PHOSPHORYLATIVE AND NON-PHOSPHORYLATIVE OXIDATION IN ACETOBACTER SUBOXYDANS*

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It has been shown that glycerol can be oxidized beyond the dihydroxyacetone (DHA) stage by resting Acetobacter suboxydans cells (1). Through washing and centrifuging of the cells at room temperature, most of the oxidative activity was destroyed, which, however, could be restored by the addition of inorganic phosphate and diphosphopyridine nucleotide (DPN). 2,4-Dinitrophenol (DNP), a phosphate "uncoupling agent," inhibited the further oxidation of dihydroxyacetone but had no effect on the first step oxidation of glycerol or sorbitol, nor of ethanol to acetic acid. These and other facts described herein suggested that oxidative dissimilation in A. suboxydans may be classified into two categories: one is independent of inorganic phosphate uptake, and the other couples with phosphate esterification, perhaps at the coenzyme level.

EXPERIMENTAL

The preparation of the resting cells and the arrangement of other experimental details were the same as previously described (1). Washing was carried out in a Servall centrifuge at room temperature. Usually about 90 minutes were required for three washings. The washed cells were resuspended in a small amount of water and dried in vacuo from the frozen state. The dried cells maintained their activity after 6 months storage at -15°. Inorganic phosphate was determined according to the method of Lowry and Lopez (2).

DPN of 75 per cent purity was purchased from the Schwarz Laboratories, Inc.

RESULTS AND DISCUSSION

Rôle of DPN in Oxidation of Glycerol, Dihydroxyacetone, and Ethanol—The thoroughly washed cells showed quantitatively different oxidative

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behavior toward different substrates. The oxidation of DHA was greatly impaired. It appeared that during washing and centrifuging at room temperature the DPN-linked enzymes had been dissociated and DPN was, in turn, removed by washing. Table I illustrates the restoration of oxidation of DHA by the addition of DPN.

In the absence of external DPN, the oxidation of glycerol virtually ceased after slightly more than 1 atom (occasionally up to 2 atoms) of oxygen had been consumed. However, in the presence of the coenzyme,

**Table I**

*Effect of DPN upon Oxidation of DHA, Glycerol, and Ethanol by Resting A. suboxydans Cells*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Substrate</th>
<th>Amount added</th>
<th>DPN added</th>
<th>Duration of experiment</th>
<th>Oxygen uptake</th>
<th>Carbon dioxide produced</th>
<th>R. Q.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DHA</td>
<td>20 µM</td>
<td>0 γ</td>
<td>360 min</td>
<td>15 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>25</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>35</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Glycerol</td>
<td>40 µM</td>
<td>0 γ</td>
<td>480 min</td>
<td>8 µM</td>
<td>5</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>20</td>
<td>9.4</td>
<td></td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>30</td>
<td>15</td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3000</td>
<td>62</td>
<td>30</td>
<td></td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Ethanol</td>
<td>90 µM</td>
<td>0 γ</td>
<td>415 min</td>
<td>62 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>118</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>136</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000</td>
<td>149</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The system contained 0.05 M phosphate, 0.01 M MgCl₂, and 10 mg (dry weight) of resting cells per flask washed at room temperature; total volume 2.8 ml.; pH 6.0.

it proceeded further, in line with the concept that the oxidation of glycerol proceeds via DHA.

The restoration of full activity required rather high concentrations of the external coenzyme. This is parallel to results in animal tissues in which the dissociated oxidative enzymes often require far greater amounts of the coenzymes than do the original intact systems (cf. (3)). Since at least 3 atoms of oxygen were required per mole of dihydroxyacetone, the exact points of participation of DPN remain to be determined.

