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FINAL PROJECT REPORT

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Principal Investigator: Dr. Gerald R. Seaman
Title of Project: Metabolism of Vitamins, with particular emphasis on Thiamin and Thiocetic Acid.

Report Prepared By: Gerald R. Seaman

Date: November 22, 1954
For Period: 1, Nov. 1953
          31, Oct. 1954
I INTRODUCTION

The finding (1) that thioctio acid (ThA) is removed from extracts of the ciliated protozoan, Tetrahymena pyriformis, by treatment with adsorption alumina suggested that this procedure may provide a tool for investigations of functions of ThA other than the established participation in α-keto acid oxidations (2, 3). The role of the cofactor in these oxidations suggests participation in additional acyl transfer reactions. The animal acetate-activating system (reaction 1) seemed a likely suspect.

\[
\text{acacetate} + \text{ATP} + \text{Co A} \rightarrow \text{acetyl-Co A} + \text{AMP} + \text{PP}
\]  

II RESULTS

**Thioctio acid requirement of the acetate system**

Alumina treatment of pigeon liver acetone powder extracts decreases the rate of acetyl-Co A formation from acetate; the thioctio acid content of the extract is also decreased (Table I). Addition of synthetic thioctio acid restores activity to mixtures containing treated enzyme.

**Table I. Alumina Treatment of Acetone Powder Extracts**

<table>
<thead>
<tr>
<th>Enzyme treatment and additions</th>
<th>System 1</th>
<th>System 2</th>
<th>System 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hydroxamate formed</td>
<td>Acetyl phosphate formed</td>
<td>Citrate formed</td>
</tr>
<tr>
<td>None</td>
<td>1.23</td>
<td>1.17</td>
<td>1.20</td>
</tr>
<tr>
<td>+ 0.1 μg ThA</td>
<td>1.19</td>
<td>1.16</td>
<td>1.21</td>
</tr>
<tr>
<td>Alumina treated</td>
<td>0.11</td>
<td>0.20</td>
<td>0.16</td>
</tr>
<tr>
<td>+ 0.1 μg ThA</td>
<td>1.17</td>
<td>1.16</td>
<td>1.16</td>
</tr>
</tbody>
</table>

1. The following abbreviations are used: ATP, adenosine triphosphate; AMP, adenosine monophosphate; PP, inorganic pyrophosphate; Co A, coenzyme A; BAL, 2,3-dimercaptopropanol; GSH, glutathione; Tris, tris-(hydroxymethyl)-aminomethane; ThA, thioctio acid; DPN, diphosphopyridine nucleotide; DPT, diphosphothiamine; Pi, inorganic phosphorus.
As anticipated in a ThA dependent system (4), arsenite inhibits the acetate-activating reaction; arsenenate is without effect. Adjustment of the thioctic acid content of extracts to just optimal levels by alumina treatment and the addition of synthetic thioctic acid results in preparations which are much more sensitive to the inhibitor than are untreated extracts which contain excess cofactor. The arsenite inhibition is reversed by BAL, but not by such monothiol compounds as cysteine, thiglycollate, or additional amounts of GSH (Table II).

TABLE II. Arsenite Inhibition of Acetate Activating System

The incubation mixture was as described in System 1 of Table I. The extract containing 15 mg. of protein was incubated for 90 min. at 32° C. The ThA content of this amount of untreated extract was 0.466 μg. After alumina treatment the cofactor content was 0.055 μg.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Hydroxamate formed</th>
<th>Untreated extract</th>
<th>Alumina treated extract 0.1 μg ThA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>μM</td>
<td>μM</td>
<td></td>
</tr>
<tr>
<td>2 μM arsenite</td>
<td>0.88</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>4 μM arsenite</td>
<td>0.71</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>8 μM arsenite</td>
<td>0.62</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>8 μM arsenate</td>
<td>0.91</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td>4 μM arsenite + 20 μM cysteine</td>
<td>0.68</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>4 μM arsenite + 20 μM thiglycollate</td>
<td>0.70</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>4 μM arsenite + 10 μM additional GSH</td>
<td>0.66</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>4 μM arsenite + 5 μM BAL</td>
<td>0.90</td>
<td>0.79</td>
<td></td>
</tr>
</tbody>
</table>
Reversal of the alumina effect by added ThA is stimulated by sodium ions and is inhibited by potassium ions (Fig. 1). However, in the presence of high

