Adenine Nucleotide Pools During Starvation of *Beneckea natriegens*

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The marine bacterium *Beneckea natriegens* was grown in batch culture on a glucose/ NH_4^+ /salts medium; growth terminated due to either carbon or nitrogen depletion from the medium. Nitrogen-limited cultures converted part of the excess glucose into glycogen whereas the carbon-limited cultures formed little glycogen. Glycogen-rich cultures survived longer than glycogen-poor cultures during starvation. Little protein was utilized during starvation and RNA was degraded as the primary endogenous source of energy. Glycogen was consumed only when the RNA content had decreased to about a third of the growth value.

The adenine nucleotide content of nitrogen-limited cultures increased at the start of the stationary phase but the energy charge remained at the growth value of 0.9 to 0.95. The maximum size of the adenine nucleotide pool depended on the concentration of glucose remaining in the medium at the start of the stationary phase but a limiting value of about $60 \mu\text{mol ATP (g protein)}^{-1}$ was attained, compared with 12 to $14 \mu\text{mol ATP (g protein)}^{-1}$ in exponentially growing cultures. During extended starvation of both glycogen-rich and glycogen-poor cultures, there was a large decrease in adenine nucleotide content, but the energy charge remained above 0.6 even when viability was very low.

INTRODUCTION

There are many factors that can cause loss of viability of bacteria (Dawes, 1976). During long-term starvation, endogenous metabolites such as non-essential RNA, protein and reserve polymers (glycogen, poly- β -hydroxybutyrate, triacylglycerols and polyphosphate) act as energy sources permitting essential maintenance processes to occur, thereby preserving viability (Dawes & Senior, 1973). Under these circumstances loss of viability may be related to the inability to regenerate ATP due to depletion of endogenous metabolites and reserve polymers. This would be seen as a decrease in the adenine nucleotide content or as a decrease in the ATP:ADP and ATP:AMP ratios, which may be measured as a decrease in energy charge (Atkinson, 1968, 1978; Chapman & Atkinson, 1977; Knowles, 1977, 1979).

There have been few studies on the adenylate pools of starving bacteria. Chapman *et al.* (1971) showed that, following nitrogen depletion from a glucose-containing growth medium, there was a gradual decrease in the intracellular energy charge of *Escherichia coli* strain B from a growth value of about 0.8 to between 0.6 and 0.5 after 60 to 80 h, without loss of viability. The intracellular adenine nucleotide content also decreased during this period. After 60 to 80 h, a rapid decrease in energy charge occurred, coincident with a loss of viability. Montague & Dawes (1974) showed a similar relationship of decrease in energy charge and loss of viability during starvation of *Peptococcus prvotii*, which does not form reserve polymers but utilizes RNA as the sole endogenous source of energy.

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The effect of termination of growth on the adenine nucleotide composition of batch cultures, due to depletion of carbon or NH_4^+ from the medium, has been examined in *E. coli* strain B (Chapman *et al.*, 1971; Walker-Simmons & Atkinson, 1977) and *Beneckea natriegens* (Niven *et al.*, 1977a). (For simplicity, we will refer to these as 'carbon-limited' and 'nitrogen-limited' cultures.) Considerable differences are observed in maintenance of the adenylate pools of these two bacteria following the transition from exponential growth into the stationary phase. These effects have been reviewed by Knowles (1979).

Chapman *et al.* (1971) reported that the adenylate pool of *E. coli* strain B fell by 50% following glucose-limitation and the energy charge decreased from 0.8 to 0.6. The energy charge values were later revised to 0.9 to 0.93 and 0.9 to 0.8, respectively (Walker-Simmons & Atkinson, 1977). The latter energy charge value was maintained for 5 to 7 h until the acetate, which had been formed during the growth on glucose, had been utilized. The energy charge then gradually decreased. In succinate-limited cultures, the energy charge immediately dropped to 0.6 at the start of the stationary phase (Andersen & von Meyenburg, 1977). In contrast to these results, glucose- or succinate-limitation of *B. natriegens* did not cause a measurable decrease in energy charge and there was a slight increase in the adenylate pool size (Niven *et al.*, 1977a). During growth on glucose, but not on succinate, volatile acids were produced. They were consumed during a second, post-growth respiratory phase. The energy charge was maintained at 0.9 to 0.95 for at least 3 h after their oxidation.

