Viability and Endogenous Substrates Used During Starvation Survival of Rhodospirillum rubrum†

JOHN A. BREZNAK,* C. J. POTRIKUS,1 NORBERT PFENNIG,2 AND JERALD C. ENSIGN3

Department of Microbiology and Public Health, Michigan State University, E. Lansing, Michigan 48824; Institut für Mikrobiologie der Universität und GSF, 34 Göttingen, West Germany; and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 23 November 1977

Cells of Rhodospirillum rubrum were grown photoorganotrophically and chemooorganotrophically and then starved for organic carbon and combined nitrogen under four conditions: anaerobically in the light and dark and aerobically in the light and dark. Illumination prolonged viability and suppressed the net degradation of cell material of phototrophically grown cells, but had no effect on chemotrophically grown cells that did not contain bacteriochlorophyll. The half-life survival times of carbohydrate-rich phototrophically grown cells during starvation anaerobically or aerobically in the light were 17 and 14.5 days, respectively. The values for starvation aerobically and anaerobically in the dark were 3 and 0.5 days, respectively. Chemotrophically grown cells had half-life survival times of 3 and 4 days during starvation aerobically in the light and dark, respectively, and 0.8 day during starvation anaerobically in the light or dark. Of all cell constituents examined, carbohydrate was most extensively degraded during starvation, although the rate of degradation was slowest for phototrophically grown cells starved anaerobically in the light. Phototrophically grown cells containing poly-β-hydroxybutyrate as carbon reserve were less able to survive starvation anaerobically in the light than were carbohydrate-rich cells starved under comparable conditions. Light intensity had a significant effect on viability of phototrophically grown cells surviving anaerobically. At light intensities of 320 to 650 lx, the half-life survival times were 17 to 24 days. At 2,950 to 10,500 lx, the survival times decreased to 1.5 to 5.5 days. The kinetics of cell death correlated well with the rate of loss of cell mass of starving cells. However, the cause of death could not be attributed to degradation of any specific cell component.

Most bacteria are periodically subjected in their natural habitat to periods of starvation stress. Bacteria starving for exogenous energy-yielding substrates depend on endogenous metabolism of intracellular substrates to provide the energy required for performing life-sustaining processes such as control of intracellular pH and osmotic pressure, maintenance of selective permeability, and turnover synthesis of macromolecules. This energy, derived from endogenous metabolism for the purpose of survival, is termed energy of maintenance (5, 7).

The subjects of the response of bacteria to starvation stress and the role of endogenous metabolism in survival have been extensively reviewed (5–7, 15, 21, 22, 31). Usually, specialized cellular reserves such as carbohydrate or poly-β-hydroxybutyric acid (PHB) are the first substrates for endogenous metabolism. As these are depleted, RNA and sometimes protein are utilized. Intracellular amino acid pools are quickly depleted, and incompletely digested products of RNA, protein, and amino acid degradation may be released into the suspending fluids.

There is a wide variation in the ability of bacteria to survive the stress of starvation (2). However, a direct correlation seems to exist between the rates of endogenous metabolism and death rates of cells in suspension. Boylen and Ensign (1, 2) attributed the longevity of starving Arthrobacter crystallopoietes (extrapolated half-life = 120 days) in part to the extremely low basal rate of endogenous metabolism of cells (0.03% cell carbon respired per h). A similar inference was made by Robertson and Batt (23) for starving suspensions of Nocardiocoralina (half-life = 20 days). Conversely, Hespell et al. (11) correlated the rapid death of starving cells of Bdellovibrio bacteriovorus (half-life = 10 h) with their high rate of endogenous respiration (3% cell carbon respired per h during the first 6 h of starvation).
Although many investigations have been made of starvation survival of chemotrophic bacteria, few such studies have been made of phototrophic bacteria. We began this study of the nutritionally diverse phototrophic bacterium *Rhodospirillum rubrum* to determine its response to starvation under a variety of conditions. We particularly wished to examine the source of maintenance energy during starvation of cells in the light and dark. The energy of maintenance of chemotrophic bacteria has been estimated under conditions where the organisms are starving or where growth is limited by an organic substrate (9, 16–18, 20). Such measurements are complicated, however, by the energy source (endogenous or exogenous) serving the demands for both cell carbon and energy of maintenance (9). By contrast, a phototrophically grown organism starved for carbon in the light would be expected to have two possible sources of energy for maintenance: photophosphorylation and oxidation of intracellular substrates. In the dark, only the latter could feed energy of maintenance. If light (photophosphorylation) supplies energy for maintenance of starving cells, illuminated cells should exhibit lower rates of endogenous metabolism and therefore should survive longer than non-illuminated cells.

