JOURNAL OF BACTERIOLOGY, Mar. 1975, p. 975-982

Vol. 121, No. 3
Printed in U.S.A.

Adenylate Energy Charge in *Saccharomyces cerevisiae* During Starvation

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Received for publication 22 November 1974

Bakers' yeast cells, *Saccharomyces cerevisiae*, if grown aerobically on ethanol or if grown aerobically on glucose and allowed to pass into stationary phase, with utilization of accumulated ethanol, maintain a normal value (0.8 to 0.9) of the adenylate energy charge during prolonged starvation. In contrast, cells grown anaerobically on glucose and cells in the early stages of aerobic growth on glucose exhibit a rapid decrease of energy charge if transferred to medium lacking an energy source. These results suggest that functional mitochondria or enzymes of the glyoxylate pathway, or both, are required for maintenance of a normal balance of adenine nucleotides during starvation. Yeast cells remain viable at energy charge values below 0.1, in marked contrast to results previously obtained with *Escherichia coli*. In other respects, the energy charge responses of yeast to starvation and refeeding are generally similar to those previously reported for *E. coli*.

The importance of the adenine nucleotides as major regulatory factors in controling metabolic processes as a result of their influence on key regulatory enzymes has been well established. Comparison of the effects exerted on individual enzymes in vitro with regulation of metabolism by the energy state of the cell is facilitated by use of the adenylate energy charge. This parameter, the mole fraction of adenosine 5'-triphosphate (ATP) plus half the mole fraction of adenosine 5'-diphosphate (ADP), (ATP + $\frac{1}{2}$ ADP)/(ATP + ADP + AMP), is a linear measure of the amount of metabolically available energy stored in the adenylate pool (2). Enzymes responding to adenylate nucleotide control that are involved in biosynthetic or other ATP-utilizing pathways have been found to have high activities when the energy charge is high. Enzymes involved in the regeneration of ATP show a sharp decrease in their activity as the energy charge increases to a high level (1). These enzymes all have their sharpest regulatory responses in the region above an energy charge value of 0.7. The half-maximal responses for enzymes of both types occur at an energy charge of 0.8 to 0.9, and it is in this region that the cell would be expected to maintain its energy charge. Nearly all recent determinations of the levels of ATP, ADP, and AMP in metabolizing cells or tissues of various organisms do in fact correspond to energy charge values in this range (7, 8).

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We report here that *Saccharomyces cerevisiae* cells grown on different carbon sources and under either aerobic or anaerobic conditions maintained energy charge values above 0.8. Differences were observed, however, in the response to starvation by cells grown under different conditions. These results complement, for a eukaryotic organism, the generally similar results previously reported from this laboratory from experiments with *Escherichia coli* (8) and *Azotobacter vinelandii* (15). Although there are some interesting differences between the responses of yeast and those of *E. coli* to some conditions of nutritional deficiency, there is good agreement between the energy charge values that have been found by direct analysis of metabolizing cells of both species and that predicted from enzyme studies in vitro.

MATERIALS AND METHODS

Growth conditions. A *Saccharomyces cerevisiae* strain, isolated from commercially available Fleischmann's bakers' yeast, was grown aerobically and anaerobically in batch cultures. Cultures were inoculated with 5 ml of stationary-phase cells, grown anaerobically on 5% glucose, per liter of medium. Aerobic cultures were grown in 2-liter flasks, closed with cotton plugs, with rotatory shaking at 25 C. Anaerobic cultures were grown in 500-ml volumes in 500-ml flasks. These cultures were initially flushed with N_2 and then allowed to grow under their own evolved CO_2. Cultures that were to be changed between anaerobic and aerobic conditions were grown with air or N_2 bubbled through the medium.

In early experiments (see Fig. 1), a complex me-

dium containing 5.0 g of Difco yeast extract, 1.0 g of KH_2PO_4, 1.2 g of K_2HPO_4, and 50 g of glucose (0.28 M) per liter was used. Generally, however, a medium with less yeast extract was employed. This medium contained 4.8 g of K_2HPO_4, 4.00 g of KH_2PO_4, 2.2 g of $(NH_4)H_2PO_4$, 2.0 g of $(NH_4)_2HPO_4$, and 0.50 g of yeast extract per liter. Metal ions and cofactors were added at twice the levels used by Olson and Johnson (17) and Phaff et al. (18).

Cell growth measurements. The growth of the cells was followed by optical absorbance readings at 600 nm, and by direct cell counts with a hemocytometer.

