REMOVAL OF THIOCTIC ACID FROM ENZYMES*

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The rôle of thioctic acid in the oxidative decarboxylation of α-keto acids is now well established (1, 2). This was accomplished largely by studies with microbial cells grown essentially free of the cofactor. In some instances (2) it has been possible to separate the coenzyme from microbial enzymes involved in thioctic acid metabolism by extensive purification procedures. Although the direct demonstration of the function of thioctic acid in vertebrate enzyme systems is lacking, the occurrence of large amounts of the cofactor in purified pyruvic (3) and α-ketoglutaric (4) oxidases from pigeon breast muscle and pig heart indicates (5) that the rôle of the cofactor in vertebrate systems parallels that in microbes. The factor is bound so tightly to the vertebrate enzymes that such procedures as dialysis, repeated washings, or ion exchange techniques do not dislodge it.

Experiments with the α-keto acid oxidases from the ciliated protozoan, Tetrahymena pyriformis S, indicate that thioctic acid can be readily removed from the enzyme by adsorption with alumina (6). It has since been recognized (7) that cofactor removal by this procedure requires the participation of an enzyme fraction which seems to catalyze the liberation of thioctic acid from protein-bound coenzyme in a reversible manner; the free thioctic acid which is liberated is then adsorbed on the alumina. Ammonium sulfate precipitation of pigeon liver acetone powder extracts separates a fraction which liberates the cofactor from enzymes. Since this fraction has not as yet been purified enough to ascertain the nature and specificity of the enzyme, the ammonium sulfate fraction has been termed (8) a "thioctic acid-splitting" fraction which contains "thioctic acid-splitting" activity.

Methods

The alumina treatments were carried out in conical centrifuge tubes. The enzyme solution was brought to room temperature and then rapidly stirred for 3 minutes with adsorption alumina. The adsorbent was used

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in a ratio of 1 gm. for each 100 mg. of protein. The tube was then cooled in an ice bath, and the alumina was allowed to settle. The supernatant fluid was pipetted and was then centrifuged at 2000 × g for 15 minutes at 4° to remove the remainder of the alumina. Treatment at room temperature effects more complete removal of thioctic acid than is accomplished at the lower temperatures used previously (6, 7).

Examination of different batches of alumina from various suppliers indicated that material with adsorptive capacities barely equivalent to a Brockmann characterization of II (9) is satisfactory; i.e., from a mixture of Sudan yellow and methoxyazobenzene (2 mg. of each dye in 2 ml. of a mixture (1:4) of benzene and light mineral oil) at least half of the methoxyazobenzene is retained in a 10 × 1.5 cm. column which is packed to a height of 5 cm. Sudan yellow should not migrate more than 10 mm. from the top of the column. Development is accomplished with 20 ml. of a mixture (4:1) of benzene and light mineral oil.

Large mesh (80 to 200) adsorbent is routinely used directly from the manufacturer's container. Acid or alkali washings do not uniformly affect the ability to adsorb thioctic acid.

Following enzyme treatment, the adsorbed thioctic acid may be removed from the alumina with a mixture containing 89 per cent benzene, 5 per cent CHCl₃, 5 per cent ethanol, and 1 per cent butanol. Thioctic acid is extracted from the organic mixture by shaking with 1/3 volume of 5 per cent aqueous NaHCO₃. Manometric assay (10) of the bicarbonate extracts indicates a recovery of 40 to 60 per cent of the thioctic acid removed from the enzyme. These assays are, however, only approximate, since the extracts contain oxidizable substrates. The enzymes were also assayed manometrically for thiotic acid before and after alumina treatment; the cofactor was liberated by hydrolysis in 6 N H₂SO₄ for an hour at 120°.

Analytical Methods—Pyruvate oxidation was measured by the rate of dismutation of pyruvate to acetyl phosphate, carbon dioxide, and lactate:1

\[
\text{(1) Pyruvate} + \text{DPN}^+ + \text{CoA} \rightleftharpoons \text{acetyl CoA} + \text{CO}_2 + \text{DPNH} + \text{H}^+ \text{ (pyruvic oxidase)}
\]

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

\[
\text{(3) Pyruvate} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{DPN}^+ \text{ (lactic dehydrogenase)}
\]

\[
\text{(4) Sum, 2 pyruvate} + \text{phosphate} \rightleftharpoons \text{acetyl phosphate} + \text{CO}_2 + \text{lactate}
\]

Acetyl phosphate formation was determined by the hydroxamic acid method (11).

1 The following abbreviations are used: oxidized and reduced diphosphopyridine nucleotide, DPN⁺ and DPNH; coenzyme A, CoA; diphosphothiamine, DPT; glutathione, GSH.
The initial step in oxidation of \( \alpha \)-ketoglutarate (Reaction 5), which is analogous to Reaction 1 of pyruvate, was measured by following the reduction of DPN by the increase in optical density at 340 \( \text{mu} \) in the Beckman DU spectrophotometer (12).