**FIGURE 1. Sodium Ion Stimulation of ThA Reversal of Alumina Treated Acetate Enzyme at various levels of Potassium Ion Concentration**

The incubation mixtures were as described for System 3 in the legend of Table I. All acidic components were converted to their Tris salts. Pigeon liver acetone powder was extracted with 0.1 M Tris buffer, pH 8.2. Activity before alumina treatment in each of the various mixtures was taken as 100%. ThA (0.1 µg) was included in mixtures after alumina treatment. KCl and NaCl were added as indicated.
concentrations of potassium ions, where ThA is inactive, the reduced form of the cofactor, dihydrothiociotic acid, increases the rate of acetyl-CoA formation (Fig. 2). For activity in the acetate activating reaction then, the disulfide linkage of ThA must be reductively split by a system which is stimulated by sodium ions and inhibited by potassium ions. This system is inactivated by repeated freezing and thawing.

**FIGURE 2. Stimulation of Alumina Treated Extracts by Dihydrothiociotic Acid in the Presence of Large Amounts of Potassium Ion**

The incubation mixture was as described for system 1 in the legend of Table I, except that 100 μM of KCl were included. The extract (18 mg of protein) was alumina treated and incubated 90 min. at 32°C.

The 8-methyl derivative of ThA which is antagonistic to the cofactor for the growth of several microorganisms (5), also inhibits the acetate activating
reaction; the inhibition is reversed by ThA (Fig. 3). In the presence of high concentrations of potassium ion the analog is not inhibitory (Table III). To be

**FIGURE 3. Effect of 3-methyl ThA on Acetate Activating Activity of Acetone Powder Extracts at Various Levels of ThA**

The incubation mixture was as described for system 1 in the legend of Table I. Fifteen mg of protein containing 0.533 µg of thioctic acid were incubated for 90 min. at 32° C.

**TABLE III. Effect of Potassium Ions on 3-methyl ThA Inhibition of Acetate Activation**

The incubation was as described for system 2 in the legend of Table I. KCl and 3-methyl ThA added as indicated. The acetone powder extract containing 13 mg protein and the *S. faecalis* extract containing 0.2 mg protein were incubated at 32° C. for 90 min.

<table>
<thead>
<tr>
<th>KCl Concentration (µM)</th>
<th>Acetyl phosphate formed (µM)</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Inhibitor</td>
<td>1.06</td>
<td>51</td>
</tr>
<tr>
<td>40</td>
<td>1.07</td>
<td>42</td>
</tr>
<tr>
<td>50</td>
<td>1.10</td>
<td>24</td>
</tr>
<tr>
<td>70</td>
<td>1.13</td>
<td>13</td>
</tr>
<tr>
<td>100</td>
<td>1.23</td>
<td>0</td>
</tr>
</tbody>
</table>
antagonistic to ThA in the acetate activating reaction the 8-methyl compound must be converted to the dithiol form. This activation is apparently achieved by the same potassium sensitive system which activates ThA.

Jones, et. al. (6) demonstrated that the initial step in acetate activation is the formation of an enzyme-AMP complex (reaction 2). This may be measured by the rate of exchange between isotopic PP

\[
\text{Enzyme} + \text{ATP} \xrightarrow{\text{reaction 2}} \text{PP} + \text{Enzyme-AMP}
\]

and ATP. Co A inhibits this exchange, indicating a subsequent exchange of Co A for AMP. Table IV shows that dithiol ThA also inhibits this pyrophosphate exchange; the disulphide compound is without effect.

