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# Endogenous Metabolism of *Azotobacter agilis*

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ABSTRACT

SOBEK, J. M. (University of Southwestern Louisiana, Lafayette), J. F. CHARBA, AND W. N. FOUST. Endogenous metabolism of *Azotobacter agilis*. J. Bacteriol. **92**: 687–695. 1966—Ribonucleic acid, deoxyribonucleic acid, cellular carbohydrate, and the cold trichloroacetic acid and acidic alcohol fractions of the cell do not appear to function as endogenous reserves for *Azotobacter agilis*. The immediate endogenous reserve of cells grown on glucose, acetate, or succinate was poly-β-hydroxybutyric acid (PHB). Viability of the cells during starvation was dependent upon the initial levels of PHB and the growth substrate. Cells with high initial PHB levels survived longer than cells with lower levels. Cells from succinate-grown cultures had lower PHB levels than cells from glucose-grown cultures, but were capable of maintaining their viability longer. Cellular protein may also serve as a secondary endogenous reserve substrate for this organism.

The relationship between endogenous metabolism and survival of starvation has been studied with relatively few microorganisms (for review, *see* 3). In *Aerobacter aerogenes*, possession of glycogen reserves favors survival of this organism during starvation (19). In *Escherichia coli*, glycogen reserves are rapidly utilized during starvation, and net degradation of protein does not occur until the glycogen is almost exhausted (14). Viability remains unchanged for the first 12 hr of starvation, during which time glycogen serves as the principal endogenous substrate.

Survival of *Micrococcus halodenitrificans* is related to the poly-β-hydroxybutyric acid (PHB) content of the cells (15). In cells with high PHB levels, endogenous $Q_{O_2}$ values and viability remain unaltered for 96 hr. When the PHB content declines to a critical level, $Q_{O_2}$ values and viability fall rapidly. In cells with low PHB content, endogenous $Q_{O_2}$ values and viability decline from the beginning of starvation.

*Azotobacter agilis* has a high rate of oxygen consumption in the presence of an oxidizable exogenous substrate, but has a low efficiency of assimilation and a low endogenous metabolism (7). Low endogenous activity, as well as the ability to form cysts, may represent survival advantages for this organism. This study was designed to identify the substrates utilized by *A. agilis* for endogenous metabolism and to relate these, if possible, to the ability to survive starvation.

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## MATERIALS AND METHODS

*Preparation of resting cell suspensions for starvation. A. agilis* (*A. vinelandii*), strain O was grown in Burk's medium (21) containing 1.0% glucose, sodium acetate, or sodium succinate as substrates. The inoculum was prepared by growing the organism for 24 hr in Burk's medium containing the appropriate substrate. The cell suspension was adjusted to 0.3 optical density (OD) at 540 mμ with phosphate buffer, and 2.0 ml was added per 100 ml of medium. In some experiments, the inoculum was 2.0 ml of an 0.6 OD suspension. This is referred to in the text and tables as a double inoculum. For experiments with $C^{14}$-labeled cells, the growth medium was supplemented with 1.5 mc per 100 ml of medium of either uniformly labeled glucose-$C^{14}$, uniformly labeled sodium acetate-$C^{14}$, or a mixture of sodium succinate-*1,4*-$C^{14}$, and sodium succinate-*2,3*-$C^{14}$. Cells were harvested after 16, 20, or 24 hr of incubation at 30 C, washed twice with sterile 0.05 M phosphate buffer (*p*H 7.2), and, after the final wash, resuspended in the desired buffer to 0.6 OD.

*Selection of a buffer.* The ability of *A. agilis* grown on glucose to survive starvation was tested in four buffer systems: 0.05 M potassium phosphate buffer containing 0.001 M $MgCl_2$ (*p*H 7.2); Burk's N-free salts (*p*H 7.2); and the tris(hydroxymethyl)aminomethane (Tris) salts buffer (*p*H 7.2), both with and without sodium chloride, described by Postgate and Hunter (14). Amounts of 75 ml of each buffered cell suspension were placed in 300-ml flasks and shaken at 200 cycles per min for 72 hr on an incubator shaker at 30 C.

*Starvation systems.* For starvation studies, 400 ml of the cell suspension in buffer was transferred to a sterile 1.0-liter gas wash bottle immersed in a water bath (30 C). In later experiments, 50 ml of cell suspension was transferred to a Nesbit absorption bulb. Starvation studies with $C^{14}$-labeled cells were carried out in a sterile 500-ml gas wash bottle containing 150 ml of the labeled cell suspension or in absorption bulbs. Starving suspensions were aerated with the rate adjusted to give a $K_LaC$ value (millimoles of dissolved $O_2$ per liter per min) of 0.60 as measured by the method of Cooper et al. (2).