Under the conditions used, DPN did not appear to be required either in the oxidation of glycerol to DHA or of ethanol to acetic acid. As shown in Table I, the washed cells showed similar rates of oxidation in the presence and absence of external DPN. Since it has not yet been possible to carry out similar experiments with cell-free preparations, it is difficult to
tell whether this enzyme acted independently of pyridine nucleotides or whether the activity reflected the presence of a relatively stable DPN-apoenzyme linkage which survived the washing procedure. The latter view is favored by the results of Lutwak-Mann (4), who found that alcohol dehydrogenase activity was dependent upon added DPN after \textit{A. suboxydans} suspensions had been saturated with ammonium sulfate and exhaustively dialyzed.

\textbf{Effect of Phosphate on Oxidative Dissimilation by A. suboxydans—Cells}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Substrate} & \textbf{Amount added} & \textbf{Buffer} & \textbf{Duration of experiment} & \textbf{O}_2 uptake \textbf{Total} \\
\hline
\textbf{Substrate} & \textbf{mM} & \textbf{mM} & \textbf{min.} & \textbf{micro-} \\
\hline
Glycerol & 40 & Phosphate & 192 & 100 \text{ micromoles} \\
& & Phthalate & & 44 \text{ micromoles} \\
\hline
Ethanol & 50 & Phosphate & 85 & 79 \text{ micromoles} \\
& & Phthalate & & 100 \text{ micromoles} \\
\hline
\end{tabular}
\end{table}

The system contained the indicated buffer at 0.05 mM, 0.01 mM MgCl$_2$, and 10 mg. (dry weight) of resting cells per flask washed in a cold room; pH 6.0; total volume 2.8 ml. Phthalate was shown in separate experiments to exert practically no effect on the oxidation.

washed in a cold room showed retarded oxidation of glycerol in the later steps when no external phosphate was present. However, as may be seen in Table II, no additional phosphate was needed for ethanol oxidation or for the initial step of glycerol oxidation. Substrate-linked phosphates therefore do not appear to be formed from ethanol in \textit{A. suboxydans}, at least by resting cells. This is in contrast to such organisms as \textit{Clostridium kluyveri}, where it has been shown (5) that oxidation of ethanol depends upon phosphate; stoichiometric yields of acetyl phosphate were obtained. Non-phosphorylative oxidation has, however, been observed with glucose in \textit{Pseudomonas} (6) and pyruvate in animal tissues (7).

\textbf{Dinitrophenol Inhibition in DPN-Linked Enzyme System—DNP has been used frequently as a phosphate “uncoupling” agent in various de-
hydrogenase systems (8, 9). The unusual non-participation of phosphate suggested that DNP might affect only the reactions beyond DHA from glycerol. Accordingly, the effect of DNP was tested in glycerol and DHA oxidations, as well as those of sorbitol and ethanol. The data, presented in Table III and Fig. 1, show clearly that the first atom of oxygen was rapidly consumed in glycerol oxidation either with or without DNP. However, after 1 atom of oxygen had been used, the reaction virtually ceased in the flasks containing $1 \times 10^{-4}$ M DNP. With DHA, the oxygen uptake with DNP was practically zero. The oxidation of sorbitol followed a similar pattern to that of glycerol: $1 \times 10^{-4}$ M DNP inhibited the process beyond the first oxygen atom. However, as shown in Fig. 2, this initial step was not affected by higher levels of the reagent. Ethanol oxidation was not affected at all by DNP.

Several workers have shown (10-13), with a variety of systems, that in the oxidation of fatty acids and members of the citric acid cycle, a high phosphate esterification ratio exists in terms of oxygen consumption (up to 4 atoms of P per atom of O in the α-ketoglutarate → succinate transformation). These observations formed the basis for Lipmann's theory of coenzyme-linked phosphorylation (14), which, with recent documentation by Lehninger (13) and expansion by others (15, 16), has pointed to the

