Effect of Oxygen on Viability and Substrate Utilization in Chromatium

R. E. HURLBERT
Department of Bacteriology and Public Health, Washington State University, Pullman, Washington

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Chromatium D can be exposed to oxygen for prolonged periods without any loss in motility or viability. Oxygen did not affect the rate of thiosulfate disappearance from the medium, the oxidation of the inner sulfur atom of thiosulfate to sulfate, or the conversion of the outer sulfur atom of thiosulfate to intracellular sulfur, but it did inhibit the oxidation of intracellular sulfur to sulfate. Oxygen partially inhibited the uptake of pyruvate from the medium, but had little effect on the uptake of acetate. The distribution of label from pyruvate-2-14C into various cell fractions under aerobic conditions differed only slightly from that obtained under anaerobic conditions. Cells utilizing acetate-2-14C aerobically converted the majority of the metabolized acetate into a cell fraction with the solubility characteristics of poly-β-hydroxybutyric acid, whereas under anaerobic conditions the acetate was distributed throughout the other cell fractions. Oxygen completely prevented the synthesis of bacteriochlorophyll.

The purple sulfur bacteria (Thiorhodaceae) are strict photoaerobes, requiring both light and the absence of molecular oxygen for growth (12, 13). However, many species of Thiorhodaceae are found in nature in extremely turbid environments (e.g., sewage digestion ponds) where they must reside close to the surface to obtain light. Under these conditions, they are frequently exposed to molecular oxygen by actions which agitate the surface waters, i.e., wind, etc.

Smith and Lascelles (10) have shown that the purple sulfur bacterium, Chromatium sp. strain D, is able to oxidize thiosulfate in the presence of oxygen. Breuker (1) has reported that C. vinosum is able to fix carbon dioxide and oxidize intracellular sulfur under aerobic conditions, and to take up oxygen in the presence and absence of light.

During studies on Chromatium D, I have frequently observed motility in cells exposed to oxygen for periods up to 8 hr. Gherma (American Type Culture Collection, Rockville, Md.; personal communication) has reported similar behavior in other species of Thiorhodaceae. These observations suggest that oxygen, for some species of Thiorhodaceae, is a bacteriostatic rather than bacteriocidal agent.

In the present report, the effect of oxygen upon viability and several aspects of metabolism of Chromatium D was determined.

Materials and Methods

Organism and culture media. The culture of Chromatium sp. strain D, its stock maintenance, and its growth in liquid thiosulfate-bicarbonate-salts medium, as well as the growth conditions, were as previously described (5). When cells devoid of intracellular sulfur were desired, cultures were grown with sodium pyruvate as the substrate (0.02 M). In these instances, bicarbonate was omitted and the pH was adjusted to 7.2 prior to sterilization of the medium.

Preparation and incubation of suspensions. Cultures were incubated until growth was complete (3 to 6 days) or, in the case of thiosulfate-grown cells, until the organisms were mostly free from intracellular sulfur (5 to 10 days), at which time the cells were sedimented at room temperature by centrifugation at 2,500 × g for 15 min. The cells were immediately resuspended to their original volume in fresh bicarbonate-salts medium. Prior to its use, the bicarbonate-salts medium was flushed with a chromous chloride-scrubbed N2 plus 5% CO2 gas mixture to remove oxygen. The cell suspension was distributed in glass vessels which were placed in a 34 C water bath 25 cm from a 300-w incandescent bulb. The final pH of the medium was 7.3. Gassing was begun immediately. With air plus 5% CO2 or with 95% O2 + 5% CO2, gassing was always continuous; however, with N2 plus 5% CO2 (anaerobic conditions), the vessels, unless otherwise specified, were tightly stoppered after an initial gassing of 10 to 15 min. Subsequently, anaerobic vessels were gassed only during sample removal. All gas mixtures were passed through distilled water to saturate them with water prior to contact.
with the experimental material. Because of a marked tendency to settle on standing, the cells in anaerobic vessels were kept suspended by a magnetic stirring system. After a 10- to 15-min equilibration period, substrate was added to the cell suspension and sampling was begun. Dark conditions were achieved by wrapping the vessels in a double layer of aluminum foil.

**Analytical methods.** The concentration of thiosulfate and pyruvate was determined by use of samples of the supernatant medium freed from organisms by centrifugation. Thiosulfate was determined titrimetrically with standardized iodine. Pyruvate was determined by the method of Friedeman and Haugen (3). Bacteriochlorophyll was extracted and estimated by the method of Cohen-Bazire et al. (2).