*Viability of starving cells.* Samples were removed from the starvation systems at 0, 24, 48, and 72 hr and were diluted in 0.05 M phosphate buffer (*p*H 7.2). Each sample and subsequent dilution was mixed vigorously on a cyclomixer to give even dispersion and was plated in triplicate in Burk's medium containing the substrate on which the cells were grown. Colony counts were made after 48 and 72 hr of incubation at 30 C. Viability was also determined by the slide culture method described by Postgate et al. (13). Bacterial objects and microcolonies were counted after 8-hr incubation at 30 C for cells starved 0 and 24 hr, and after 12 hr for cells starved 48 and 72 hr.

*Warburg respirometer studies.* Cells grown for 20 hr on unlabeled media were starved for 72 hr. At 0, 24, 48, and 72 hr of starvation, 50-ml samples were removed from the liter wash bottles, centrifuged, and resuspended in 7.0 ml of phosphate buffer. A 2-ml amount of this concentrated cell suspension was placed in the main compartments of standard Warburg respirometer flasks, and oxygen consumption and carbon dioxide production were measured for a 4-hr period at 30 C by use of conventional techniques. Respiratory quotients (RQ) and $Q_{O_2}$ (microliters of $O_2$ per hr per mg of cell, dry weight) values were determined from the manometric data and cell (dry weight) measurements.

*Chemical fractionation of cells.* At 0, 24, 48, and 72 hr, samples of labeled or unlabeled cell suspensions were removed from the starvation systems and the cells were packed by centrifugation. The cell pellets were then fractionated by the procedure described by Clifton and Sobek (1). This yielded a cold trichloroacetic acid fraction, an acidic alcohol fraction, a hot trichloroacetic acid fraction, and a residue fraction. A duplicate cell pellet of labeled or unlabeled cells was digested for 90 min at 37 C with 5% sodium hypochlorite, and the residue was collected by centrifugation before assaying for poly-β-hydroxybutyric acid.

*Analytical methods.* Ammonia in acidified cell suspensions from Warburg flasks was determined by nesslerization of steam distillates after release of the ammonia with 20% NaOH in a microstill. Ribonucleic acid (RNA) was determined by the orcinol method (5) with purified yeast RNA as the standard. Deoxyribonucleic acid (DNA) was determined by the method described by Snell and Snell (16) with use of salmon sperm DNA as the standard. Protein was measured by the method of Lowry et al. (10) on the hot trichloroacetic acid-insoluble residue and on whole cell pellets dissolved in 1.0 N NaOH at 100 C for 10 min. Purified serum albumin fraction V was used as a standard. Total cell carbohydrate of ice-cold aqueous suspensions of cells was estimated by the anthrone method (9). Amino acids, peptides, and other ninhydrin-reacting material in the cold trichloroacetic acid and alcohol fractions and the supernatant fraction were measured by the method of Moore and Stein (12) with glutamic acid as the standard. PHB was determined gravimetrically (20) and spectrophotometrically (8) on the residue obtained from alkaline hypochlorite digests of cells. Purified polymer was used as the standard.

Radioactivity measurements were made on the dried chemical fractions obtained from 5- or 10-ml samples of labeled cells by combustion to $CO_2$ and measurement in a Dynacon (Nuclear-Chicago Corp., Des Plaines, Ill.) electrometer system.

## RESULTS

*Selection of a buffer.* The survival of glucose-grown *A. agilis* aerated at 30 C on an incubator shaker was tested in four buffer systems. The viable count decreased 40% in phosphate buffer and 95% in Burk's salts by the end of 72 hr. An increase in viable count was observed when cells were suspended in Tris buffer, with or without saline. Growth curves with 1% Tris in Burk's salts confirmed the presence of materials in the buffer capable of supporting growth. Of the suspending media tested, phosphate buffer containing $MgCl_2$ seemed the most suitable for starvation experiments, although $Mg^{++}$ ions may delay cell death (6, 14).

*Cell viability during starvation.* Loss of viability was related to the age of the cultures at the beginning of starvation, and, to some extent, to the growth substrate. Viable cell counts, as determined by both plate counts and slide cultures, declined more rapidly in starved cells from cultures grown on glucose and succinate for 16 hr than from 24-hr cultures. The decrease in viability of cells from both 16- and 24-hr acetate-grown cultures was similar. Preliminary growth experiments showed that 16-hr-old cultures of this organism grown on all three substrates were in the logarithmic phase of growth. Cultures grown on glucose or succinate were in the late logarithmic phase of growth at 24 hr. Glucose-grown cultures had attained the stationary phase by 28 hr, and succinate-grown cultures by 32 hr. Cultures grown on acetate did not reach the stationary phase of growth until 36 hr. The time of incubation necessary to reach the early stationary phase of growth could be shortened to 28 hr in acetate-grown cultures, and to 24 hr in succinate-grown cultures by doubling the size of the initial inoculum.