In this paper we report the effects of illumination and of aerobic versus anaerobic conditions on survival and metabolism of intracellular substrates during starvation of phototrophically and chemotrophically grown *R. rubrum*.

**MATERIALS AND METHODS**

**Organism and growth conditions.** *R. rubrum* Ha was used for all experiments and was grown at 30°C in LLY, LHY, or ALY medium (Table 1).

Cells were grown phototrophically in LLY or ALY medium under a gas phase of Ar or 95% Ar–5% CO₂, respectively. Media were inoculated with 1% (vol/vol) of an exponential-phase culture growing phototrophically in homologous medium. Illumination (2,690 to 3,220 lx) was provided by standard 100-W incandescent light bulbs, and cultures were agitated by use of a magnetic stirrer.

Cells were grown chemotrophically in aluminum foil-wrapped 2-liter Erlenmeyer flasks containing 500 ml of LHY medium. The flasks were inoculated with 1% (vol/vol) of an exponential-phase culture of chemotrophically grown cells and were shaken at 300 rpm.

Growth was monitored turbidimetrically by measuring the absorbance at 720 nm (phototrophically grown cells) or 660 nm (chemotrophically grown cells) with a Beckman model DB spectrophotometer or a Bausch & Lomb Spectronic 20 colorimeter. Absorbance readings were converted to cell mass equivalents by means of standard curves relating the readings to micrograms (dry weight) per milliliter.

**Starvation procedure.** All glassware used was thoroughly cleaned with a detergent solution, rinsed, and then treated for 8 h with a solution of Chromerge (Manostat, New York) in concentrated H₂SO₄. A thorough rinsing with distilled water and deionized, glass-distilled water followed.

A buffered starvation salts solution (BSS) was used to wash and resuspend cells for starvation. BSS contained all the inorganic salts used in LLY medium (Table 1) except that NH₄Cl was omitted. The final pH of BSS was 7.0.

Cells were harvested during exponential growth by centrifugation at 4,080 × g for 10 min at 15 to 20°C, washed once with sterile BSS, and resuspended in a sterile aspirator bottle containing a stirring bar. The cell suspension was vigorously mixed with a magnetic stirrer for 10 min at 25°C. This freed the suspension of clumps of cells as evidenced by microscopic examination. Lowering the temperature to 30°C, the cell suspension was washed and resuspended in a 7-mm gas inlet tube with a tip tapered to a 1-mm opening 1 to 2 mm from the bottom. The top of the tube was fitted with a glass lid containing a gas outlet tube and 25-mm (OD) sampling port. The sampling port was sealed with a removable screw cap.

The starvation tubes were incubated in a glass water bath maintained at 30°C. Tubes incubated in the light were positioned as close to the inside surface of glass as possible (approximately 5 mm) to minimize absorption of long-wavelength light by water. A bank of 15-
W incandescent light bulbs was used to give light intensities varying from 320 to 650 lx. When greater illumination was desired, light bulbs of higher wattage were used, and the light intensity was controlled by placing layers of cotton cheesecloth between the lights and water baths. Starvation tubes to be incubated in the dark were wrapped with aluminum foil and black plastic tape.

Anaerobic conditions were obtained by bubbling Ar (99.99% purity) through the starvation tubes. Compressed air was used for starvations under aerobic conditions. The gases were passed first through a sterile cotton filter and then through sterile distilled water of 30°C before entering the starvation tubes. The flow rate of the gases was about 500 ml/min per tube.