**Table III**

*Effect of DNP on Oxidation of Glycerol, Ethanol, Dihydroxyacetone, and Sorbitol by Resting A. suboxydans Cells*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Substrate</th>
<th>Amount added</th>
<th>DNP</th>
<th>Duration of experiment</th>
<th>$O_2$ uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glycerol</td>
<td>40</td>
<td>-</td>
<td>400</td>
<td>147</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>43</td>
</tr>
<tr>
<td>3</td>
<td>DHA</td>
<td>20</td>
<td>-</td>
<td>320</td>
<td>61</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>Sorbitol</td>
<td>40</td>
<td>-</td>
<td>450</td>
<td>158</td>
</tr>
<tr>
<td>6</td>
<td>Ethanol</td>
<td>20</td>
<td>+</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>90</td>
<td>+</td>
<td></td>
<td>170</td>
</tr>
</tbody>
</table>

The system contained $1 \times 10^{-4}$ M DPN, 0.05 M phosphate, 0.01 M MgCl$_2$, and 10 mg. (dry weight) of cells per flask washed at room temperature; DNP (where added) $1 \times 10^{-4}$ M; total volume 2.8 ml.; pH 6.0. Substrate was tipped into the main compartment containing DNP after 5 minutes equilibration.
coupling of phosphorylation to electron transfer in coenzymes as well as to substrate oxidation. DNP has been reported to inhibit only the phosphorylations that are coupled to pyridine nucleotide oxidation (16, 17).

Fig. 1. The effect of DNP on the rate of oxidation of glycerol by resting A. suboxydans cells. The system contained $1 \times 10^{-4}$ M DPN, 0.05 M phosphate, 0.01 M MgCl$_2$, and 10 mg. (dry weight) of resting cells per flask washed at room temperature; total volume 2.8 ml.; pH 6.0. Each flask in Curve 2 contained, in addition, $1 \times 10^{-4}$ M DNP in the main compartment. 40 µM of glycerol were tipped into the main compartment after 5 minutes equilibration.

Fig. 2. The effect of various concentrations of DNP on the rate of oxidation of sorbitol by resting A. suboxydans cells.
In view of these observations, the present results are consistent with the idea that the oxidation in *A. suboxydans* of glycerol to DHA, or sorbitol to sorbose, or of ethanol to acetic acid does not depend upon coenzyme-linked phosphorylation. However, since the organism grows well in the glycerol medium to give virtually quantitative yields of DHA, the energy for the various synthetic processes must be derived from this one-step oxidation. If the prevalent ideas described for DNP action are correct, the further observation that *A. suboxydans* growth proceeds equally well in the presence of $2 \times 10^{-4}$ M DNP indicates either that (1) a substrate-linked phosphorylation operates during growth (but not during oxidation by resting cells, as shown above), or if not, that (2), additional energy storage mechanisms must be sought in this organism. Several attempts have been made to establish these points by correlating phosphate esterification with oxidation in the presence and absence of DNP. No phosphorus uptake could be demonstrated; however, a high, fluoride-resistant phosphatase activity was observed, particularly toward adenosinetriphosphate and adenosine-5-phosphate, and it is possible that labile phosphate esters existed. A final decision on the mechanisms of oxidation and phosphorylation in this organism will be sought with suitable cell-free systems.

The authors wish to thank Mr. L. R. Hopping for technical assistance.

**SUMMARY**

Repeated washing and centrifuging of *Acetobacter suboxydans* cells at room temperature destroyed most of their oxidative activity toward glycerol and virtually all toward dihydroxyacetone (DHA). Full activity could be restored by the addition of inorganic phosphate and diphosphopyridine-nucleotide. However, under the conditions used, these agents were not required in the oxidation of glycerol to DHA or of ethanol to acetic acid.

2,4-Dinitrophenol at concentrations of $10^{-4}$ M completely inhibited the oxidation of glycerol beyond the DHA stage. No effect of this inhibitor was observed on the oxidation of ethanol, of glycerol to DHA, or of sorbitol to sorbose. It was concluded that oxidative dissimilation in *A. suboxydans* may be differentiated into phosphate-dependent and phosphate-independent types, with the former probably coupled to phosphorylation at the coenzyme level.

**BIBLIOGRAPHY**


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