**TABLE IV.** $^{32}P$ Exchange between Inorganic PP and ATP

<table>
<thead>
<tr>
<th>Additions</th>
<th>pyrophosphate</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ots/min/ml.</td>
<td>ots/min/μM</td>
</tr>
<tr>
<td>None</td>
<td>92,970</td>
<td>27,700</td>
</tr>
<tr>
<td>11 units enzyme</td>
<td>63,100</td>
<td>18,600</td>
</tr>
<tr>
<td>+ 2 μM Co A</td>
<td>76,610</td>
<td>23,520</td>
</tr>
<tr>
<td>+ 2 μM Co A</td>
<td>62,960</td>
<td>19,350</td>
</tr>
<tr>
<td>+ 2 μM dihydro ThA</td>
<td>76,340</td>
<td>25,210</td>
</tr>
<tr>
<td>+ 10 μM KCN</td>
<td>60,630</td>
<td>19,550</td>
</tr>
<tr>
<td>+ 2 μM dihydro ThA + 10 μM KCN</td>
<td>68,340</td>
<td>21,180</td>
</tr>
</tbody>
</table>
Cyanide accelerates the pyrophosphate exchange and when in combination with dithiol ThA, it reverses the dihydrothiolic acid inhibition. This is compatible with the proposal that in the acetate system, ThA is bound to the enzyme protein by a S-C linkage, which is readily broken by cyanide. Inhibition of the pyrophosphate exchange by dithiol ThA is then the result of formation of large amounts of this enzyme-dithiol thiolic acid complex which speeds reaction 2 to the right. The reaction sequence of acetate oxidation may then be visualized to proceed as:

\[
\begin{align*}
\text{Enz} + \text{ATP} & \rightleftharpoons \text{Enz-AMP} + \text{PP} \\
\text{S} + \text{SH} & \rightleftharpoons \text{S} + \text{SH} \\
\text{Enz-AMP} + \text{HSCoA} & \rightleftharpoons \text{Enz-SCoA} + \text{AMP} \\
\text{S} + \text{H} & \rightleftharpoons \text{S} + \text{SH} \\
\text{Enz-SCoA} + \text{CH}_3 \text{COOH} & \rightleftharpoons \text{Enz-SCoA} + \text{HOH} \\
\text{S} + \text{SH} & \rightleftharpoons \text{S} + \text{SCOCH}_3 \\
\text{Enz-SCoA} + \text{HOH} & \rightleftharpoons \text{Enz} + \text{CH}_3 \text{COSCoA} \\
\text{S} + \text{SCOCH}_3 & \rightleftharpoons \text{S} + \text{SH}
\end{align*}
\]

It is possible that reactions 4 and 5 may be reversed in order; that is, that acetate may combine with the enzyme-thiolic complex prior to the exchange between CoA and AMP. The sequence of these changes are being investigated at present.

**The Thiolic Acid Splitting Enzyme**

The enzyme fraction of pigeon liver acetone powder extracts which is precipitated between 35-70% ammonium sulfate saturation contains most of the acetate activating activity of the extract. This fraction however does not respond to the alumina treatment for the removal of ThA. However, combination with the lower ammonium sulfate fraction (0-35% saturation) which contains only slight acetate activating activity, results in a mixture which responds to the alumina procedure.
The mixture of the two fractions responds to arsenite inhibitions as does the unfractionated extract (of Table II). It seems that the low ammonium sulfate fraction functions by splitting protein bound ThA; liberated ThA is then adsorbed on the alumina.

**TABLE V. Alumina Treatment of Ammonium Sulfate Fractions of Pigeon Liver Acetone Powder Extracts**

The incubation mixture was as described for system 3 in the legend of Table I. The ThA content of the 35-70% fraction amounted to 0.299 µg before alumina treatment. After treatment in combination with the 0-35% fraction, the cofactor content was 0.043 µg. Alumina treatment of the 35-70% fraction alone did not alter the ThA content. Incubations were for 90 min. at 37° C.

