The third hemoprotein isolated in the hydroxylapatite chromatography (cytochrome b-562) could better be purified from the soluble fraction obtained from the sonicate of intact cells by DEAE-cellulose column chromatography followed by ammonium sulfate fractionation and hydroxylapatite column chromatography. As shown in Fig. 2, the pigment thus purified showed absorption bands at 562 nm (α), 532 nm (β) and 427 nm (Soret) in the reduced form and at 420 nm (Soret) in the oxidized form. These spectra resembled those reported for "cytochrome b" of Bacterium antiratnum. The prosthetic group of this cytochrome was identified as protoheme from its pyridine hemochromogen spectrum. This pigment was definitely different from cytochrome b₁ (ref. 7) not only in its spectral properties but also in its unusual non-autoxidizability. Cyanide and CO again showed no affinity to this cytochrome.

The three soluble cytochromes described in this paper seemed to be present also in E. coli B, E. coli var communior and Serratia marcescens grown under similar conditions.

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Received September 16th, 1963


PN 10077

Citryl-CoA and the citrate condensing enzyme

Previous work from this laboratory¹,² has shown that a preparation of synthetic citryl-CoA which was cleaved by the citrate cleavage enzyme to acetyl-CoA and oxaloacetate, inhibited the citrate condensing enzyme. It is the purpose of this paper to extend our earlier observations and present evidence which indicates that the inhibition by citryl-CoA is competitive for both acetyl-CoA and oxaloacetate. It is further shown that the slow cleavage of citryl-CoA catalyzed by condensing enzyme, which was first reported by Eggerer and Remberger³, occurs at the same site as acetyl-CoA and oxaloacetate condensation.

Abbreviation: DTNB, 5,5'-dithio-bis-2-nitrobenzoic acid.
ELLMAN\textsuperscript{4} has reported that when DTNB reacts with free sulfhydryl groups 2-nitrobenzoate mercaptide is formed which has a strong absorption maximum at 412 mu. If DTNB is added to a reaction mixture containing condensing enzyme and its substrates, the rate of appearance of CoASH can be assayed spectrophotometrically by observing the rate of change of absorption at 412 mu\textsuperscript{5,6}.

Since the conversion of DTNB to the mercaptide has an isosbestic point at 355 mu, it is possible to follow the utilization of oxaloacetate generated from malate, NAD\textsuperscript{+} and malate dehydrogenase (EC 1.1.1.37) by the appearance of NADH at 355 mu and simultaneously the appearance of CoASH by its reaction with DTNB at 412 mu. Table I shows the change in absorbancy occurring simultaneously at 412 mu and 355 mu in a citrate condensing enzyme assay system. Upon the addition of citryl-CoA an inhibition of oxaloacetate utilization and CoASH liberation occurs. These results are in agreement with our early work\textsuperscript{1,2} and with the data reported by EGGERER AND REMBERGER\textsuperscript{3}. The data shown in Table II confirm the observations of these authors that in the presence of large quantities of condensing enzyme a hydrolysis of citryl-CoA occurs. The rate of this reaction is about one-tenth the rate of the citrate condensing reaction.

As pointed out by EGGERER AND REMBERGER\textsuperscript{3} one would expect the $v_{\text{max}}$ for hydrolysis of citryl-CoA to be at least as high as that for the condensing reaction. Since the chemical synthesis of citryl-CoA leads to the formation of diastereomers

\begin{table}[h]
\centering
\caption{Inhibition of Condensing Enzyme by Citryl-CoA}
\begin{tabular}{lll}
\hline
Contents of cuvette & AA per min & \\
\hline
Complete system & 0.155 & 0.034 \\
+ 6 mM moles citryl-CoA & 0.015 & 0.000 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Hydrolysis of Citryl-CoA by Condensing Enzyme}
\begin{tabular}{ccc}
\hline
Enzyme (units) & Citryl-CoA (mM moles) & $\Delta A$ per min (412 mu) \\
\hline
0.35 & 1.2 & 0.560 \\
0.35 & 3.0 & 0.095 \\
0.35 & 1.2 & 0.030 \\
0.035 & 3.0 & 0.033 \\
0.035 & 2.4 & 0.028 \\
0.035 & 1.8 & 0.017 \\
\hline
\end{tabular}
\end{table}

and since the condensing enzyme is highly stereospecific, these results could be explained if the unnatural isomer is an inhibitor for the reaction.

The results in Table III show that inhibition of condensing enzyme reaction by synthetic citryl-CoA can be overcome by increasing either the acetyl-CoA or oxaloacetate concentration. The synthetic citryl-CoA preparation contains, therefore, not only a substrate but a potent competitive inhibitor for both substrates.

**TABLE III**

**EFFECT OF ACETYL-CoA AND OXALOACETATE ON CITRYL-CoA INHIBITION**

Cuvettes contained in addition to the components listed 200 μmoles Tris-HCl (pH 8.1), 0.1 μmole DTNB and 0.007 unit condensing enzyme in a total volume of 1 ml.