Viable counts obtained by the slide-culture method were in a general agreement with those obtained by plate counts for the first 24 to 48 hr of

starvation of cells from 24-hr cultures. In cells from 24-hr cultures starved for 72 hr, the individual division lags were more variable, and up to 24 hr of incubation was necessary to observe division of some organisms in the slide cultures. The method did not prove entirely satisfactory for cells from 16-hr cultures after the first 24 hr of starvation. Division lags were long, and many of the individual organisms and even the microcolonies themselves were of low contrast, and filamentous, pointed, and round cells were observed. This problem was most pronounced with cells from acetate-grown cultures. In cells from 16- and 24-hr acetate-grown cultures, the viabilities at the beginning of starvation as determined by the slide-culture method were 50 to 60% (live versus total organisms). In comparable glucose- or succinate-grown cultures, the initial viabilities were >99% as determined by this method. Starvation was not prolonged beyond 72 hr to avoid the problem of a cryptic growth response in suspensions with low viabilities.

Smears of the starving suspensions made at various times and stained by the method described by Socolofsky and Wyss (17) did not reveal the presence of cysts at any time during starvation. No significant degree of clumping was noted in slide cultures or in stained smears taken from the plate count dilution tubes.

*Warburg studies.* Data obtained from Warburg studies on cells obtained from 20-hr cultures are presented in Table 1. RQ values of glucose-grown cells ranged from 0.72 to 0.97 during the course of starvation, suggesting the oxidation of different compounds at different times. The oxidation of carbohydrates or PHB by succinate-grown cells is suggested by RQ values ranging from 0.88 to 0.97. Values for acetate-grown cells were more constant throughout starvation, and ranged from 0.76 to 0.83. In general, RQ values were of little value in predicting the compound or compounds serving as the endogenous substrates in *A. agilis*. This was probably due to the simultaneous occurrence of multiple reactions. $Q_{O_2}$ values of glucose- and acetate-grown cells were low (4.0 to 6.0), but were three to four times higher than the values obtained from succinate-grown cells. Cells from 24-hr cultures had a slightly higher initial $Q_{O_2}$ value. However, after 24 hr of starvation, the $Q_{O_2}$ values were almost identical in all experiments with each substrate.

TABLE 1. *Effect of starvation of cells from 20-hr cultures on RQ, oxygen consumption, $Q_{O_2}$, and ammonia production*

| Growth substrate | Time of starvation | RQ | $O_2$ consumed[a] | $NH_3$ produced[a] | $O_2$-$NH_3$ ratio | $Q_{O_2}$ |
|---|---|---|---|---|---|---|
| | *hr* | | *μmoles* | *μmoles* | | |
| Glucose | 0–4 | 0.83 | 10.6 | 0.1 | 106.0 | 4.6 |
| | 24–28 | 0.79 | 2.9 | 0.1 | 29.0 | 1.8 |
| | 48–52 | 0.97 | 2.3 | 0.3 | 7.0 | 1.4 |
| | 72–76 | 0.72 | 1.7 | 0.3 | 5.3 | 1.0 |
| Acetate | 0–4 | 0.81 | 10.7 | 0.3 | 35.7 | 5.8 |
| | 24–28 | 0.83 | 3.2 | 0.6 | 5.3 | 1.8 |
| | 48–52 | 0.83 | 2.1 | — | — | 1.2 |
| | 72–76 | 0.76 | 1.8 | 0.3 | 6.0 | 0.9 |
| Succinate | 0–4 | 0.94 | 3.0 | 0 | — | 1.5 |
| | 24–28 | 0.88 | 3.1 | 0 | — | 1.5 |
| | 48–52 | 0.97 | 1.9 | 0.3 | 6.3 | 0.9 |
| | 72–76 | 0.90 | 1.2 | 0.3 | 4.0 | 0.6 |

[a] Expressed as micromoles per 10 mg of cells (dry weight) per 4-hr time period.

Ammonia production by starved cells from 20-hr cultures was low, and $O_2$-$NH_3$ ratios indicated little oxidation of nitrogenous compounds until after 24 to 48 hr of starvation. When concentrated suspensions of cells were starved in Warburg flasks, ammonia production per 10 mg of cells (dry weight) was .8 μmoles of $NH_3$ by glucose-grown cells, 0.3 μmole of $NH_3$ by acetate-grown cells, and 2.6 μmoles of $NH_3$ by succinate-grown cells during the first 24 hr. Between 24 and 72 hr, 3.4 μmoles of $NH_3$ was produced by glucose-grown cells, 0.7 μmole of $NH_3$ by acetate-grown cells, and 2.0 μmoles of $NH_3$ by succinate-grown cells.