<table>
<thead>
<tr>
<th>Additions and treatments</th>
<th>Citrate Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-35% fraction (18 mg protein)</td>
</tr>
<tr>
<td></td>
<td>µM</td>
</tr>
<tr>
<td>None</td>
<td>0.03</td>
</tr>
<tr>
<td>+ 0.1 µg ThA</td>
<td>0.02</td>
</tr>
<tr>
<td>+ 4 µM arsenite</td>
<td>----</td>
</tr>
<tr>
<td>Alumina treated</td>
<td>----</td>
</tr>
<tr>
<td>+ 0.1 µg ThA</td>
<td>----</td>
</tr>
<tr>
<td>+ 0.1 µg ThA + 2 µM arsenite</td>
<td>----</td>
</tr>
<tr>
<td>+ 0.1 µg ThA + 2 µM arsenite + 5 µM BAL</td>
<td>----</td>
</tr>
</tbody>
</table>

It seems possible then that ThA can be removed from highly purified enzymes by the alumina procedure—in conjunction with the 0-35% ammonium sulfate fraction from pigeon liver extracts. The large amounts of cofactor in purified pyruvic oxidase (6) and α-ketoglutaric oxidase (7) are so tightly bound to the protein that such procedures as dialysis, repeated washing, or ion-exchange techniques do not dislodge it.
Table VI shows that the procedure is effective in removing ThA from highly purified pyruvic oxidase from pigeon breast muscle. The alumina procedure, also in conjunction with the splitting fraction (0-35\% ammonium sulfate) from pigeon liver extracts, removes the cofactor from purified α-ketoglutaric oxidase (Fig. 4).

**TABLE VI. Removal of ThA from Purified Pyruvic Oxidase**

Inoculation mixture contained in 1.0 ml: 60 \(\mu\)M phosphate buffer, pH 7.4; 2.4 \(\mu\)M MgCl\(_2\); 1.6 \(\mu\)M MnCl\(_2\); 1 \(\mu\)M GSH; 0.15 \(\mu\)M DPN; 0.47 \(\mu\)M DPT; 28 units CoA; 50 \(\mu\)M Na pyruvate, and 0.24 mg protein of *S. faecalis* extract. The pyruvic oxidase was of specific activity 42. The liver fraction was added in amounts containing 35 mg of protein. Incubations were carried out for 60 min. under nitrogen at 38\°C.

<table>
<thead>
<tr>
<th>Enzyme mixture and treatment</th>
<th>ThA content of added enzyme mixture</th>
<th>Acetyl phosphate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu)g</td>
<td>(\mu)M</td>
</tr>
<tr>
<td>Pyruvic oxidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.178</td>
<td>1.7</td>
</tr>
<tr>
<td>Alumina treated</td>
<td>0.183</td>
<td>1.7</td>
</tr>
<tr>
<td>Pyruvic oxidase + liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-35% fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.380</td>
<td>1.6</td>
</tr>
<tr>
<td>+ 1.0 (\mu)g ThA</td>
<td>------</td>
<td>1.7</td>
</tr>
<tr>
<td>Alumina treated</td>
<td>0.044</td>
<td>0.2</td>
</tr>
<tr>
<td>+ 1.0 (\mu)g ThA</td>
<td>------</td>
<td>1.8</td>
</tr>
</tbody>
</table>
FIGURE 4. Effect of Alumina Treatment of $\alpha$-ketoglutarate Oxidase on Rate of
Reduction of DPN

Incubation mixture contained in 3.0 ml: 75 units of Co A; 50 $\mu$M GSH; 0.3 $\mu$M
DPN; 100 $\mu$M glyoxylglycine, pH 7.2, and enzyme mixture. The enzyme mixture con-
tained 0.08 mg protein of $\alpha$-ketoglutarate oxidase, specific activity 66, and
10.9 mg of 0-38% liver fraction. The ThA content of the untreated mixture was
0.263 $\mu$g, and in the alumina treated mixture the cofactor amounted to 0.016 $\mu$g.