Sampling and assay procedures. Samples of approximately 28 ml were periodically removed from starvation tubes with sterile pipettes. A portion of the cell suspension was diluted to 10^-7 with LHY medium, and 0.1-ml amounts were spread on each of five plates of LHY medium solidified with 1.5% agar. The plates were incubated aerobically at 30°C in the dark for 7 days, after which viable cell counts were determined. No increase in the number of colonies was observed after 7 days of more incubation. Microscopic examination of the starving-cell suspensions revealed no cell clumping.

A 3.0-ml sample was used for turbidity measurement and for determination of bacteriochlorophyll (Bchl). The method of Cohen-Bazire et al. (4) was used for Bchl extraction except that a 3.0-ml volume of acetone-methanol (7:2, vol/vol) was used. The amount of Bchl in the extract was estimated spectrophotometrically by using an extinction coefficient of 75 mM^-1 cm^-1 at 770 nm in acetone-methanol (3).

Exactly 25.0 ml of the cell suspension was centrifuged at 12,000 × g for 10 min at 5°C. The supernatant fluid was then aspirated and filtered through a cellulose ester disk (0.45-μm average pore diameter; Millipore Corp., Bedford, Mass.), and its absorbance at 260 and 280 nm was determined. These measurements were used as an index of leakage of intracellular materials from the starving cells.

The cell pellet was resuspended in distilled water to a final volume of 5.0 ml. A 1.0-ml sample of the concentrated cell suspension was used to determine cellular carbohydrate by the anthrone method (24) or to assay for PHB content (10). Glucose and crotonic acid were used as standards, respectively. The protein of a 0.9-ml sample of the concentrated cell suspension was determined by using the Folin phenol reagent after hydrolysis of cells with NaOH (10), using egg white lysozyme or bovine serum albumin as standard.

To 3.5 ml of concentrated cell suspension was added 0.5 ml of 2 N HClO₄. After incubation at 3°C for 30 min, the suspension was centrifuged at 12,000 × g for 10 min at 5°C. The supernatant fluid was then removed and discarded. The cell pellet was suspended in 3.0 ml of 0.67 N HClO₄ and incubated at 70°C for 15 min. After centrifugation, the supernatant fluid was assayed for RNA and DNA content by the orcinol and diphenylamine methods, respectively (10). Escherichia coli tRNA and salmon sperm DNA were used as standards.

Chemicals. Lysozyme (grade I), bovine serum al-

bumin (fraction V), and DNA (type III; Na salt) were obtained from Sigma Chemical Co., St. Louis, Mo. Crotonic acid was obtained from K & K Laboratories, Inc., Plainview, N.Y. E. coli tRNA was a gift from L. R. Snyder. Agar and yeast extract were obtained from Difco Laboratories, Detroit, Mich.

RESULTS

Composition of cells grown under different conditions. The chemical compositions of cells grown phototrophically and chemotrophically in three media are shown in Table 2. The variations in the major macromolecules, RNA, DNA, and protein, were not appreciable. As expected, the carbohydrate content of LLY- and LHY-grown cells and PHB content of ALY-grown cells were high (25). These values for the three growth conditions will serve as reference points for the starvation studies.

Effect of light intensity on survival of starving cells. Light intensity had a significant effect on the survival of phototrophically grown cells starving anaerobically (Fig. 1). Cells starving in the dark had a half-life survival time of 0.5 day. Starvation at low light intensity (320 to 650 lx) extended the half-life survival time to 17 to 24 days. Light intensities greater than 650 lx resulted in a rapid decrease in viability of the starving cells. The half-life survival time remained nearly constant (approximately 4 days) at light intensities greater than 2,950 lx.

Decreased viability of cells starved at high light intensities was not accompanied by extensive net degradation of cell material. Cells starved for 1.5 days at 10,230 lx (half of the cells were nonviable) still retained greater than 95% of their initial protein, RNA, and carbohydrate content and 115% of their initial DNA content.