Labeled cells (14C) were fractionated according to the method of Roberts et al. (8). With this fractionation procedure, recovery of radioactivity was low (Table 2); however, no attempt was made to account for the lost material. Hoare and Gibson (4) reported similar findings by use of this procedure.

**Radioactive determinations.** For determination of radioactivity in the organisms, samples of cell suspensions were drawn through membrane filters (0.45-μm pore diameter; Millipore Filter Corp., Bedford, Mass.) by vacuum. For intracellular 35S determination, the cells were washed twice with 0.05 M phosphate buffer (pH 7.0), whereas for 14C incorporation they were washed twice with cold 5% trichloroacetic acid. For the determination of 35S in sulfate, samples of cell-free supernatant fluids were treated with sufficient iodine to convert any remaining thiosulfate to tetrathionate and were placed in a 100 C water bath. The iodine treatment was necessary to prevent coprecipitation of residual 35S-thiosulfate. A 1-ml amount of a solution of 0.5 M BaCl2 in 0.1 N HCl was added with mixing. After 30 min at 100 C, the precipitated BaSO4 was collected on membrane filters (Millipore Filter Corp.). The tubes were rinsed twice into the filters with anticeep solution (Schleicher & Schuell Co., Keene, N.H.; 2 ml in 500 ml of water). The filter pads containing the samples were immediately placed on aluminum planchettes containing 0.1 ml of glue (Elmer’s Glue-All, The Borden Chemical Co., New York, N.Y.; 1 oz diluted to 50 ml with water) and were dried under an infrared lamp. Radioactivity was determined with an end-window gas-flow counter. Self-adsorption was negligible, and all counts were corrected for background.

**Viability determinations.** Viable counts were made in quadruplicate in the thiosulfate-bicarbonate-salts medium by the method of Sistrom (9), except that the tubes were sealed with a mineral oil-paraffin mixture previously described (5).

**Chemicals.** Sodium pyruvate was obtained from the Sigma Chemical Co., St. Louis, Mo. Inner- and outer-labeled 35S-thiosulfate and sodium pyruvate-2-14C were obtained from the New England Nuclear Corp., Boston, Mass. Sodium acetate-2-14C was obtained from Calbiochem, Los Angeles, Calif.

### Results

**Effect of oxygen on viability.** The effect on viable counts of long-term exposure to aerobic conditions is given in Table 1. The viable-cell count neither decreased nor increased upon prolonged exposure to oxygen.

**Effect of oxygen on thiosulfate utilization.** The observation of Smith and Lascelles (10) and Breuker (1) that some *Chromatium* metabolism continues in the presence of oxygen suggested that a study of the effect of oxygen on specific metabolic activities might be useful in locating the site or sites of oxygen inhibition.

Representative results of the effect of oxygen on thiosulfate utilization are illustrated in Fig. 1. There was a slight stimulation of thiosulfate utilization by oxygen when thiosulfate-grown cells were used; however, with pyruvate-grown cells, no differences could be detected. There was no thiosulfate disappearance in the dark under anaerobic conditions, but thiosulfate was slowly utilized in the dark under aerobic conditions (Fig. 1). In the absence of cells or with heat-killed cells, no thiosulfate disappeared under any conditions.

In preliminary experiments, it was noted that cells metabolizing thiosulfate in the light under both aerobic and anaerobic conditions took on a chalky appearance due to intracellular-sulfur accumulation. This change was more marked in aerobic cultures. Furthermore, sulfur accumulation was transitory in anaerobic cultures but permanent under aerobic conditions; i.e., anaerobic cells lost their chalky appearance during the experimental period but aerobic ones did not. This suggested that oxygen may interfere with the conversion of intracellular sulfur to sulfate. Smith and Lascelles (10) have shown...
anaerobic conditions. Furthermore, the higher the oxygen concentration the more inhibitory it was on the conversion of intracellular sulfur to sulfate (Fig. 4).

Effect of oxygen on pyruvate and acetate utilization. Molecular oxygen inhibited pyruvate utilization but had little effect on acetate utilization (Fig. 5). In the particular experiment described in Fig. 5 and Table 2, all the vessels were flushed continuously throughout the experimental period. To determine whether any of the substrates were converted to volatile products (e.g., carbon dioxide), samples of each of the cell suspensions were collected at the beginning and conclusion of the experiment, and total radioactivity was determined. In all cases, the final total radioactivity was the same as the initial total radioactivity.

