OXIDATIVE DISSIMILATION IN ACETOBACTER SUBOXYDANS*

BY TSOO E. KING AND VERNON H. CHELDELIN

(From the Department of Chemistry, Oregon State College, Corvallis, Oregon)

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Interest in this laboratory has focused upon the metabolism of the acetic acid bacteria since the superior activity of bound forms of pantothenic acid (PAC) ((1, 2); coenzyme A (3, 4)) over the free vitamin was discovered. The disclosure that pantetheine (Lactobacillus bulgaricus factor) is a mercaptan capable of coexisting with an oxidized disulfide form (5) has led to speculation as to whether coenzyme A and other bound forms of pantothenic acid might contribute to oxidative metabolism in Acetobacter suboxydans. However, even though this organism has been extensively used in industrial fermentations (cf. (6–8)), knowledge is generally lacking concerning the mechanisms of its oxidative patterns. The present study has therefore been designed to obtain initial pertinent information.

EXPERIMENTAL

Organisms—Three strains of A. suboxydans were used, viz. ATCC 9322 and 621, and another obtained from Dr. L. A. Underkofler of Iowa State College. The latter, which is designated herein as strain 621U, had been maintained in his laboratory since its procurement in 1935.

Preparation of Cells—Cells were grown in 2 liters of single strength medium (Table I) in a 5 liter Erlenmeyer flask. The composition of the medium was the same as that used in previous studies (2, 9, 10), except for the omission of glucose and addition of calcium pantothenate. For growth of organisms, the medium was inoculated with about 25 ml. of a 24 to 48 hour-old culture that had been grown on the basal medium plus 10 mg. of yeast extract.

The inoculated culture was incubated for about 36 to 44 hours at 30°, with aeration through a glass sparging tube. The air flow was maintained at 1 liter per minute during the first 16 hours, and then increased to 5 to 10 liters per minute. The cells were centrifuged and washed four times.

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in cold distilled water, twice in a Sharples supercentrifuge, and twice in a Servall angle centrifuge. The washed cells were then dried in vacuo from the frozen state. Although aseptic conditions were not strictly maintained, electron microscopic examination revealed practically no contamination of the cultures, either at this point or after their use in Warburg flasks (see below).

Materials—Coenzyme A samples containing 1.2 per cent bound pantothenic acid activity were prepared as previously described (11, 12). All other chemicals used, except where otherwise noted, were commercial preparations.

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Growth Medium (Double Strength) for A. suboxydans*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>100 gm.</td>
</tr>
<tr>
<td>Peptone, treated with Norit A</td>
<td>5 &quot;</td>
</tr>
<tr>
<td>Casein, vitamin-free, acid-hydrolyzed</td>
<td>5 &quot;</td>
</tr>
<tr>
<td>Liver concentrate, Wilson's 1:20, treated with Norit A</td>
<td>2 &quot;</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>200 mg.</td>
</tr>
<tr>
<td>Cystine</td>
<td>100 &quot;</td>
</tr>
<tr>
<td>Adenine</td>
<td>10 &quot;</td>
</tr>
<tr>
<td>Guanine</td>
<td>10 &quot;</td>
</tr>
<tr>
<td>Uracil</td>
<td>10 &quot;</td>
</tr>
<tr>
<td>Salt Solution A</td>
<td>10 ml.</td>
</tr>
<tr>
<td>&quot; &quot; B</td>
<td>10 &quot;</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>200 γ</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>200 &quot;</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>5000 &quot;</td>
</tr>
<tr>
<td>Water, distilled, to</td>
<td>1 liter</td>
</tr>
<tr>
<td>pH</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* For details of the preparation of the medium, see King et al. (2).

Methods—Oxygen uptake was determined manometrically in a Warburg apparatus at 29°. CO₂ production was measured by the "direct method." Air was used as the gas phase in all experiments.

Acetic acid was determined by titration (13) after steam distilling a 2 ml. aliquot of the supernatant solution from the centrifuged contents of the Warburg flasks, with 2.5 gm. of MgSO₄·7H₂O and 1 ml. of 10 per cent HPO₄. Recoveries of known amounts of added acetic acid were made with each series of determinations, as well as suitable blanks. When these determinations could not be made immediately after the oxidation period, the samples were stored in the frozen state.

Dihydroxyacetone (DHA) was determined by the method of Underkoffler et al. (14) with the following adaptation to a micro scale. The sugar reagent, which was prepared at a concentration of 1.7833 gm. of
KI\textsubscript{3} per liter, was filtered through a fine sintered glass funnel before use. 2 ml. of the sugar reagent were mixed with 2.2 ml. of sample in a 25 ml. Erlenmeyer flask covered with a 10 ml. Pyrex beaker. The mixture was steamed in an autoclave for 30 minutes. After cooling to slightly above room temperature, 0.4 ml. of KI-oxalate reagent and 0.8 ml. of 7.5 N H\textsubscript{2}SO\textsubscript{4} were then added. The mixture was titrated with approximately 0.02 N sodium thiosulfate. A standard curve was made in each run, including samples containing 100 to 1000 \gamma of DHA. The curve so obtained (ml. of thiosulfate as a function of DHA added) was nearly a straight line (see Table II).