*Cell-fractionation studies.* Changes in cellular RNA, DNA, and ninhydrin-reacting material in the cold trichloroacetic acid- and alcohol-soluble fractions of starving cells from 20-hr cultures are shown in Table 2. Similar results were obtained with cells from cultures grown for 24 hr. In early experiments, lipid, as triglyceride, was determined in an ether-soluble fraction following extraction with acidic alcohol, but the lipid content of this fraction was less than 1% of the cell dry weight and did not change during starvation, so this extraction and determination was dropped in subsequent experiments. Glucose-grown cells showed little change or a slight decline in RNA content. Cells from acetate- and succinate-grown cultures showed a small loss in RNA in each experiment. There was a net increase in orcinol-positive material in the supernatant fractions in all experiments with each substrate. DNA values remained unchanged or showed only minor fluctuations in all experiments. Ninhydrin-reacting material, i.e., amino acids, peptides and "protein," in the cold trichloroacetic acid- and alcohol-soluble fractions showed a small loss in all experiments, although the level of ninhydrin-reacting material in these fractions could have been maintained by degradation of protein reserves.

Typical data from experiments with starved cells from 16- and 24-hr cultures in which changes in cellular and supernatant carbohydrate were followed are shown in Table 3. There was a decrease in cellular carbohydrate in all experiments. Most of the decrease in cellular carbohydrate observed can be accounted for by an increase in supernatant carbohydrate. From the small amounts of carbohydrate lost during starvation, it seems unlikely that this fraction serves as an endogenous reserve in this organism.

TABLE 2. *Changes in cellular RNA, DNA, and ninhydrin-reacting materials during starvation of cells from 20-hr cultures*

| Growth substrate | Time of starvation | RNA[a] | DNA[a] | Ninhydrin-reacting material | |
|---|---|---|---|---|---|
| | | | | Cold trichloroacetic acid fraction[a] | Alcohol fraction[a] |
| | *hr* | | | | |
| Glucose | 0 | 76.4 | 12.6 | 35.4 | 13.6 |
| | 24 | 80.9 | 10.7 | 35.1 | 10.2 |
| | 48 | 76.6 | 15.2 | 35.4 | 10.2 |
| | 72 | 78.0 | 13.7 | 29.1 | 8.7 |
| Acetate | 0 | 74.2 | 15.3 | 24.5 | 11.1 |
| | 24 | 73.3 | 13.9 | 29.4 | 10.8 |
| | 48 | 68.3 | 13.8 | — | 8.4 |
| | 72 | 61.7 | 13.6 | 25.4 | 9.8 |
| Succinate | 0 | 118.2 | 14.7 | 32.0 | 10.9 |
| | 24 | 99.2 | 17.3 | 25.8 | 8.3 |
| | 48 | 100.8 | 15.1 | 30.4 | 7.9 |
| | 72 | 101.6 | 19.4 | 25.5 | 10.5 |

[a] All values are expressed as micrograms per milligram of cells (dry weight).

Changes in cellular protein and supernatant ninhydrin-reacting material during starvation are shown in Table 4. A decrease in protein occurred in all experiments, except in cells from 24-hr succinate-grown cultures. In all cases, cells from cultures grown for 16 hr showed more extensive degradation of cellular protein than those grown for 24 hr. Much of this loss from the cells could be accounted for, however, by a corresponding increase in ninhydrin-reacting material in the supernatant fraction. The contributions of the cold trichloroacetic acid- and alcohol-soluble fractions to the supernatant ninhydrin-reacting material, as seen in Table 2, are relatively minor. These observations, coupled with the observation of low ammonia production during the first 24 hr of starvation and high $O_2$-$NH_3$ ratios, suggest that protein does not serve as an endogenous reserve for glucose-, acetate-, or succinate-grown *A. agilis* until after 24 hr of starvation.