To determine the fate of the metabolized organic compounds, cells exposed to labeled substrates under both aerobic and anaerobic conditions were harvested and fractionated (Table 2). The distribution pattern for the two substrates was very different. Although oxygen inhibited pyruvate utilization, it had no significant effect that only the outer atom of thiosulfate appears as intracellular sulfur in Chromatium D cells. I have confirmed these observations. This specificity provides a means of following the path of metabolism of the individual sulfur atoms of thiosulfate in Chromatium D.

The effect of oxygen on the metabolism of each of the sulfur atoms of thiosulfate is seen in Fig. 2 and 3. Oxygen had no significant effect on the conversion of the inner sulfur atom to sulfate; the slightly smaller amount of sulfate produced under anaerobic conditions was not unexpected as the cells grow under these conditions and fix thiosulfate sulfur into protein (Fig. 2). However, oxygen did inhibit the oxidation of intracellular sulfur to sulfate, the rate of sulfate formation from the outer sulfur atom of thiosulfate under aerobic conditions (air plus 5% CO₂) being one-half that under anaerobic conditions (Fig. 3).

![Fig. 1. Utilization of thiosulfate under aerobic and anaerobic conditions. A suspension of thiosulfate-grown cells, prepared as described in Materials and Methods, was incubated in the presence of thiosulfate (11 mM) under aerobic (air + 5% CO₂) and anaerobic (N₂ + 5% CO₂) conditions. Thiosulfate disappearance under aerobic (○) and anaerobic (●) conditions in the light (solid lines) and dark (broken lines) was determined.](image1)

![Fig. 2. Oxidation of the inner sulfur atom of thiosulfate to sulfate under aerobic and anaerobic conditions. A suspension of thiosulfate-grown cells, prepared as described in Materials and Methods, was incubated with inner-labeled thiosulfate (16 mM; 525 counts per min per μmole) under aerobic (air + 5% CO₂) and anaerobic (N₂ + 5% CO₂) conditions. Sulfate production under aerobic (○) and anaerobic (●) conditions was determined.](image2)
on its pattern of incorporation. However, the reverse was true with acetate, in that oxygen had little inhibitory effect on acetate utilization, but did affect the distribution pattern (Table 2). The disproportionate distribution of label in the protein fraction, particularly with acetate, was surprising, and suggested that this fraction might be contaminated with some other cell component. It is known that poly-β-hydroxybutyric acid is readily synthesized from acetate by photosynthetic bacteria (11) and that it would fractionate with the protein in the fractionation procedure used. To determine whether poly-β-hydroxybutyric acid was a component of the protein fraction, samples of this fraction were extracted with boiling chloroform (7) and the hot-chloroform extractable radioactivity was determined (Table 2). The findings indicated that a material with the solubility characteristics of poly-β-hydroxybutyric acid is a significant contaminant of the protein fraction.

Effect of oxygen on bacteriochlorophyll synthesis. Several studies have shown that bacteriochlorophyll synthesis is strongly inhibited by oxygen in a variety of photosynthetic bacteria (2, 5, 9). Bacteriochlorophyll synthesis in Chromatium D showed a similar sensitivity to oxygen (Fig. 6). The data of Fig. 6 are representative of several similar experiments, and in no case was bacteriochlorophyll synthesized in the presence of molecular oxygen. Cells exposed to aerobic conditions for as long as 24 hr were able to begin bacteriochlorophyll synthesis upon the establishment of anaerobic conditions.

**DISCUSSION**

The observations that Chromatium D can maintain its viability, motility, and ability to take up and metabolize both inorganic and organic sub-

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**FIG. 3.** Metabolism of the outer sulfur atom of thiosulfate under aerobic and anaerobic conditions. A suspension of thiosulfate-grown cells, prepared as described in Materials and Methods, was incubated with outer-labeled thiosulfate (10 mM; 155 counts per min per μmole) under aerobic (air + 5% CO₂) and anaerobic (N₂ + 5% CO₂) conditions. The distribution of ³⁵S in the cell (solid lines) fraction and the sulfate fraction (broken lines) under aerobic (●) and anaerobic (○) conditions was followed.