Cell viability. The number of viable cells was determined by plating 1 ml of a cell suspension (diluted 10^5- to 10^7-fold) in 12 ml of nutrient agar and counting the number of colonies formed after incubation at room temperature for 3 to 4 days.

Starvation medium. The starvation salts medium generally used was that of Williamson and Scopes (25) buffered at pH 6.0 with 3.6 g of KH_2PO_4 and 1.0 g of K_2HPO_4 per liter. When the cells were starved in the presence of NH_4^+, the buffer consisted of 2.0 g of $(NH_4)_2HPO_4$ and 5.0 g of KH_2PO_4 per liter in a solution containing the salts and cofactors of the growth medium at half their usual concentration.

Starvation cells. The cells used for the starvation experiments were collected by centrifugation at 4,000 × *g* for 5 min at 20 C in the Sorvall centrifuge. They were then washed with the salts starvation medium, centrifuged again for 5 min and resuspended in the starvation medium.

Extraction of the adenine nucleotides. Nucleotides were extracted from the yeast cells by two methods, using perchloric acid or hot ethanol. The adenine nucleotide levels and ratios recovered from yeast cells extracted by the two procedures were essentially the same. Thus, both procedures were used, and the results obtained with both are presented interchangeably in this paper.

(i) Ethanol extraction procedure. A sample of the cell culture was obtained by inserting a small tube into the culture and withdrawing a sample of about 2 ml. One ml of the removed sample was pipetted into 1 ml of hot 95% ethanol. The entire operation required 8 to 12 s. The sample was incubated in a hot water bath at 78 C for 5 min, cooled in ice, and brought to 4 ml with water. The sample was then frozen and stored at −20 C until thawed for use.

(ii) Perchloric acid extraction procedure. In early experiments, a 1-ml sample of the yeast culture was added to 0.20 ml of 35% perchloric acid. The solution was rapidly mixed and then kept on ice for 5 min. The extract was then neutralized with 0.54 ml of cold 2.5 N KOH, and buffered with 0.36 ml of 0.2 M phosphate buffer at pH 7.3. The 2.10-ml sample was brought to 4.10 ml with water and then frozen and thawed before assaying. The denatured protein and $KClO_4$ were removed by centrifugation after thawing. In later experiments, the extraction procedure was changed to a 6-min room temperature extraction in perchloric acid. The freezing and storage temperature was lowered to −70 C.

Luciferase assay. The amount of ATP in standards and cell samples was determined by means of the luciferase reaction (24) using a Luminescence Biometer (E. I. DuPont de Nemours and Co.). A brief description of this instrument has been given by Johnson et al. (13).

One vial of crystalline luciferin-luciferase mixture obtained from DuPont was dissolved in 3.0 ml of buffer as described by Chapman et al. (8). Immediately before each ATP measurement was made, 10 μl of cold, buffered luciferin-luciferase was added to a DuPont biometer tube containing 100 μl of buffer consisting of 40 mM glycylglycine and 3 mM $MgSO_4$ (replacing $MgCl_2$ to relieve Cl^- inhibition) at pH 7.4 and room temperature. The tube was then placed in the instrument, and 10 μl of the cell sample or standard was injected into the mixture with a Hamilton injection syringe. The adenine nucleotides gave linear responses over a concentration range of 0.01 to 24 μM.

(i) Preparation of samples for the assay. The cell extract samples and standard samples were prepared for assay by the method described by Pradet (20) and Chapman et al. (8) except that 15 mM $MgSO_4$ was substituted for 15 mM $MgCl_2$, and that both the pyruvate kinase and adenylate kinase were dialyzed against 1 liter of 0.10 M potassium phosphate buffer, pH 7.3, for 5 to 8 h before use.

For determination of ATP, 200 μl of cell extract was added to 50 μl of 75 mM potassium phosphate buffer (pH 7.3) containing 15 mM $MgSO_4$.

For determination of ATP + ADP, 200 μl of cell extract was added to 50 μl of 75 mM potassium phosphate buffer (pH 7.3) containing 15 mM $MgSO_4$, 0.5 mM phosphoenolpyruvate, and 20 μg of pyruvate kinase.

For determination of ATP + ADP + AMP, 200 μl of cell extract was added to 50 μl of 75 mM potassium phosphate buffer (pH 7.3) containing 15 μM $MgSO_4$, 0.5 mM phosphoenolpyruvate, 20 μg of pyruvate kinase, and 25 μg of adenylate kinase.