A comparison between viable-cell count and PHB contents during starvation of cells which had been grown for 16 and 24 hr with glucose or succinate as the substrate is shown in Fig. 1 and 2. In all cases, cells from 16-hr cultures had low initial PHB levels, usually less than 5% of the cell dry weight. PHB levels increased rapidly after this time, and, by 24 hr, amounted to almost 18% of the cell dry weight in glucose-grown and 10 to 12% in succinate-grown cells. Other workers, using different substrates, have shown a rapid increase in PHB levels in *Azotobacter* beginning around 24 hr and reaching a maximum at 48 to 72 hr (18). Twenty-four hour cultures were free

TABLE 3. *Changes in cellular carbohydrates during starvation of cells from 16- and 24-hr cultures*

| Growth substrate | Time of starvation | Cells from 16-hr cultures | | Cells from 24-hr cultures | |
|---|---|---|---|---|---|
| | | Cells[a] | Supernatant fraction[a] | Cells[a] | Supernatant fraction[a] |
| | *hr* | | | | |
| Glucose | 0 | 147.2 | 2.7 | 93.8 | 4.3 |
| | 72 | 115.0 | 14.5 | 75.4 | 14.7 |
| | | (−32.2)[b] | (+11.8) | (−18.4) | (+10.4) |
| Acetate | 0 | 119.6 | 6.9 | 161.9 | 0.8 |
| | 72 | 110.4 | 12.3 | 153.6 | 0.9 |
| | | (−9.2) | (+5.4) | (−8.3) | (+0.1) |
| Succinate | 0 | 125.4 | 9.9 | 93.4 | 17.9 |
| | 72 | 115.9 | 17.5 | 88.3 | 18.6 |
| | | (−9.5) | (+7.6) | (−5.1) | (+0.7) |

[a] Expressed as micrograms per milligram of cells (dry weight).
[b] Numbers in parentheses show the change.

TABLE 4. *Changes in cellular protein and supernatant ninhydrin-reacting material during starvation of cells from 16- and 24-hr cultures*

| Growth substrate | Time of starvation | Cells from 16-hr cultures | | Cells from 24-hr cultures | |
|---|---|---|---|---|---|
| | | Cell protein[a] | Supernatant ninhydrin-reacting material[a] | Cell protein[a] | Supernatant ninhydrin-reacting material[a] |
| | *hr* | | | | |
| Glucose | 0 | 707.3 | 12.4 | 522.4 | 1.8 |
| | 24 | 691.6 | 53.4 | 497.5 | 2.6 |
| | 48 | 667.4 | 88.6 | 482.5 | 35.0 |
| | 72 | 587.3 | 130.5 | 475.5 | 61.6 |
| | | (−120.0)[b] | (+118.1) | (−46.9) | (+59.8) |
| Acetate | 0 | 630.1 | 9.6 | 539.7 | 2.9 |
| | 24 | 613.2 | 88.8 | 509.2 | 21.0 |
| | 48 | 547.4 | 136.9 | 471.4 | 45.3 |
| | 72 | 391.0 | 217.4 | 444.1 | 90.4 |
| | | (−239.1) | (+207.8) | (−95.6) | (+87.5) |
| Succinate | 0 | 582.1 | 5.3 | 515.2 | 1.4 |
| | 24 | 529.8 | 35.5 | — | 0.6 |
| | 48 | 540.9 | 51.4 | 515.2 | 0.0 |
| | 72 | 493.9 | 88.2 | 520.0 | 0.0 |
| | | (−88.2) | (+82.9) | (+4.8) | (−1.4) |

[a] Expressed as micrograms per milligram of cells (dry weight).
[b] Numbers in parentheses show the change.

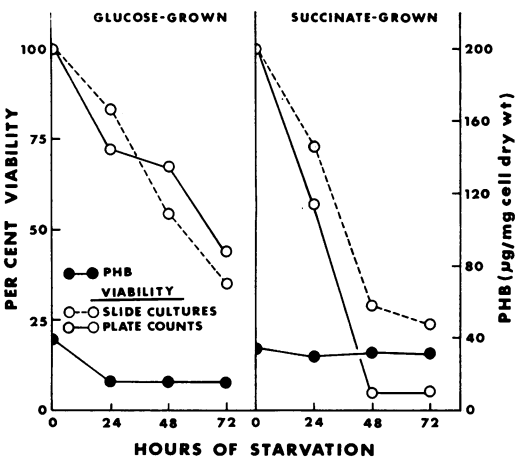


FIG. 1. *Changes in PHB levels and viability during starvation of glucose- and succinate-grown cells from 16-hr cultures of Azotobacter agilis.*