TABLE 2. Chemical composition of R. rubrum Ha cultivated in different media

<table>
<thead>
<tr>
<th>Medium</th>
<th>LLY, pho-</th>
<th>ALY, pho-</th>
<th>LHY, che-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>toor-</td>
<td>toor-</td>
<td>moor-</td>
</tr>
<tr>
<td>Protein</td>
<td>61.0</td>
<td>68.9</td>
<td>55.1</td>
</tr>
<tr>
<td>RNA</td>
<td>13.1</td>
<td>10.2</td>
<td>14.0</td>
</tr>
<tr>
<td>DNA</td>
<td>1.7</td>
<td>1.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>18.3</td>
<td>5.3</td>
<td>17.0</td>
</tr>
<tr>
<td>PHB</td>
<td>0.1</td>
<td>8.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Bchl</td>
<td>0.8</td>
<td>1.1*</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Determined for cells harvested during exponential growth. Values expressed as percentage of dry cell weight.

* Nanomoles of Bchl/milligram of protein = 15 (LLY-grown cells) and 19 (ALY-grown cells).
These values were almost identical to those obtained for cells starved at low light intensity for 1.5 days when the cells remained 100% viable (see below).

These data suggested that the lethal effect of high light intensities might be due to something other than starvation stress. Since optimum survival was observed at 320 to 650 lx, light intensities in this range were used for subsequent starvation experiments involving illuminated cells so that the results would reflect the response of cells to starvation stress without being influenced by the deleterious effects of high-intensity illumination.

Effects of starvation on viability and macromolecule content of phototrophically grown cells. Organisms grown phototrophically with lactate (LLY medium) were subjected to starvation under four experimental conditions: anaerobically in the light and dark and aerobically in the light and dark. Illumination markedly prolonged the viability of cells starving both under aerobic and anaerobic conditions (Fig. 2). Cells starved in the light remained 100% viable for 8 days, after which the viabilities declined to 50% at 17 and 14.5 days. By contrast, cells starving in the dark had viability half-lives of only 0.5 (anaerobically) or 3 (aerobically) days.

Carbohydrate accounted for 18.3% of the dry weight of LLY-grown cells (Table 2) and was extensively degraded during starvation (Fig. 3). Under either aerobic or anaerobic conditions, illumination suppressed the net rate of degradation of carbohydrate. About 80% of the initial cellular carbohydrate was ultimately degraded by all cell suspensions. The remaining 20% of anthrone-reactive material may represent cell wall hexose not degraded during starvation.

Protein and RNA were not extensively de-
260/280-nm absorbance ratio of extracellular fluids ranged from 1.5 to 1.8.

The DNA content of cells starved anaerobically in light and aerobically in the light and dark increased by 19 to 20% during the initial 1 to 2 days of starvation (data not shown). This increase was probably due to completion of rounds of DNA replication that had been initiated before starvation was begun. An initial increase in DNA during starvation was also reported for Arthrobacter crystallopoietes (1) and Aerobacter aerogenes (27). By contrast, the DNA content of cells starving anaerobically in the dark decreased rapidly, nearly half being lost after 5 days of starvation. Most of this degradation occurred after 99% of the cells were dead.

The Bchl content of anaerobic-light- and aerobic-light- and -dark-starved cells decreased slowly (data not shown). Approximately 5 to 10% of the initial contents of Bchl had been depleted after 3 to 4 weeks of starvation. The rate of Bchl decrease was much greater in anaerobic-dark-starved cells; 2% was lost after 1 day and 12% after 4 days of starvation. The cell mass, estimated by absorbance measurements of cell suspensions, decreased in parallel to the losses of macromolecules (Fig. 5).

Phase-contrast microscopic examination of cells at various times from the different starvation conditions revealed that they retained their characteristic spiral shape and size. No visual signs of lysis were evident even after considerable cell death had occurred. When viabilities had decreased by approximately 20%, many cells appeared less phase dense and contained dark granules, but maintained their normal shape.