Ammonia-limitation of glucose-grown cultures of *E. coli* strain B (Chapman *et al.*, 1971; Walker-Simmons & Atkinson, 1977) and *B. natriegens* (Niven *et al.*, 1977a) also resulted in different responses of the adenine nucleotide pools. In both bacteria the energy charge was maintained at the growth value, but in *E. coli* the adenylate pool size remained stable whereas in *B. natriegens* it increased until the excess glucose had been oxidized. In both bacteria some of the excess glucose was deposited as glycogen. It has been suggested that deposition of glycogen by *E. coli* strain W4597(K) is due to a small rise in energy charge at the start of the stationary phase (Dietzler *et al.*, 1974).

In view of the paucity of information about the adenine nucleotide composition of bacteria subjected to extended starvation, and the differences observed in adenylate pool maintenance at the start of the stationary phase in *E. coli* and *B. natriegens*, we have investigated these effects in starved *B. natriegens*.

METHODS

Organism and medium. *Beneckea natriegens* strain III (Baumann *et al.*, 1971) was grown in the synthetic medium described previously (Niven *et al.*, 1977a) but buffered with 100 mM-Tris/HCl. For carbon-limited growth, 5 mM-glucose and 25 mM- NH_4Cl were added; for nitrogen-limited growth, 8 mM-glucose and 2.5 mM- NH_4Cl were added.

Growth of bacteria. The bacterium was maintained on agar slants (Difco Bacto marine agar 2216) with monthly subculturing. Bacteria for inocula were grown in 100 ml medium in 250 ml conical flasks for 16 h at 35 °C on a rotary shaker (200 rev. min^{-1}); 20 ml of an appropriate culture was used to inoculate 1 l of the experimental culture which was grown at 35 °C in a 1 l Quickfit fermentation vessel with a sampling/injection port, as described by Niven *et al.* (1977a). The air flow rate was 1.25 l min^{-1} and the stirring rate was 10 cycles s^{-1} . During growth in the fermenter, bacteria were monitored daily for purity by microscopic examination, Gram staining, the ability to ferment rhamnose on an eosin methylene blue plate, citrate utilization and for the presence of catalase and Kovac's oxidase (Baumann *et al.*, 1971).

Estimation of adenylates and energy charge. Culture samples (0.8 ml) were collected rapidly into 0.2 ml 25% (w/v) HClO_4 precooled to 4 °C in a spring-loaded syringe unit (Niven *et al.*, 1977a). The supernatants from filtration (0.45 μm pore size filters) were also analysed. The luciferase assay procedure for analysis of ATP, ADP and AMP was used (Niven *et al.*, 1977a). Poole & Salmon (1978) reported interference of traces of HClO_4 with the enzymic conversion of ADP and AMP to ATP. Our control experiments did not reveal similar difficulties, and there was no detectable interference.

Determination of glucose and glycogen. Glycogen was extracted by a modified Pflüger procedure (Hassid & Abraham, 1957). To a 1.5 ml cell sample, 0.75 ml 90% (w/v) KOH was added, followed by digestion by heating in a boiling water bath for 15 min. After cooling, 0.23 ml 8% (w/v) Na_2SO_4 and 5.8 ml absolute

ethanol were added. The precipitated glycogen was centrifuged and stored at -22°C . This glycogen was dissolved in 1 ml distilled water, re-precipitated with 2.4 ml absolute ethanol and re-washed twice with water and ethanol. After hydrolysis in 0.5 ml 1 M- H_2SO_4 at 100°C for 3 h and subsequent neutralization to pH 7.0 with 6 M-NaOH, the liberated glucose was determined by the glucose oxidase method (Niven *et al.*, 1977a).

Total cell numbers. Bacteria were killed by adding 1 drop of formaldehyde per ml to a 1:10 dilution of the culture in fresh medium. A drop of the suspension was placed on the grating of a haemocytometer (Improved Neubauer, B.S.748, depth 0.1 mm, $1/400\text{ mm}^2$; Weber & Sons, Lancing, W. Sussex) and the coverslip was pressed down until Newton's rings were obtained. Eighty squares were counted using a Zeiss phase contrast microscope ($500\times$ magnification).

Viable cell numbers. Cells were serially diluted in fresh culture medium without glucose. To avoid cold shock, the dilution tubes were preincubated at 35°C for 30 min. Quintuplicate portions (0.1 ml) of two appropriate dilutions were spread evenly over the surface of marine agar plates which had been dried at 35°C for 3 h. The plates were incubated for 24 h at 35°C and colonies were then counted.