The three mixtures were incubated at 30 C for 30 min and then kept on ice until assayed. The incubated mixtures where shown to be stable for 2 days when kept at 0 C.

(ii) Standardization, calculations, and reliability. The assay was routinely standardized by using 200 μl of a 1 μM ATP solution mixed with 50 μl of the phosphate and $MgSO_4$ buffer. The conversion reactions were checked by assaying 200 μl of 1 μM ADP and AMP solutions with 50 μl of the appropriate reaction mixtures. Five determinations were made for each sample assayed, and the standard error of the mean was typically ± 2%.

The amounts of ADP and AMP may be determined by difference and used in calculation of the energy charge value. Alternatively, the charge value may be calculated directly from the three determinations by putting the defining equation for the energy charge in the form:

$$\text{Energy charge} = {}^1\!/_2[(\text{ATP}) + (\text{ATP} + \text{ADP})]/[\text{ATP} + \text{ADP} + \text{AMP}]$$

Use of the equation in this form simplifies the propagation of errors treatment. By this treatment

(22), the standard error of the mean for the energy charge estimations was found to be approximately ± 0.04 in most cases.

(iii) Inhibition of the assay. The luciferase assay reaction is inhibited by both ethanol and perchlorate, and the extraction samples of 2 ml were diluted to 4 ml to decrease this inhibition. At this dilution, the ethanol (25%) inhibited the reaction 26%, while the perchlorate (1.7%) inhibited 22%. The nucleotide recovery levels presented in this paper were corrected according to the amount of inhibition.

Extracellular adenine nucleotides. The extracellular levels of the adenine nucleotides were determined so that any excretion or leakage of the nucleotides from the cells could be accounted for and subtracted from the total recoveries. Cell-free medium samples obtained by centrifugation (1.5 to 2 min) or by filtration (10 to 15 s) gave comparable results.

The yeast extract used in the growth medium contained AMP at a relatively high level. In the medium containing 0.5% yeast extract, the initial AMP level was nearly 50% of the total pool recovered at low cell densities. The medium with 0.05% yeast extract contained AMP at 5 to 10% of the total pool at low cell densities. The extracellular levels of ATP, ADP, and AMP increased little during cell growth.

Intracellular adenine nucleotide levels. The intracellular adenine nucleotide level was determined as the difference between the adenylate pool measured in the complete culture and that measured in the medium. The levels are reported in the figures simply as apparent concentrations in the whole culture volume. Such values are valid for use in comparisons and in calculation of energy charge values. Intracellular concentrations of the adenylate nucleotides were calculated for cell sap volumes as estimated from dry weights on the assumption that 1 g of dry weight corresponds to 5 g of wet weight, and that 1.67 g of wet weight corresponds to 1 ml of cell sap volume (11). Average total pool concentrations were 2.0 mM for cells growing on glucose and 2.4 mM for cells growing on ethanol. These values are probably slightly low since they assume an even distribution of nucleotides throughout the cell and do not take bound water into account. They are, however, similar to other reported values as tabulated by Gancedo and Gancedo (12).

Glucose determination. The glucose level in the medium was determined after the yeast cells had been removed by centrifugation or Millipore filtration by the glucose oxidase reaction (kit supplied by Sigma Chemical Co.).

Ethanol determination. Ethanol in the medium was estimated by gas chromatography. A Varian 1200 Aerograph with a flame detector was used. A 4-foot copper tube (1/8 inch in diameter) was packed with Porapak Q (Waters Association, Inc., mesh 100 to 120) solid support. The column was used at 175 C and the retention times were standardized with water solutions of methanol, ethanol, acetone, acetic acid, and hexane. Samples of the medium filtrate (0.40 to 0.60 μl) were injected directly without further treatment after the cells had been removed.

RESULTS

Yeast cells grown anaerobically with 5% (0.28 M) glucose as the carbon source exhibited an energy charge of about 0.8 during growth. The level of ATP and of total adenine nucleotides increased in parallel with increases in optical absorbance. When the glucose supply was exhausted, the sum of adenine nucleotide concentrations did not change significantly during the next 10 or 12 h, but the concentration of ATP and the energy charge fell. The charge reached a value of about 0.4 in 12 h. The extracellular level of adenine nucleotides showed little change during the growth period, and AMP was the predominant form in the medium because of its presence in the yeast extract used.