The acetylation of sulfanilamide (15, 16) and the formation of citrate (17, 18) were tested as previously. Resting \textit{A. suboxydans} cells were used as the source of apoenzyme.

**Table II**

<table>
<thead>
<tr>
<th>Amount of dihydroxyacetone</th>
<th>0.02 N sodium thiosulfate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>\gamma</td>
<td>ml.</td>
</tr>
<tr>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>100</td>
<td>0.19</td>
</tr>
<tr>
<td>250</td>
<td>0.42</td>
</tr>
<tr>
<td>500</td>
<td>0.85</td>
</tr>
<tr>
<td>1000</td>
<td>1.68</td>
</tr>
<tr>
<td>2520</td>
<td>4.08</td>
</tr>
</tbody>
</table>

* Average of duplicate determinations.

RESULTS AND DISCUSSION

**Oxidation of Glycerol and Dihydroxyacetone**—In preliminary experiments it was noticed that the oxidation of glycerol by resting \textit{A. suboxydans} cells in phosphate buffer was always accompanied by the evolution of significant amounts of CO\textsubscript{2}. Although CO\textsubscript{2} production from glucose by this organism has been observed by Butlin (19–21), the body of information that has accumulated on the fermentation of glycerol has indicated that only DHA is regularly formed, in yields approaching 100 per cent (22–27). This one-step oxidation should proceed without the production of CO\textsubscript{2}.

Determinations were therefore made of the oxygen consumption and CO\textsubscript{2} production with glycerol and DHA as substrates. These are shown in Table III, where up to 4 atoms of oxygen were consumed per mole of glycerol. It is evident that the DHA formed was further oxidized, and that added DHA was also readily dissimilated. Approximately 3 atoms
of oxygen were consumed per mole, which is consistent with the idea that glycerol is oxidized by a pathway that includes DHA as an intermediate. Although the products of the oxidation have not been identified, it is apparent from the CO₂ yield (1.2 to 1.5 moles per mole of either substrate) that dissimilation proceeded somewhat beyond a single scission into C₁ and C₃ units. The results of DHA determination indicated that the products were able to reduce the Somogyi reagent. The recovery of total volatile acid was only about 20 per cent of the substrate added.

**Table III**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amount added</th>
<th>Organism strain No.</th>
<th>Duration of experiment</th>
<th>O₂ uptake</th>
<th>CO₂ produced</th>
<th>R. Q.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td></td>
<td>min.</td>
<td>microatoms</td>
<td>µM</td>
<td></td>
</tr>
<tr>
<td>Glycerol*</td>
<td>40</td>
<td>621U</td>
<td>260</td>
<td>140</td>
<td>47</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>621U</td>
<td>260</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>621U†</td>
<td>150</td>
<td>69</td>
<td>25</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0322</td>
<td>300</td>
<td>154</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>621</td>
<td>175</td>
<td>130</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHA</td>
<td>40‡</td>
<td>621U</td>
<td>154</td>
<td>130</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20§</td>
<td>621</td>
<td>195</td>
<td>61</td>
<td>33</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>40§</td>
<td>621</td>
<td>240</td>
<td>100</td>
<td>48</td>
<td>0.98</td>
</tr>
</tbody>
</table>

The system contained 0.05 M phosphate, 0.01 M MgCl₂, and 10 mg. (dry weight) of cells washed in a cold room per flask. Total volume, 2.8 ml.; pH 6.0.

* Other supplements added, without effect, were coenzyme A (500 γ of 1.2 per cent bound pantothenic acid content) and cytochrome C (40 γ) per flask; MgCl₂ was also omitted in some experiments without effect.

† 20 mg. cells used.

‡ DHA isolated from large scale fermentations by strain 621U (kindly supplied by Dr. H. D. Reese).

§ DHA obtained from Hoffmann-La Roche, Inc.

**Oxidation of Lactic Acid, Pyruvic Acid, and Ethanol**—Lactic and pyruvic acids were apparently oxidized as far as the acetate stage by a route similar to that occurring in other microorganisms and higher animals. Thus, as illustrated in Table IV, lactic acid was transformed nearly quantitatively into acetate and CO₂. 1 mole of CO₂ was also produced per mole of pyruvic acid (acetate production was not measured here). Ethanol oxidation was the same as that reported by other workers (6, 25); CO₂ was not produced, and the reaction was complete in a relatively shorter period.