**FIG. 4.** Effect of oxygen concentration on the conversion of intracellular sulfur to sulfate. Cells containing ³⁵S-intracellular sulfur were suspended in bicarbonate-salts medium, divided into thirds, and gassed with N₂ + 5% CO₂ (●), air + 5% CO₂ (X), or 95% O₂ + 5% CO₂ (○). ³⁵S-sulfate formation was determined.
facultative, anaerobic *Athiorhodaceae* (2; 9). *Chromatium* D would appear to respond to a similar mechanism. It is clear from the data presented that the effect of oxygen on bacteriochlorophyll synthesis is sufficient to explain the obligate, anaerobic characteristic of *Chromatium* D.

The effects of oxygen on thiosulfate metabolism by *Chromatium* D observed in this study differed from those of Smith and Lascelles (10). Smith and Lascelles found a much higher rate of thiosulfate disappearance under aerobic-light conditions compared with anaerobic-light conditions. They also observed that thiosulfate utilization was as rapid under anaerobic-light conditions as under aerobic-dark conditions (10). In the current study, however, thiosulfate utilization under aerobic-light conditions was equal to, or only slightly more rapid than, that under anaerobic-light conditions; further, thiosulfate disappearance under aerobic-dark conditions was comparatively slow. The reasons for these quantitative differences were not determined, but are probably due to differences in the experimental procedures (e.g., cell density, light intensity, or suspending medium, or a combination of these).

**TABLE 2. Distribution of pyruvate-2-\(^{14}\)C and acetate-2-\(^{14}\)C into cell fractions under aerobic and anaerobic conditions**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Pyruvate Percentage</th>
<th>Acetate Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold-trichloroacetic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>acid-soluble</td>
<td>23.0</td>
<td>12.2</td>
</tr>
<tr>
<td>Alcohol-soluble</td>
<td>14.5</td>
<td>26.9</td>
</tr>
<tr>
<td>Alcohol-ether-soluble</td>
<td>0.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Hot-trichloroacetic</td>
<td>39.0</td>
<td>34.5</td>
</tr>
<tr>
<td>acid-soluble</td>
<td>23.0</td>
<td>24.7</td>
</tr>
<tr>
<td>Residual protein</td>
<td>83.0</td>
<td>87.0</td>
</tr>
<tr>
<td>Total recovery</td>
<td>22.0</td>
<td>57.0</td>
</tr>
</tbody>
</table>

\(^{a}\) A 5-ml amount of cells from the experiment described in Fig 4 was collected after 6 hr of incubation with pyruvate (8 mM; 0.26 \(\mu\)g) or acetate (10 mM; 1 \(\mu\)g), washed once in pyruvate or acetate (10 mM)-bicarbonate-salts medium, and fractionated as described in Materials and Methods.

\(^{b}\) Initial counts per minute per milliliter of pyruvate and acetate.

\(^{c}\) Percentage of protein fraction.
The inhibitory effect of oxygen on the conversion of the outer sulfur atom of thiosulfate to sulfate and the lack of inhibition of the conversion of the inner sulfur atom to sulfate substantiate the conclusion of Smith and Lascelles (10) that these atoms are separated at an early stage of metabolism. The mechanism of oxygen inhibition on intracellular sulfur oxidation is still to be determined.

The different effects of oxygen on substrate metabolism illustrate the complexity of the oxygen effect. Whether these observed effects represent differing degrees of response to a single action of oxygen or are the result of multiple actions (e.g., a number of oxygen-sensitive enzymes) cannot be determined from these data.

Sistrom observed that cultures of the obligate anaerobe R. molischianaum increased in optical density in growth media in the presence of oxygen. However, he did not determine whether this change was due to cell division (9). Although Chromatium D is able to metabolize thiosulfate under aerobic conditions, it apparently is unable to divide (Table 1). This observation raises the possibility that oxygen inhibits some process essential to cell division (e.g., deoxyribonucleic acid synthesis).

The observation (Table 2) that under aerobic conditions acetate is almost entirely incorporated into a fraction with the solubility characteristics of poly-β-hydroxybutyric acid implies that oxygen inhibits an early stage in acetate metabolism.

The foregoing experiments are obviously inadequate for a complete understanding of the effect of oxygen on Chromatium D. However, it would appear that oxygen may be a useful probe for investigating various aspects of Chromatium D metabolism. Certain aspects of these observations are currently under investigation.

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LITERATURE CITED