The drop in the intracellular ATP level and the energy charge when glucose was exhausted was studied further in a long-term starvation experiment. During the starvation period, the energy charge steadily decreased until it reached 0.14 (Fig. 1). Direct cell counts remained constant and plate counts showed no apparent loss in cell viability during the first 24 h after exhaustion of carbon source. At the end of 7 days, 66% of the cells were still viable, yet the energy charge was 0.03. During this long-term starvation period, the total intracellular adenine nucleotide pool remained steady. In addition, the extracellular nucleotides remained relatively unchanged except for a 25% decrease in the AMP level near the end of the experiment.

Yeast cells grown aerobically on 1% glucose (55 mM) had an average energy charge of 0.9 both during the log phase growth and the stationary phase. The growth curve under these conditions was biphasic, showing a slow rate of growth for several hours immediately after the glucose was consumed (Fig. 2). Plots of the level of intracellular adenine nucleotides and of cell number gave biphasic curves resembling those obtained from the turbidity measurements. The extracellular levels of the nucleotides increased only slightly above those of the initial medium. Assays for ethanol in the medium showed accumulation at an exponential rate until the glucose supply was exhausted, followed by consumption during the second phase of growth.

Batch cultures were also grown aerobically with ethanol (0.22 M) as the carbon source. The energy charge value was 0.84 throughout the growth period, and it appeared to drop to about 0.7 during the stationary phase (Fig. 3).

When yeast cells growing aerobically on glucose were subjected to anaerobic conditions, there was no detectable effect on the energy

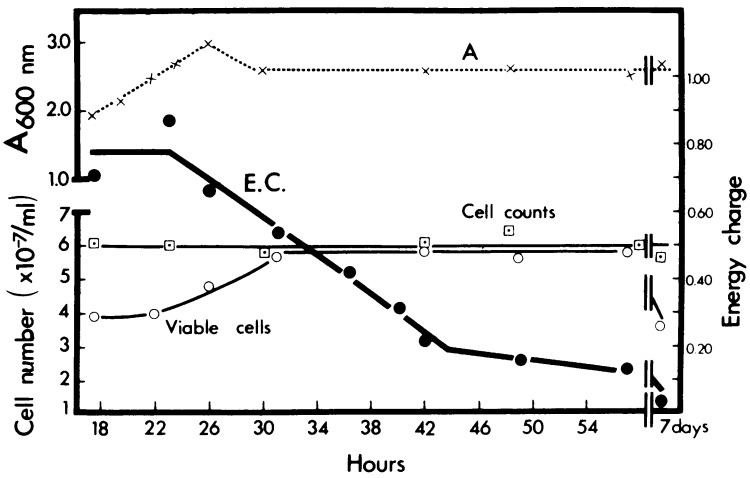

FIG. 1. *Decline in energy charge in S. cerevisiae cells during prolonged starvation. The cells were grown in the complex medium containing 0.28 M glucose. The curves represent: A, absorbance at 600 nm; E.C., adenylate energy charge; Cell counts, direct hemocytometer counts; Viable cells, number of cells forming colonies when plated.*

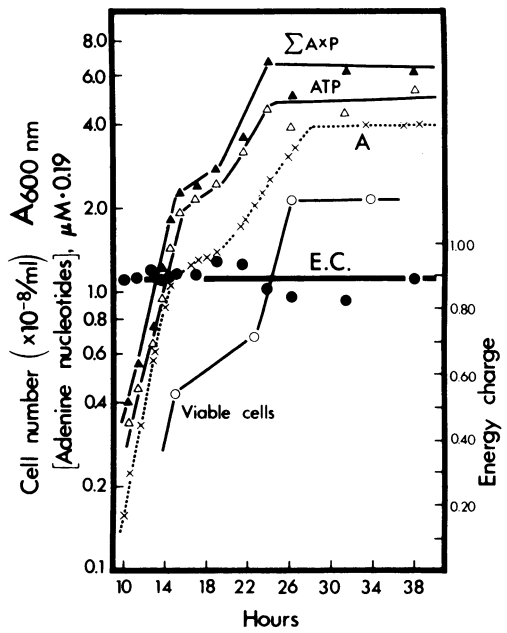

FIG. 2. *Stability of energy charge values in S. cerevisiae cells during aerobic growth on glucose. The cells were grown in the second medium, as specified in the text, with 55 mM glucose as the carbon source. Identification of curves are as in Fig. 1;* $\Sigma A \times P$, *relative concentration of total adenine nucleotides (ATP + ADP + AMP); ATP, relative concentration of ATP.*