Viability. The percentage viability of cultures was estimated by a slide culture method similar to that of Postgate *et al.* (1961). Stainless-steel annuli on glass microscope slides were filled with marine agar from which solid debris had been removed and placed in Petri dishes containing fresh marine agar to keep the slides moist. Culture samples were diluted 10-fold, spread in duplicate on the surface of the agar film and excess fluid was removed with a Pasteur pipette. Coverslips were omitted and the cultures were incubated at 35°C for 90 min and inspected using a Zeiss phase contrast microscope ($500\times$ magnification).

Measurement of RNA. A 10 ml sample was removed from the fermentation vessel and centrifuged at 3000 g for 20 min. The pellet was stored at -22°C until required. RNA was extracted as described by Blobel & Potter (1968) and measured by the orcinol method (Herbert *et al.*, 1971), using D-ribose as the standard. The RNA content was calculated using a correction factor of 4.28, assuming equal concentrations of each of the nucleotides.

Other assays. For protein determination, a 2 ml sample from the fermentation vessel was centrifuged at 3000 g for 20 min, and the pellet was resuspended in 2 ml 5 M-NaOH and boiled for 2 min; protein was assayed by the method of Lowry. Growth was monitored by the absorbance at 680 nm, using 1 cm cuvettes in a Pye Unicam SP500 spectrophotometer. Poly- β -hydroxybutyrate was estimated by the method of Carter & Dawes (1979), using a 5 ml culture sample. For estimation of polyphosphate, a complete 1 l culture was centrifuged, polyphosphate was extracted and hydrolysed as described by Herbert *et al.* (1971), and the phosphate released was estimated by the method of Fiske & Subbarow (1925).

Chemicals. Adenylate kinase, firefly lantern extract (FLE-250), glucose oxidase, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate phosphokinase, ATP, ADP, AMP, NADP (grade III), phosphoenolpyruvate, glycylglycine and Tris (Trizma base) were obtained from Sigma. Pyruvate kinase [as an $(\text{NH}_4)_2\text{SO}_4$ suspension] was obtained from Boehringer. Whenever possible, all other reagents were of analytical grade; glass-distilled water was used throughout.

RESULTS

Figure 1 shows the effect of extended starvation on cultures of *B. natriegens* in which growth had terminated due to depletion of glucose from the medium. Growth, as measured by the absorbance and protein content of the culture, finished about 3 h after inoculation, when glucose was no longer detectable in the medium (Fig. 1a). During growth there was a high level of respiratory activity. Following glucose depletion there was a second respiratory phase of 2 h due to oxidation of excreted organic acids (not shown), in agreement with previous observations (Niven *et al.*, 1977a), before oxygen uptake declined to a low level. The pH was between 7.2 and 7.5 throughout the experiment.

Total and viable cell numbers continued to increase (by about 50%) after the maximum absorbance had been attained, indicating that a round of division continued after termination of growth (Fig. 1b). Viability remained at 90 to 100% for 22 h, then declined rapidly. However, the total cell numbers remained high for at least 50 h, indicating that loss of viability was not associated with cell lysis. The apparent fourfold discrepancy in total and viable cell numbers was due to a systematic error in the former counts. There was an increase in the thickness of the haemocytometer (Mallette, 1969). Also, there was probably incomplete division of cells seen under the phase contrast microscope, so that a single colony on a plate may have represented two undivided but viable cells.

Fig. 1. Growth and starvation of *B. natriegens* under conditions where growth terminated due to depletion of glucose from the medium. The initial glucose and ammonium concentrations were 5 mM and 25 mM, respectively. Absorbance ($\circ$), protein ($\bullet$), glucose ($\square$), percentage viability ($\blacksquare$), total cell numbers ($\triangle$), viable cell numbers ($\blacktriangle$), RNA (∇), glycogen, expressed in terms of its glucose content ($\blacktriangledown$), energy charge ($\diamond$), bacterial ATP ($\blacklozenge$).

Little glycogen was formed in carbon-limited cells (Fig. 1c). The RNA content decreased gradually during starvation, to about one-third of the optimum level at 22 h after inoculation, when viability started to decrease. Thereafter there was little change in RNA content. The protein content of the culture decreased only slightly during starvation for up to 50 h (Fig. 1a).

Fig. 2. Growth and starvation of *B. natriegens* under conditions where growth terminated due to depletion of ammonia from the medium. The initial glucose and ammonium concentrations were 8 mM and 2.5 mM, respectively. Symbols as in Fig. 1.