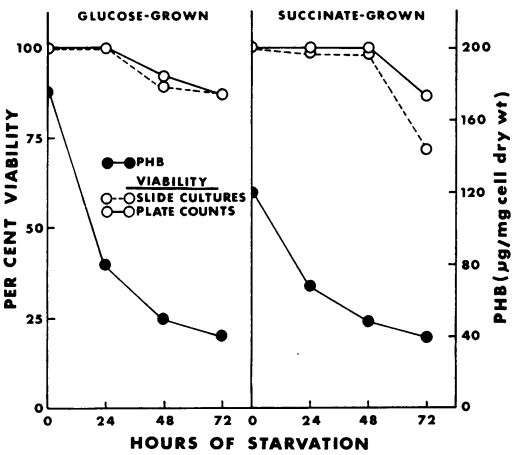


FIG. 2. *Changes in PHB levels and viability during starvation of glucose- and succinate-grown cells from 24-hr cultures of Azotobacter agilis.*

from cysts. Cells from 24-hr acetate-grown cultures did not show a marked increase in PHB polymer levels over those of 16-hr cells. These cells grew more slowly than cells grown on the other two substrates, and longer incubation or a double initial inoculum was necessary to obtain the same levels of PHB.

Viability of starving cell suspensions appears to be related to the initial PHB levels. Those cells with higher PHB levels survived better than cells with low initial polymer levels. In cells from 24-hr glucose-grown cultures, viability did not decrease until after 24 hr of starvation, at which time the PHB content had declined from 176 to 80 μg/mg of cells (dry weight). At the end of 72 hr of starvation, the loss in viability was only 13 %, as meas-

ured by the slide-culture method. Cells from 16 hr cultures with much lower initial polymer contents showed a more rapid loss in viability amounting to 65% by 72 hr. Viability may also be dependent to some extent on the growth substrate. Twenty-four-hour succinate-grown cultures had grown more slowly, and the cells consistently had lower polymer levels than glucose-grown cells of the same age, but succinate-grown cells utilized the polymer at a slower rate and survived longer. However, cells from 16-hr succinate-grown cultures with low polymer contents died more rapidly during starvation than cells from 24-hr cultures.

Initial PHB levels in cells from 16- and 24-hr acetate-grown cultures, and the loss in viability of these cells during starvation, were similar. If a double inoculum were used, higher PHB levels in acetate-grown cells could be obtained in 24 hr of incubation. The loss in viability when the initial polymer level was 150 $\mu$g/mg of cells (dry weight) was less rapid than in cells with lower polymer contents. The initial polymer levels of cells from 24-hr acetate- and succinate-grown cultures were similar [150 and 120 $\mu$g/mg of cells (dry weight), respectively], but the loss in polymer was 60% greater in acetate-grown cells during the first 24 hr of starvation. Further incubation of the acetate cultures resulted in PHB levels of 190 to 220 $\mu$g/mg of cells (dry weight), and the loss in viability of these cells during starvation was similar to that observed in cells from cultures grown for 24 hr on glucose. These results are summarized in Fig. 3.

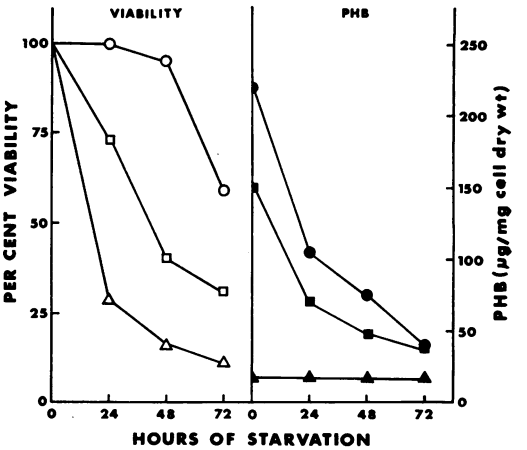


FIG. 3. *Changes in PHB levels and viability during starvation of acetate-grown Azotobacter agilis. Viability was measured by plate count. Symbols:* ○, ●, *cells from 28-hr culture, double inoculum;* □, ■, *cells from 24-hr culture, double inoculum;* △, ▲, *cells from 16- or 24-hr culture, single inoculum.*

*Labeled-cell experiments.* Changes in the radioactivity of the different cellular fractions of $C^{14}$-labeled cells from cultures grown for 16 and 24 hr and starved for 24 hr are shown in Tables 5 and 6. In all xperiments, it was found that the death rate was faster and the different cellular fractions seemed to be degraded more rapidly with labeled than with unlabeled cells. A cryptic growth response was noted between 48 and 72 hr; when the viability of the suspensions was $<1\%$. Presumably, this was due to the utilization of materials released by the dead cells. For this reason, starvation experiments with labeled cells were not extended beyond 24 hr.

