehyde-

\[
\begin{align*}
\text{O} & \quad \text{Mg}^{+} \quad \text{cocoxyrase} \\
\\
\text{CH}_2\text{CCH}_2\text{COOH} \quad \text{CH}_2\text{C} \quad \text{CO}_2
\end{align*}
\]

enzyme complex and leading to acetoin formation as a side reaction has been previously discussed (4, 16). Lynen et al. (13) have also considered the possibility of condensation of CoA with the carbonyl group of pyruvate or acetaldehyde before oxidation.

**EXPERIMENTAL**

**Methods**—The reduction of DPN was followed in the Beckman DU spectrophotometer at 340 m\(\mu\). DPNH was estimated in the chemical balance studies (Table I) by reoxidation with acetaldehyde and alcohol dehydrogenase (17). Pyruvate was analyzed as the 2,4-dinitrophenylhydrazone (18) and —SH by the nitroprusside reaction (19) modified for use in the Beckman spectrophotometer. CoA was estimated by arsenolysis in the phosphotransacetylase system (7) or by measuring the rate of DPN reduction in the presence of the \(\alpha\)-ketoglutaric oxidase system (1, 20) and succinyl CoA deacylase (5).4

**Preparations**—The preparative methods used were as follows: lithium pyruvate (21), alcohol dehydrogenase (17), condensing enzyme (14), lactic dehydrogenase (22), and CoA (23). DPN (about 90 per cent pure) was supplied by Pabst Laboratories.

Pyruvic oxidase was prepared by a modification of the method of Jaganathan and Schweet (2). The preparation at the Fraction 3 stage was diluted with 0.05 \(\text{m}\) phosphate so that the protein concentration was about 30 mg. per ml. The \(\text{pH}\) of the solution was taken to 5.9 with 1 per cent acetic acid and the precipitate removed. The supernatant was adjusted to \(\text{pH}\) 7.2 and 23 gm. of ammonium acetate were added per 100 ml. of solution.

After standing in an ice bath for 10 minutes, the precipitate was collected by centrifuging for 5 minutes at 18,000 \( \times \) g. The precipitate was dissolved in 0.01 M phosphate buffer, pH 7.2, dialyzed against the same buffer for 6 hours, and the small precipitate removed. The activity of the oxidase prepared by this method was between 15 and 25 units per mg. of protein.

**SUMMARY**

The single step oxidation of pyruvate by DPN in the presence of CoA has been studied. The product of the reaction was isolated and shown to be acetyl CoA. Thus, at least four cofactors are required for pyruvate oxidation; namely, DPN, CoA, Mg\(^{++}\), and cocarboxylase.

"The authors appreciate the continued interest and encouragement of Dr. David E. Green as well as the technical assistance of Mrs. Alfons Hilbe in this study.

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J. W. Littlefield and D. R. Sanadi


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