During growth, the ATP content was about $14 \mu\text{mol (g protein)}^{-1}$, equivalent to approximately $7 \text{ nmol (mg dry wt)}^{-1}$, in reasonable agreement with Niven *et al.* (1977a). Following termination of growth, the ATP pool increased slightly and then declined gradually from 13 h after inoculation (Fig. 1d). The energy charge was about 0.95 during growth and decreased slowly from 13 h onwards, but was still 0.6 at 50 h when the total adenylate content was less than 10% of the maximum level and viability was only 6%.

When *B. natriegens* was grown in a medium containing 8 mM-glucose and 2.5 mM-

Fig. 3. Effect of variation of initial glucose concentration in the medium on the maximum ATP pool size and glycogen content in the stationary phase. The initial ammonium concentration was 2.5 mM and the initial glucose concentration was varied as indicated. Bacteria were grown until glucose disappeared from the medium and then the following parameters were measured: culture absorbance (○), medium pH (●), total cell numbers (□), bacterial ATP (■), glucose used (Δ), glycogen formed (▲).

NH_4Cl , growth was limited by depletion of nitrogen from the medium. About 4.5 mM-glucose remained in the medium at this time; it was oxidized during the first 6 h of the stationary phase (Fig. 2a). About 8% of the excess glucose was converted to glycogen during this period (Fig. 2c). No poly- β -hydroxybutyrate or polyphosphate was detected during the early-stationary phase. Accumulated organic acids were also oxidized during this period, during a second respiratory phase (not shown), as described by Niven *et al.* (1977a).

Total and viable cell numbers continued to increase, by about 50%, after the maximum absorbance had been reached (Fig. 2b). In contrast to carbon-limited cultures, there was only a gradual decrease in viability during starvation of nitrogen-limited cultures, with a viability of 30% at 50 h after inoculation (Fig. 2b). Total cell numbers remained relatively constant up to at least 90 h, again indicating that loss of viability was not associated with cell lysis. The pH remained between 7.2 and 7.5 throughout the experiment.

The RNA content of the culture started to decrease immediately after growth had terminated, declining to about 50% of the maximum level at 50 h (Fig. 2c). Surprisingly, the glycogen content of the cells did not start to decrease until 36 h after inoculation and was still 30% of the maximum level after 90 h. The protein content of the cells decreased only slightly during the experiment (Fig. 2a).

During growth, the ATP content was $12 \mu\text{mol (g protein)}^{-1}$. The ATP pool then increased massively to about $60 \mu\text{mol (g protein)}^{-1}$ at 10 h after inoculation (Fig. 2d). After

a period of stability, the ATP content gradually decreased, although at 50 h it was still greater than the growth value. The energy charge was about 0.95 both during growth and in the stationary phase until approximately 22 h after inoculation. It then declined slowly to 0.65 at 90 h, when both the viability and ATP content of the culture were extremely low.

A post-growth increase in adenylate content of nitrogen-limited cultures of *B. natriegens* has been reported previously by Niven *et al.* (1977a), but the initial glucose content of the cultures was 5 mM compared with 8 mM used in this study. The effect of variation of the initial glucose concentration on the maximum glycogen synthesis and adenylate content is shown in Fig. 3. With an initial NH_4Cl concentration of 2.5 mM, growth terminated due to carbon depletion from the medium when the initial glucose concentration was less than 3.5 mM. At higher glucose concentrations, growth was terminated by nitrogen limitation. The maximum glycogen content increased with increasing initial concentration of glucose, up to 10 mM. Of the glucose in excess of the requirement for growth, 6 to 8% was converted to glycogen at the start of the stationary phase. There was, however, an apparent maximum size of the adenine nucleotide pool, during the post-growth phase. At an initial glucose concentration of 6 mM, equivalent to about 2.5 mM residual glucose at the start of the stationary phase, the maximum ATP content noted during the post-growth increase was $60 \mu\text{mol (g protein)}^{-1}$. Further increases in initial glucose concentration did not result in higher concentrations of adenine nucleotides.

DISCUSSION

RNA was the preferred endogenous source of energy for maintenance of starving *B. natriegens*. The RNA content decreased progressively during starvation, whereas the protein content was relatively unaffected. It was only when the RNA content had decreased considerably, presumably to the minimum level essential for survival, that glycogen-rich cultures utilized glycogen to extend the period of viability. In glycogen-poor cultures, loss of viability occurred when the RNA content fell to about a third of the level found during growth.

Unlike *E. coli* (Chapman *et al.*, 1971) and *P. prévotii* (Montague & Dawes, 1974), loss of viability during starvation of *B. natriegens* was not associated with a decrease in energy charge below 0.6. No direct correlation was obtained between the size of the adenine nucleotide pool and loss of viability, although in both carbon- and nitrogen-limited cultures the ATP content decreased during starvation. The actual ATP pool in nitrogen-limited cultures was very much greater during starvation than that in carbon-limited cultures.