Preparation of Enzymes—\( T. pyriformis \) S was grown in mass culture in 21 liter Pyrex carboys containing 10 liters of 2 per cent "bacteriological peptone" (Nutritional Biochemicals Corporation), 0.5 per cent yeast extract (Nutritional Biochemicals), and approximately 5 parts per million of Antifoam A (Dow Corning Corporation). The carboys were well aerated on a vacuum line, and after 48 hours of growth at 25° ± 1.5° the cells were harvested by passage through a Sharples supercentrifuge. The collected cells were suspended in 0.01 M phosphate buffer, pH 7.4, and washed three times with the same buffer. The concentrate was ground with twice its weight of powdered quartz on a mechanical grinder for 30 minutes at 4° and extracted with 5 volumes of 0.01 M phosphate buffer, pH 7.4, for an additional 30 minutes. The suspension was clarified by centrifuging at 5000 \( \times \) g for 15 minutes in the cold.

Pyruvic oxidase activity of \( S. faecalis \) was measured with extracts prepared according to Korkes et al. (13); the cells were grown in the complete AC.3 medium (10). Pyruvic oxidase from pigeon breast muscle was prepared as described by Jagannathan and Schweet (14) from muscle which had been stored at \(-20^\circ\) for 8 to 12 months. The material obtained at Step 1 and at Step 6 of this procedure was examined. The dialyzed \( S. faecalis \) extracts added to incubation mixtures as a source of phosphotransacetylase and lactic dehydrogenase were prepared (13) from acetone powders of cells grown in synthetic, thioctic acid-deficient medium (10).

Purification of \( \alpha \)-ketoglutaric oxidase followed the procedure described by Sanadi et al. (4). The specific activity of the ammonium acetate precipitate was 63, expressed as units per mg. of protein.

The thioctic acid-splitting fraction was prepared from extracts of pigeon liver acetone powder as described previously (7).

Results

Treatment of extracts of \( T. pyriformena \) with alumina removes large quantities of thioctic acid from the enzyme. This is accompanied by almost complete loss of activity. The activity of alumina-treated enzyme is almost entirely restored by the addition of 6,8-thioctic acid (Table 1). Similar results are obtained with extracts of \( S. faecalis \) prepared from either lyophilized or acetone-dried cells (Table 11).

The adsorption procedure, however, does not remove thioctic acid or
alter the pyruvic oxidase activity of phosphate extracts of washed pigeon breast muscle minces. However, extracts prepared from acetone powders of breast muscle, although containing only a fraction of the enzymatic activity of the washed mince extracts, do respond to alumina treatment. The thioctic acid-splitting activity is thus separated from the minced preparation during the washing and extraction procedure. Addition of the thioctic acid-splitting fraction from pigeon liver to these phosphate ex-

### TABLE I

**Effect of Alumina on Pyruvate Dismutation by Extracts of *T. pyrijormis***

The incubation mixture contained, in 1.4 ml., 100 μmoles of Na pyruvate, 10 μmoles of MgCl₂, 0.22 μmole of DPT, 20 units of CoA, 10 μmoles of GSH, 0.1 μmole of DPN⁺, dialyzed *S. faecalis* extract containing 0.28 mg. of protein as the source of phosphotransacetylase and lactic dehydrogenase, 100 μmoles of phosphate buffer, pH 7.4, and 10 mg. of *Tetrahymena* extract. Incubation was for 60 minutes at 32° in nitrogen.

<table>
<thead>
<tr>
<th>Enzyme treatment and additions</th>
<th>Thiocytic acid concentration of enzyme mixture</th>
<th>Acetyl phosphate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>None..................................... 0.370</td>
<td>1.1 μmoles</td>
<td></td>
</tr>
<tr>
<td>&quot; + 0.5 γ thioctic acid..............</td>
<td>0.021</td>
<td>0.1</td>
</tr>
<tr>
<td>Alumina-treated ................. 0.021</td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE II

**Effect of Alumina Treatment on Pyruvate Dismutation by Extracts of *S. faecalis***

The incubation mixture contained, in 1.0 ml., 50 μmoles of phosphate buffer, pH 7.4, 2.4 μmoles of MgCl₂, 1.6 μmoles of MnCl₂, 11 μmoles of GSH, 0.15 μmole of DPN⁺, 0.47 μmole of DPT, 28 units of CoA, 50 μmoles of Na pyruvate, and dialyzed *Streptococcus* extract containing 7 mg. of protein. Incubation was for 40 minutes at 38° under nitrogen.