Effects of starvation on viability and macromolecule content of cells grown phototrophically on acetate. Phototrophic growth of R. rubrum with acetate (ALY medium) resulted in the intracellular accumulation of relatively large amounts of PHB and low levels of carbohydrate (Table 2). Studying the behavior of these cells during starvation provided a comparison of the relative importance of PHB versus carbohydrate as intracellular substrates for endogenous metabolism.

As shown in Fig. 6, the numbers of viable cells increased by 10% during the first day of starvation anaerobically in the light and then decreased rapidly for the next 4 to 5 days. Death then continued at a slower rate. The cells degraded PHB rapidly for 6 days and then more slowly. The half-life survival time of 9 days compares with 24 days for LLY-grown cells starving anaerobically at the same light intensity (Fig. 1).

The net rate and extent of RNA and protein degradation were nearly the same as those for LLY-grown cells starved anaerobically in the light (see Fig. 4). The DNA content of the cells remained constant, whereas the Bchl content declined by 9% during 24 days of starvation.

Effect of starvation on viability and macromolecular content of chemotrophically grown cells. The composition of chemotrophically grown cells was similar to that of cells grown phototrophically in LLY medium except that the former were devoid of Bchl (Table 2). Cells starved anaerobically in the light or dark died very rapidly, the half-life survival time being less than 1 day (Fig. 7). Cells starved aerobically survived longer: 3.2-day half-life for those in the light and 4.2 days for those in the dark. No Bchl was formed by the cells during any of the starvation conditions. The slightly decreased viability of cells starved aerobically in light compared with dark could be due to lethal photooxidations, perhaps resulting from the absence of protecting carotenoid pigments (14).

Carbohydrate, accounting for 17% of the dry weight of the growing cells (Table 2), was degraded rapidly during starvation (Fig. 8). The initial rates were greater in aerobically starved cells. Degradation of carbohydrate ceased after approximately 85% of the initial content had been depleted.

![Fig. 5. Cell mass (absorbance) of photoorganotrophically grown cells during starvation. Symbols and illumination as in legend to Fig. 2.](http://jb.asm.org/)

![Fig. 6. Viability (○) and cellular PHB content (△) of photoorganotrophically grown cells starving anaerobically. Growth medium was ALY. Illumination was 650 lx.](http://jb.asm.org/)
Relatively little net protein degradation occurred during starvation, and illumination had no significant effect on net protein degradation by either aerobic or anaerobic cell suspensions (Fig. 9).

Cells starved anaerobically degraded RNA to a greater extent than did aerobically starved cells (Fig. 9). However, the rate of RNA degradation during starvation was unaffected by light. An 18% increase in the DNA content of cells occurred during the first 2 days of aerobic starvation (data not shown). The levels of DNA remained nearly constant thereafter. After 10.5 days, when less than 5% of the cells were viable, 110 to 113% of the initial DNA remained. By contrast, DNA was degraded from the onset of starvation anaerobically, and after 7 days only 62% of the initial DNA remained. Nearly all the cells were nonviable after 2 days of starvation anaerobically.

The absorbance of cell suspensions decreased in parallel with the overall decrease in cell macromolecules (Fig. 10). Most of the decrease in cell mass occurred during the first 2.5 days of starvation. Anaerobic suspensions showed a more rapid initial rate of decrease in cell mass (1.88%/h) than did aerobic suspensions (1.18%/h). The rates of decrease were the same in the light or dark.

**DISCUSSION**

The data in this paper show that illumination markedly prolongs the survival of phototrophically grown starving cells and suppresses the net degradation of cell materials. Photophosphorylation apparently supplies energy for maintenance and thereby spares the utilization of endogenous reserves.

Starvation of phototrophically grown cells during anaerobic dark incubation proved to be the most deleterious of the conditions tested. Cells lost viability and cell material rapidly. The only energy-yielding mechanism available to cells under such conditions is fermentation of intracellular reserve materials. Although *R. rubrum* is capable of fermentative ATP generation (13, 29, 30), the amount of ATP available for energy demands is minimal compared with that from photophosphorylation. The viability of
phototrophically grown cells starved in the dark was extended by aerobic incubation, presumably because more energy became available to cells through oxidative phosphorylation. This explanation seems tenable, since Thore et al. (28) reported that a major part of the respiratory pathways of phototrophically and chemotrophically grown cells is identical, and the capacity for oxidative phosphorylation is similar in both types of cells.