<table>
<thead>
<tr>
<th>Acetyl-CoA (μmoles)</th>
<th>Oxaloacetate (μmoles)</th>
<th>Citryl-CoA (μmoles)</th>
<th>Rate (ΔA at 412 nm for min)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.06</td>
<td>0.1</td>
<td>0.0</td>
<td>0.130</td>
<td>—</td>
</tr>
<tr>
<td>0.06</td>
<td>0.1</td>
<td>6.0</td>
<td>0.037</td>
<td>72</td>
</tr>
<tr>
<td>0.06</td>
<td>1.0</td>
<td>0.0</td>
<td>0.175</td>
<td>—</td>
</tr>
<tr>
<td>0.06</td>
<td>1.0</td>
<td>6.0</td>
<td>0.053</td>
<td>70</td>
</tr>
<tr>
<td>0.3</td>
<td>0.1</td>
<td>0.0</td>
<td>0.280</td>
<td>—</td>
</tr>
<tr>
<td>0.3</td>
<td>0.1</td>
<td>6.0</td>
<td>0.145</td>
<td>48</td>
</tr>
<tr>
<td>0.3</td>
<td>1.0</td>
<td>0.0</td>
<td>0.340</td>
<td>—</td>
</tr>
<tr>
<td>0.3</td>
<td>1.0</td>
<td>6.0</td>
<td>0.335</td>
<td>1.5</td>
</tr>
</tbody>
</table>

By allowing large amounts of citrate condensing enzyme to cleave consecutive aliquots of citryl-CoA the natural diastereomer is hydrolyzed and one can accumulate the "unnatural" isomer. The results of such an experiment shown in Table IV indicate the accumulation of an inhibitor, since each successive addition of citryl-CoA is hydrolyzed more slowly than the preceding one.

Further evidence which shows that the site that catalyzes the condensation reaction is probably the same as the site which catalyzes the hydrolysis of citryl-CoA. The crystalline condensing enzymes from moth muscle and pigeon breast muscle also catalyzed the hydrolysis of citryl-CoA. The ratios of the rate of condensing enzyme reaction to the rate of citryl-CoA hydrolysis for the three enzymes are very similar. Condensing enzyme is inactivated under mild acetylation.

**TABLE IV**

**CUMULATIVE INHIBITORY EFFECT OF CITRYL-CoA ON ITS HYDROLYSIS**

Cuvettes contained 0.175 unit of condensing enzyme, 200 μmoles Tris–HCl and 0.1 μmole DTNB in a total volume of 1 ml. In each case the reaction proceeded to completion before the next addition of substrate.

<table>
<thead>
<tr>
<th>Citryl-CoA</th>
<th>Time for 0.050 ΔA at 412 nm (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First addition, 6.0 μmoles</td>
<td>35</td>
</tr>
<tr>
<td>Second addition, 6.0 μmoles</td>
<td>53</td>
</tr>
<tr>
<td>Third addition, 6.0 μmoles</td>
<td>85</td>
</tr>
<tr>
<td>Fourth addition, 6.0 μmoles</td>
<td>155</td>
</tr>
</tbody>
</table>

*Biochim. Biophys. Acta, 77 (1963) 693–696*
conditions. When 3-4 acetyl groups are introduced per mole of enzyme the condensation activity is reduced 70%. Inactivation of condensing enzyme by acetylation results in a parallel loss of condensing enzyme activity against acetyl-CoA and oxaloacetate and against citryl-CoA (Table V).

In a recent report from this laboratory we have shown that citryl-CoA is cleaved by the citrate cleavage enzyme. The rate of cleavage of citryl-CoA to acetyl-CoA and oxaloacetate was slightly greater than the rate of the overall reaction from citrate, ATP, and CoA. In the cleavage of citryl-CoA by cleavage enzyme no evidence was obtained for an inhibition by the unnatural diastereomer.

The results indicate therefore that citryl-CoA can serve as a substrate for both

<table>
<thead>
<tr>
<th>Acetic anhydride addition* (µl)</th>
<th>Condensing enzyme (% activity)</th>
<th>Citryl-CoA hydrolysis (% activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>35</td>
<td>27</td>
</tr>
<tr>
<td>40</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td>50</td>
<td>9</td>
<td>2.0</td>
</tr>
<tr>
<td>60</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>70</td>
<td>0.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* Diluted 1:25 in tetrahydrofuran.

The results indicate therefore that citryl-CoA can serve as a substrate for both
the citrate cleavage and citrate condensing enzymes. There is no direct evidence, however, that indicates it is an intermediate in the catalyzed reactions.

I would like to thank Mrs. L. Gonen and Mrs. B. Torp for technical assistance. This work was supported in part by a grant from the U. S. Public Health Service (H5328).

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