III METHODS

Pigeon liver acetone powder extracted as described by Kaplan and Lipmann
(8) served as the source of acetate activating enzyme. Insoluble materials were
removed by centrifugation at 18,000 x g for 15 min. Co A was removed from the
extracts by treatment with Dowex-1 (9). Pyruvio oxidase was purified from pigeon
breast muscle according to Jagannathan and Schwest (10). Purification of $\alpha$-keto-
glutarate oxidase from pig heart followed the procedure of Sanadi, et al. (7).
Acetate activating activity was measured by either of three assay systems: (a) the acetyl-Co A formed by reaction 1 was non-enzymatically converted to acetylhydroxamate in the presence of high concentrations of hydroxylamine (reaction 2)(11); (b) inorganic phosphate served as acyl acceptor to form acetyl

\[
\text{acetyl-Co A + hydroxylamine} \quad \rightarrow \quad \text{CoA} + \text{acetylhydroxamate} \quad (7)
\]

phosphate in the presence of phosphotransacetylase (reaction 3)(9); (c) oxaloacetate served as acceptor to form citrate (reaction 4)(12)(sufficient condensing enzyme is present in the extracts (13).

\[
\text{acetyl-Co A + Pi} \quad \rightarrow \quad \text{acetylphosphate + Co A} \quad (8)
\]

\[
\text{acetyl-Co A + oxaloacetate} \quad \rightarrow \quad \text{citrate + Co A} \quad (9)
\]

Pyruvate oxidation was measured by following the rate of dismutation to acetyl phosphate, CO₂ and lactate:

\[
\text{pyruvate + DPN + Co A} \quad \rightarrow \quad \text{acetyl-Co A + CO₂ + DPNH} \quad (10)
\]

\[
\text{acetyl-CoA + Pi} \quad \rightarrow \quad \text{acetylphosphate + Co A} \quad (11)
\]

\[
\text{pyruvate + DPNH} \quad \rightarrow \quad \text{lactate + DPN} \quad (12)
\]

\[
\text{SUM: 2 pyruvate + Pi} \quad \rightarrow \quad \text{acetyl phosphate + CO₂ + lactate} \quad (13)
\]

The initial step in the oxidation of α-ketoglutarate (reaction 9) which is analogous to the oxidation of pyruvate (reaction 5), was measured by following

\[
\text{α-ketoglutarate + DPN + Co A} \quad \rightarrow \quad \text{succinyl-Co A + CO₂ + DPNH} \quad (14)
\]
the reduction of DPN by the increase in optical density at 340 μm in the Beokman
DU spectrophotometer.

Acetyl phosphate and acetyhydroxamate were determined according to
Lipmann and Tuttle (14, 15). Citrate was determined according to Matelson, et
al. (16). An ammonium sulfate fraction of an extract of Streptococcus faecalis
strain 10C1 which had been growing in media deficient in ThA (17) served as
source of phosphotransacetylase and of lactic dehydrogenase.

Protein was determined turbidimetrically (9). The ThA content of
enzymes was determined manometrically (17) following hydrolysis in 6 N H₂SO₄
at 120° C. for 1 hour.

Alumina treatments of enzymes were carried out in conical centrifuge
tubes. The enzyme solution was brought to room temperature and was then rapidly
stirred for 3 min. with adsorption alumina. The adsorbant was used in a ratio
of 1 gram 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 off
and was then centrifuged at 2000 × g for 15 min. at 4° C. to remove the remainder
of the alumina. Treatment at room temperature appears to effect more complete
splitting of ThA from the enzyme protein and adsorption than is accomplished at
lower temperatures. Adsorption alumina with adsorptive capacity barely equivalent
to a Beokman characterization of II is satisfactory. Large mesh (80-200)
adsorbant is routinely used directly from the manufacturer's container. Acid or
alkali washings do not uniformly affect the ability to adsorb ThA.

Isotopic exchanges between pyrophosphate and ATP were carried out with
fraction 4 of the acetate enzyme from yeast (19), as described by Jones, et al.
(6).
IV REFERENCES

V PUBLICATIONS RESULTING FROM GRANT

1. Participation of thioctic acid in the acotato-activating reaction. *J. Am. Chem. Soc.*, 76, 1712

2. Discussion on the requirement for thioctic acid in the acotato activating system. Presented at the "Symposium on B-Vitamins" sponsored by the Biochemical Institute of the University of Texas and the Clayton Foundation for Research, December 3, 1953, Austin, Texas.


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