Light intensities of 1,300 to 10,760 lx, which normally support maximal growth rates of _R. rubrum_ (12), were quite lethal to phototrophically grown cells starving anaerobically (Fig. 1). A possible explanation for this is that high light intensities promote an accumulation of intracellular ATP accompanied by a severe depletion of ADP and AMP required as phosphate acceptors for endogenous metabolism. Alternatively, exposure of starving cells to high light intensities might result in formation of free radicals, leading to lethal events.

In contrast to phototrophically grown cells, illumination had no significant effect on viability or endogenous metabolism of starving, chemotrophically grown cells. This would be expected since these cells did not contain Bchl during either growth or starvation. It would seem advantageous for chemotrophically grown _R. rubrum_ to synthesize Bchl during starvation since, if radiant energy were available, prolonged survival would result. However, Oelze and Kammen (19) correlated a significant decrease in cellular ATP content with Bchl synthesis during transitions of _R. rubrum_ from chemotrophic to phototropic growth. It may be that photopigment synthesis is too costly an expenditure for the starving cells, or that net protein and RNA degradation does not provide sufficient nitrogen for this purpose.

The viability half-lives of starving _R. rubrum_ correlated favorably with the overall rate of loss of cell mass of both phototrophically and chemotrophically grown cells (Fig. 2, 5, 7, and 10). The faster cell mass was lost, the shorter were the survival times. However, the ultimate death of starving cells could not be directly attributed to degradation of any one particular cell constituent. Although greater viability was observed for cells containing large amounts of carbohydrate relative to PHB (Table 2; Fig. 2 and 6), the net rate and extent of carbohydrate degradation appeared unrelated to viability. For example, half of the carbohydrate content of phototrophically grown cells starved under anaerobic light and aerobic light conditions was depleted at 11.5 and 2 days, respectively, yet the half-life survival times of the cells differed by only 2.5 days (Fig. 2 and 3).

Net protein degradation was not extensive in either the phototrophically or chemotrophically grown starving cells (Fig. 4 and 9). These cells contained either carbohydrate or PHB or both as reserve materials. Experiments with other bacteria (1, 8, 23, 26) have shown that degradation of non-nitrogenous polymers suppresses net protein degradation by starving cells.

About 20 to 30% of the initial content of RNA was degraded during starvation. This magnitude is similar to, but slightly lower than, that observed in other bacteria (1, 7). Although the products of RNA degradation were not determined, the high 260/280-nm ratios of extracellular fluids suggested that some purine and/or pyrimidine bases were excreted.

Starving cells of chemotrophic bacteria meet the demands for maintenance energy at the cost of degrading intracellular substrates. Yet in theory, radiant energy could supply the entire maintenance requirement of starving, phototrophically grown cells of _R. rubrum_, and the cells should survive indefinitely. The cells do not, however, survive indefinitely, and although illumination suppresses the degradation of cell material, it does not do so completely. The cause of death of phototrophic cells starving in the light is not apparent. Death could result from one or a combination of events such as loss of functional integrity of the photophosphorylation system, the inherent thermodynamic instability of cell constituents that cannot be resynthesized during starvation, or photochemical events.

**ACKNOWLEDGMENTS**

We thank R. L. Uffen and H. van Gemerden for helpful discussions.

This work was supported by Public Health Service fellowship GM 50618 from the National Institute of General Medical Sciences, awarded to J.A.B.; by the Michigan Agricultural Experiment Station of Michigan State University; by the College of Agriculture and Life Sciences of the University of Wisconsin; and by Public Health Service grant AI 6506 from the National Institute of Allergy and Infectious Diseases. J.C.E. held a NATO senior postdoctoral fellowship during part of the research.

**LITERATURE CITED**


J. Bacteriol.