The major decrease in the activity of cells from 16-hr cultures with low PHB levels occurred in the residue fraction which contains protein, structural carbohydrates, and, probably, capsular material. A loss from the alcohol-soluble and hot trichloroacetic acid-soluble fractions of the cell was noted in all cases, probably due to some loss of lipids, nitrogenous substances, and RNA. The per cent contribution of both these fractions to the total loss in activity was almost the same in cells from cultures grown for 16 or 24 hr. In cells from 16-hr glucose and succinate cultures, most of the activity of the initially low PHB levels is lost during starvation, suggesting almost complete utilization of the polymer. A 12-hr lag before extensive degradation of the PHB in acetate-grown cells was noted in most experiments with this substrate. In cells with higher PHB levels from cultures grown for 24 hr, this polymer was apparently the most expendable reserve. This fraction accounted for most of the loss in activity from cells from 24-hr glucose-grown cultures during the first 12 hr of starvation. PHB also accounted for most of the loss from cells from cultures grown for 24 hr on acetate and succinate during the whole 24-hr starvation period. In cells from 24-hr glucose-grown cultures, the loss in activity from the residue fraction is not significant until after 12 hr, when almost 80% of the PHB has been utilized. A similar sparing effect by PHB on the residue fraction is seen with cells from 24-hr acetate and succinate-grown cultures, and is more pronounced with the latter. During the first 12 hr of starvation, some sparing effect by PHB on the alcohol-soluble fraction of the cell is also noted in cells from 24-hr cultures. Data from experiments with unlabeled and $C^{14}$-labeled cells are not directly comparable. The only conclusion suggested by experiments with labeled cells was that, in cells with high PHB levels, the PHB is preferentially degraded and exerts a sparing effect on the degradation of other cellular fractions.

TABLE 5. *Changes in various fractions during starvation of $C^{14}$-labeled cells from 16-hr cultures*[a]

| Growth substrate | Time of starvation | Cold trichloroacetic acid-soluble | Alcohol-soluble | Hot trichloroacetic acid-soluble | PHB | Residue[b] | Supernatant fraction | Whole cells |
|---|---|---|---|---|---|---|---|---|
| | *hr* | | | | | | | |
| Glucose | 0 | 0.47 | 1.00 | 1.49 | 0.81 | 8.97 | 0.31 | 12.74 |
| | 24 | 0.53 | 0.60 | 0.92 | 0.04 | 7.02 | 0.79 | 9.24 |
| | | (+0.06) | (−0.40) | (−0.57) | (−0.77) | (−1.95) | (+0.48) | (−3.50) |
| | 0–24 | +2.0% | 10.8% | 15.4% | 20.5% | 51.8% | | |
| Acetate | 0 | 0.80 | 1.13 | 1.15 | 0.72 | 7.02 | 0.0 | 11.29 |
| | 12 | 0.69 | 0.87 | 0.83 | 0.73 | 6.48 | 0.0 | 9.74 |
| | 24 | 0.53 | 0.49 | 0.83 | 0.25 | 5.64 | 0.13 | 8.17 |
| | | (−0.27) | (−0.64) | (−0.32) | (−0.48) | (−1.38) | (+0.13) | (−3.12) |
| | 0–12 | 8.7% | 21.5% | 26.4% | +1.0% | 43.0% | | |
| | 12–24 | 8.6% | 20.5% | 0.0% | 25.8% | 45.1% | | |
| | 0–24 | 8.7% | 20.8% | 10.4% | 15.3% | 44.8% | | |
| Succinate | 0 | 0.24 | 0.84 | 0.92 | 0.36 | 4.82 | 0.0 | 6.71 |
| | 12 | 0.26 | 0.69 | 0.66 | 0.03 | 4.50 | 0.0 | 6.41 |
| | 24 | 0.28 | 0.37 | 0.55 | 0.02 | 3.98 | 0.06 | 5.39 |
| | | (+0.04) | (−0.47) | (−0.37) | (−0.34) | (−0.84) | (+0.06) | (−1.32) |
| | 0–12 | +2.0% | 14.1% | 24.5% | 31.1% | 30.3% | | |
| | 12–24 | +2.0% | 33.3% | 11.4% | 1.0% | 52.3% | | |
| | 0–24 | +2.0% | 23.3% | 18.3% | 16.8% | 41.6% | | |

[a] Activity is expressed as nanocuries, with the amount of the change shown in parentheses. Percentages are the percentage of total loss in activity by all fractions.

[b] The value for the residue fraction is corrected by subtracting the value for PHB which is determined on a separate sample of cells.