**Oxidation of Krebs Cycle Intermediates**—The complete oxidation of carbohydrates and related compounds in many organisms is believed to proceed via the Krebs cycle. The key condensations of pyruvate and acetate are catalyzed by coenzyme A (28, 29). However, in this organism,
the fact that acetate accumulates as an end-product of ethanol, lactic, and pyruvic acid oxidations suggests that the former may not be readily metabolized. Acetate was indeed found to be completely inert in this system, even in the presence of glycerol as a potential "sparker." No acetylation of sulfanilamide or citrogenase activity could be demonstrated with resting cells, with or without adenosinetriphosphate and coenzyme A. Attempts to oxidize citrate, α-ketoglutarate, succinate, or fumarate failed consistently, either alone or in the presence of glycerol. A soluble enzyme mixture and an insoluble fraction from A. suboxydans cells were prepared\(^1\) and found active for the oxidation of ethanol, glycerol, DHA,

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amount added</th>
<th>Organism strain No.</th>
<th>Duration of experiment</th>
<th>O₂ uptake</th>
<th>CO₂ produced</th>
<th>Acetic acid formed</th>
<th>R. Q.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic acid</td>
<td>40 µM</td>
<td>621U</td>
<td>120 min.</td>
<td>73</td>
<td>48 µM</td>
<td>38 µM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40 µM</td>
<td>621U</td>
<td>120 min.</td>
<td>88</td>
<td>48 µM</td>
<td>40 µM</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>9322</td>
<td>180 min.</td>
<td>97</td>
<td>48 µM</td>
<td>52 µM</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>40 µM</td>
<td>621</td>
<td>120 min.</td>
<td>74</td>
<td>41 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>100 µM</td>
<td>621U</td>
<td>70 min.</td>
<td>98</td>
<td>100 µM</td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>621U</td>
<td>120 min.</td>
<td>109</td>
<td>99 µM</td>
<td></td>
<td>1.8</td>
</tr>
<tr>
<td>Ethanol</td>
<td>50 µM</td>
<td>621U</td>
<td>60 min.</td>
<td>99</td>
<td>1 µM</td>
<td>52 µM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>621U</td>
<td>120 min.</td>
<td>182</td>
<td>8 µM</td>
<td>106 µM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>621</td>
<td>100 min.</td>
<td>98</td>
<td>0 µM</td>
<td>49 µM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>621</td>
<td>120 min.</td>
<td>193</td>
<td>2 µM</td>
<td>99 µM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>9322</td>
<td>100 min.</td>
<td>94</td>
<td>48 µM</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 washed in a cold room. Total volume 2.8 ml.; pH 6.0.

sorbitol, and glucose, but they did not oxidize the members of the citric acid cycle, even in the presence of a hot water extract of A. suboxydans or methylene blue, or both. It would appear from these experiments that neither a C₆ nor a C₄ cycle operates in this organism to any significant extent. Also, as stated in a preliminary report (30), the absence of acetylating power as noted leaves the rôle of coenzyme A still unestablished in A. suboxydans. Hot water extracts of A. suboxydans contained liberal quantities of coenzyme A, as determined by acetylation and bound pantothenate assay.

\(^1\) The procedure for obtaining cell-free active enzyme preparations from A. suboxydans will be described in detail elsewhere. Since the insoluble portion still contained many intact cells (as revealed by electron microscopic examination at magnification of 5000 to 13,000 X), the observed activity of this fraction may have been due to them.
Strain Differences—Since the results described above were markedly different from those usually obtained in large scale fermentations, it was felt that they might be due to strain peculiarities. However, strains 621, 621U, and 9322 all possessed the same oxidative pattern, although growth was slower in strain 9322 than in the others. Finally, the assignment of these effects to strain differences seems out of the question when it is considered that strain 621U produced DHA in high yield from glycerol when grown in large scale fermenters, yet oxidized this product of its own metabolism when the cells were washed and transferred to the phosphate buffer medium. The implications of this relationship to such factors as oxidative phosphorylation, as well as the possible role of coenzyme A, are being investigated further.

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

SUMMARY

Resting cells of Acetobacter suboxydans in phosphate buffer oxidized glycerol with an oxygen consumption of approximately 4 atoms per mole of substrate. Oxidation of dihydroxyacetone required about 3 atoms per mole. Yields of CO₂ ranged from 1.2 to 1.5 moles per mole of either substrate.

1 mole of pyruvic acid consumed 1 atom of oxygen and produced 1 mole of CO₂ (R. Q. = 2). Lactic acid was completely oxidized to acetic acid and CO₂ (R. Q. = 1). Ethanol was oxidized to acetic acid without CO₂ formation.

Intermediates of the Krebs cycle, including citrate, α-ketoglutarate, succinate, fumarate, and acetate, were not oxidized in either the presence or the absence of small amounts of glycerol as a potential “sparker.” No acetylation of sulfanilamide or formation of citrate from acetate and oxalacetate could be shown, even with added coenzyme A and adenosinetriphosphate. It was concluded that neither a C₆ nor a C₄ cycle operates to a significant degree in this organism and that the role of coenzyme A remains to be established.

BIBLIOGRAPHY

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