<table>
<thead>
<tr>
<th>Enzyme treatment and additions</th>
<th>Lyophilized cells</th>
<th>Acetone powder</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thiocytic acid concentration of enzyme mixture</td>
<td>Acetyl phosphate formed</td>
</tr>
<tr>
<td>None..................................... 0.199 2.5</td>
<td>0.232 2.7</td>
<td></td>
</tr>
<tr>
<td>&quot; + 0.5 γ thioctic acid............ 0.021 0.2</td>
<td>0.042 0.3</td>
<td></td>
</tr>
<tr>
<td>Alumina-treated................... 0.021 2.1</td>
<td>0.042 2.6</td>
<td></td>
</tr>
</tbody>
</table>
TABLE III

Removal of Thiocytic Acid from Pyruvate Oxidase of Pigeon Breast Muscle

The incubation mixture contained, in 1.0 ml., 50 μmoles of phosphate buffer, pH 7.4, 2.4 μmoles of MgCl₂, 1.6 μmoles of MnCl₂, 11 μmoles of GSH, 0.15 μmole of DPN⁺, 0.47 μmole of DPT, 28 units of CoA, 50 μmoles of Na pyruvate, and dialyzed S. faecalis extract containing 0.24 mg. of protein as the source of phosphotransacetylase and lactic dehydrogenase. In Experiments 1 and 3, 7.8 mg. of protein of breast muscle extract of specific activity 0.3 were used. 30 mg. of enzyme protein were used in Experiment 2; the powder had a specific activity of <0.1. 10 mg. of 0 to 37 per cent liver fraction were used in Experiment 3. When indicated, the liver fraction was inactivated by heating at 70° for 6 minutes. Experiments 4 and 5 contained 0.316 mg. of protein of pyruvic oxidase, specific activity 42. In Experiment 5, 35 mg. of protein of the 0 to 37 per cent liver fraction were added. All incubations were carried out under nitrogen for 60 minutes at 38°.

<table>
<thead>
<tr>
<th>Experiment No</th>
<th>Enzyme fraction</th>
<th>Treatment</th>
<th>Thiocytic acid content of added enzyme mixture (μmoles)</th>
<th>Acetyl phosphate formed (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Minced breast muscle extract</td>
<td>None</td>
<td>0.263</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>“ + 0.5 γ thiocytic acid</td>
<td>0.274</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alumina-treated</td>
<td>1.230</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>Extract of acetone powder of breast muscle</td>
<td>None</td>
<td>0.084</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>“ + 0.5 γ thiocytic acid</td>
<td>0.330</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alumina-treated</td>
<td>0.024</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>“ + 0.5 γ thiocytic acid</td>
<td>0.341</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
<td>Minced breast muscle extract + 0 to 37% liver fraction</td>
<td>None</td>
<td>0.178</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>“ + 0.5 γ thiocytic acid</td>
<td>0.183</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alumina-treated</td>
<td>0.380</td>
<td>1.7</td>
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<tr>
<td></td>
<td></td>
<td>“ + 1 γ thiocytic acid</td>
<td>0.044</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alumina-treated</td>
<td>0.380</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>“ + 1 γ thiocytic acid</td>
<td>0.044</td>
<td>1.8</td>
</tr>
</tbody>
</table>
tracts results in ready response to the alumina procedure for removal of the cofactor. Table III shows that the adsorption technique is also effective in removing thioctic acid from highly purified pyruvic oxidase from pigeon breast muscle (specific activity 42). Stimulation of dismutation activity is achieved by addition of thioctic acid to alumina-treated enzymes. The alumina procedure, in conjunction with the thioctic acid-splitting fraction, also removes the cofactor from purified α-ketoglutaric oxidase (Fig. 1).

Fig. 1. Effect of alumina treatment of α-ketoglutaric oxidase on rate of reduction of DPN⁺. The incubation mixture contained, in 3.0 ml., 75 units of CoA, 50 μmoles of GSH, 0.3 μmole of DPN, 100 μmoles of glycylglycine, pH 7.2, and enzyme mixture. The CoA and GSH were preincubated at room temperature for 15 minutes before the addition of the other components. After 2 minutes 5 μmoles of α-ketoglutarate were added to start the reaction. A cuvette containing all components except the substrate served as the blank. The enzyme mixture contained 0.33 mg. of protein of α-ketoglutaric oxidase, specific activity 66, and 10.9 mg. of 0 to 37 per cent liver fraction. The thioctic acid content of the untreated mixture was 0.263 μg of thioctic acid, and in the alumina-treated mixture the cofactor amounted to 0.016 μg.

The ready removal of thioctic acid from α-keto acid oxidase from Tetrahymena, S. faecalis, pigeon breast muscle, and pig heart by alumina adsorption attests to the general applicability of the method for removing the cofactor from enzymes with negligible inactivation of the enzyme protein. It may be anticipated that the procedure will allow the direct demonstration of thioctic acid participation in additional enzyme systems suspected of requiring the cofactor.

SUMMARY

Alumina treatment of extracts of Tetrahymena pyriformis and of Streptococcus faecalis removes thioctic acid from the enzyme proteins and is...
accompanied by a decreased rate of pyruvate oxidation. Activity of the treated extracts is restored upon addition of thioctic acid.

The procedure, in the presence of an ammonium sulfate fraction from pigeon liver acetone powder extracts, also removes thioctic acid from purified pyruvic oxidase from pigeon breast muscle and from purified \( \alpha \)-keto-gluutaric oxidase from pig heart muscle.

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