## DISCUSSION

The results reported show that PHB probably serves as the immediate endogenous reserve for glucose-, acetate-, and succinate-grown cells of *A. agilis*. Decrease in cell viability is apparently related to the initial PHB content of the cells. Adequate initial PHB reserves prolong the viability of the cells, whereas cells with lower initial levels of polymer show a more rapid loss in viability (Fig. 1, 2, and 3). In almost every experiment there was also a loss of protein by the cells and, although much of this loss can be accounted for by an increase in ninhydrin-positive material in the supernatant fraction, there is probably some oxidation and utilization of protein (Table 4). PHB levels in 24-hr cells seem to exert a sparing effect on the degradation of cellular protein and may delay the utilization of the degradation products. Support for this conclusion is provided by experiments with labeled cells (Tables 5 and 6). Cells from 16-hr cultures with low polymer contents degrade their residue fraction, presumably protein, extensively. Cells from 24-hr cultures with higher polymer contents, with the exception of glucose-grown cells, show a negligible loss from this fraction until PHB levels have markedly declined. A similar sparing effect on protein degradation in *Bacillus megaterium* by PHB has been suggested (11). In labeled cells, some sparing effect by PHB on other cellular components is also noted. Protein utilization apparently does not support the viability of the cells adequately, since cells from 16-hr cultures degrade the protein fraction extensively during starvation, but lose their viability more rapidly than cells from 24-hr cultures.

It has been suggested that the actual amount of an endogenous substrate may be less important than the ability of the cells to utilize that substrate (6). Although PHB levels of unlabeled cells from 24-hr succinate-grown cultures are lower than in cells grown on glucose for the same length of time, the decline in viability is less. This suggests that succinate-grown cells may be more efficient in their use of PHB reserves, and their lower $Q_{O_2}$ values and lower rate of utilization of this polymer may be a reflection of a lower energy demand and, hence, a lower requirement for utilizable substrate.

TABLE 6. *Changes in various fractions during starvation of $C^{14}$-labeled cells from 24-hr cultures*[a]

| Growth substrate | Time of starvation | Cold trichloroacetic acid-soluble | Alcohol-soluble | Hot trichloroacetic acid-soluble | PHB | Residue[b] | Supernatant fraction | Whole cells |
|---|---|---|---|---|---|---|---|---|
| | *hr* | | | | | | | |
| Glucose | 0 | 0.61 | 1.39 | 1.84 | 2.36 | 11.56 | 0.0 | 17.76 |
| | 12 | 0.58 | 1.27 | 1.57 | 0.50 | 11.01 | 0.47 | — |
| | 24 | 0.48 | 0.63 | 1.13 | 0.18 | 8.94 | 0.77 | 11.36 |
| | | (−0.13) | (−0.76) | (−0.71) | (−2.18) | (−2.62) | (+0.77) | (−6.40) |
| | 0–12 | 1.0% | 4.2% | 9.6% | 65.7% | 19.5% | | |
| | 12–24 | 3.0% | 18.0% | 12.3% | 9.0% | 57.7% | | |
| | 0–24 | 2.0% | 11.9% | 11.1% | 34.1% | 40.9% | | |
| Acetate[c] | 0 | 0.73 | 1.88 | 0.83 | 1.49 | 6.81 | 0.22 | 10.62 |
| | 12 | 0.70 | 1.79 | 0.66 | 1.26 | 6.71 | 0.29 | 9.96 |
| | 24 | 0.76 | 1.49 | 0.65 | 0.66 | 6.31 | 0.37 | 8.96 |
| | | (+0.03) | (−0.39) | (−0.18) | (−0.83) | (−0.50) | (+0.15) | (−1.66) |
| | 0–12 | 4.9% | 14.5% | 27.4% | 37.1% | 16.1% | | |
| | 12–24 | +1.0% | 22.9% | 0.8% | 45.8% | 30.5% | | |
| | 0–24 | +1.0% | 20.5% | 9.5% | 43.6% | 26.3% | | |
| Succinate[c] | 0 | 0.73 | 1.79 | 1.19 | 3.65 | 6.31 | 0.25 | 14.94 |
| | 12 | 0.76 | 1.69 | 1.00 | 2.44 | 6.19 | 0.30 | 12.96 |
| | 24 | 0.76 | 1.14 | 0.95 | 1.39 | 6.25 | 0.29 | 11.46 |
| | | (+0.03) | (−0.65) | (−0.24) | (−2.26) | (−0.06) | (+0.04) | (−3.48) |
| | 0–12 | +1.0% | 6.2% | 11.7% | 74.7% | 7.4% | | |
| | 12–24 | 0.0% | 33.3% | 3.0% | 63.7% | +4.0% | | |
| | 0–24 | +1.0% | 20.3% | 7.5% | 70.4% | 1.8% | | |

[a] Activity is expressed as nanocuries, with the amount of the change shown in parentheses. Percentages are the percentage of total loss in activity by all fractions.

[b] The value for the residue is corrected by subtracting the value for PHB which is determined on a separate sample of cells.

[c] A double inoculum was used to grow the acetate- and succinate-grown cells.

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