The viability of the *B. natriegens* cultures decreased during starvation without a decrease in the total cell numbers. This does not mean that the non-viable intact bacteria did not become permeable, losing some of their intracellular metabolites and proteins, etc., which could act as a source of nutrients for the remaining bacteria. No appreciable increase in absorbance at 260 or 280 nm was noted, nor were any extracellular adenylates found in the cell-free medium, but the remaining viable bacteria could have utilized these nutrients too rapidly for their detection. If this was occurring, it would explain the decrease in adenine nucleotide content of the culture and simultaneous maintenance of the energy charge at a value greater than 0.6 even when there had been a dramatic decrease in percentage viability.

The size of the adenylate pool is a balance between the rate of *de novo* synthesis of AMP plus the rate of salvage of AMP or its precursors from RNA catabolism and the rate of incorporation of ADP and ATP into RNA, DNA, proteins (in histidine residues) and co-factors (e.g. NAD) plus the rate of AMP catabolism (Knowles, 1979). At the start of the stationary phase there is a dramatic decrease in the demand for adenylate supply for synthesis of macromolecules. The rise in adenylate content of *B. natriegens* at the start of the stationary phase in the presence of glucose as an energy source, compared with the

constant adenylate content of *E. coli* (Chapman *et al.*, 1971), therefore reflects a difference in the overall regulation of the pathways in the two bacteria. The increase in size of the adenine nucleotide pool of *B. natriegens* occurred only as long as there was sufficient glucose present as an exogenous source of energy (Fig. 3).

An important difference between the organisms could be the pattern of AMP catabolism. *Escherichia coli* contains AMP nucleosidase and, as a periplasmic enzyme involved in adenosine uptake, AMP nucleotidase (Schramm & Lazorik, 1975; Yagil & Beacham, 1975). Indirect assays on cell-free extracts of *B. natriegens* suggested the presence of AMP deaminase and AMP nucleotidase (Niven *et al.*, 1977*b*). However, further attempts to detect and purify the former enzyme have been unsuccessful (M. A. Pickard, P. A. Collins & C. J. Knowles, unpublished observations). The ability of intact cells of *B. natriegens* to degrade exogenous AMP (M. A. Pickard, personal communication) and to utilize AMP as a carbon source (Niven *et al.*, 1977*a*) suggests that AMP nucleotidase may be periplasmic in this bacterium. *Beneckea natriegens* does not form AMP nucleosidase.

In *Azotobacter vinelandii*, AMP nucleosidase is activated by a decrease in energy charge, and its role may be to buffer energy charge at the expense of the total adenine nucleotide pool size under conditions where the cell is unable to regenerate ATP from ADP sufficiently rapidly (Schramm & Leung, 1973). If the AMP nucleosidase of *E. coli* is regulated in a similar manner, the enzyme would not be active under the energy-rich conditions prevailing at the start of the stationary phase in the presence of excess glucose. Thus, the difference in maintenance of the size of the adenine nucleotide pools of *B. natriegens* and *E. coli* could not be explained by the presence of this enzyme in the latter organism only.

On the other hand, when growth stops due to carbon-limitation the drop in energy charge of *E. coli* could activate AMP nucleosidase, thus accounting for the decrease in adenine nucleotide pool size under these conditions (Chapman *et al.*, 1971; Walker-Simmons & Atkinson, 1977; Andersen & von Meyenburg, 1977). In *B. natriegens*, which does not form this enzyme, there was no drop in adenine nucleotides on carbon-limitation.

The increase in adenine nucleotide content at the start of the stationary phase of nitrogen-limited cultures of *B. natriegens* was not associated with a measurable decrease in the RNA content. However, the RNA content was much greater than the adenine nucleotide content and only a small change in RNA concentration, which could not be accurately measured, would significantly alter the size of the adenylate pool. Consequently, it is not possible to conclude whether the extra adenylates are derived from salvage from RNA degradation or are due to *de novo* synthesis of AMP occurring at a greater rate than the decreased demand for RNA, DNA and protein synthesis.

Conversely, the decrease in RNA content as starvation progresses would lead to a dramatic increase in adenine nucleotide content unless the adenosine residues were further catabolized, with the other nucleoside residues, as a source of energy. The concomitant decrease in adenylate content of the cultures strongly suggests that this was occurring.

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