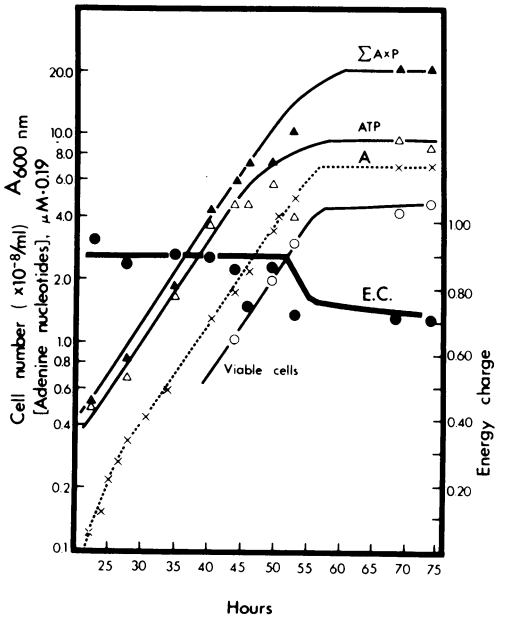

FIG. 3. *Energy charge values in S. cerevisiae during aerobic growth on ethanol. Cells were grown aerobically in medium containing 0.22 M ethanol. Identifications of curves are as in Figs. 1 and 2.*

charge (Fig. 4). There was however a general lowering of the nucleotide levels in the cells after a transient oscillation of the pool size. The normal pool size was restored rapidly after air was resupplied.

A similar experiment was done with cells growing on ethanol. Cell growth stopped immediately after nitrogen was bubbled through the culture, but again, no decrease in the energy charge was caused by the transition to anaerobic conditions. Cell growth resumed, and the adenine nucleotide pool size increased when aeration was resumed.

Cells grown aerobically on glucose in medium

in which NH_4^+ was limited showed no detectable change in the energy charge when growth stopped because of nitrogen depletion. The yeast cells were capable of maintaining an energy charge of about 0.75 under these nitrogen-starvation conditions. There was a slight increase in the adenylate pool after growth stopped.

When cells were grown anerobically in a glucose-limited culture, there was a sharp drop in ATP and in the energy charge when the glucose was exhausted (Fig. 5). The total adenine nucleotide pool decreased only slightly. The energy charge dropped to about 0.5 and recovered somewhat during the first 2 h of starvation and then decreased slowly in a manner similar to that observed in an earlier experiment (see Fig. 1). After 7.5 h of starvation, the addition of glucose caused the nucleotide pool to drop initially (probably because of resumption of nucleic acid synthesis) and then to recover to the normal level as growth resumed. At the same time, the energy charge rose rapidly to the normal value in growing cells. There was no loss in the viability of the cells throughout the starvation period.

Early log phase cells growing aerobically on glucose were centrifuged and then suspended in the same volume of complete medium. This treatment did not affect the energy charge of the cells. Also, there was no release of cellular nucleotides into the medium and only a small temporary decrease in the intracellular adenine nucleotide pool. This contrasts with the results obtained with *E. coli* by Cole et al. (10) and Chapman et al. (8), who found the ATP level to be strongly affected by centrifugation or Millipore filtration.

When cells were centrifuged and suspended in medium without glucose, the energy charge

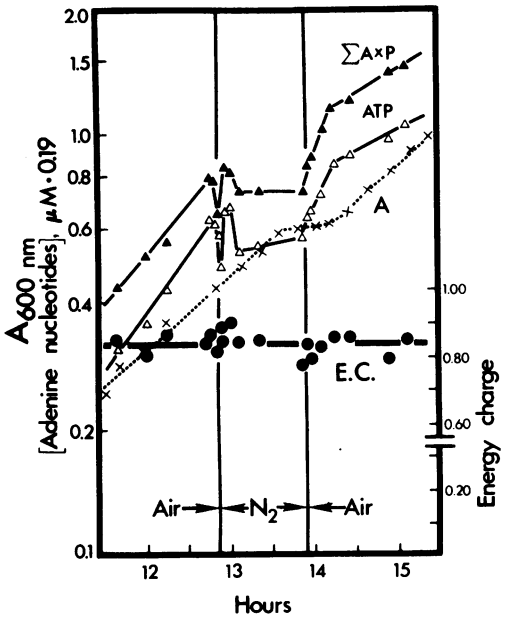

FIG. 4. *Effect of temporary anaerobiosis on the energy charge in S. cerevisiae growing aerobically on glucose. The culture was made anaerobic for 75 min during early exponential phase, and then aeration was resumed. Identifications of curves are as in Figs. 1 and 2.*

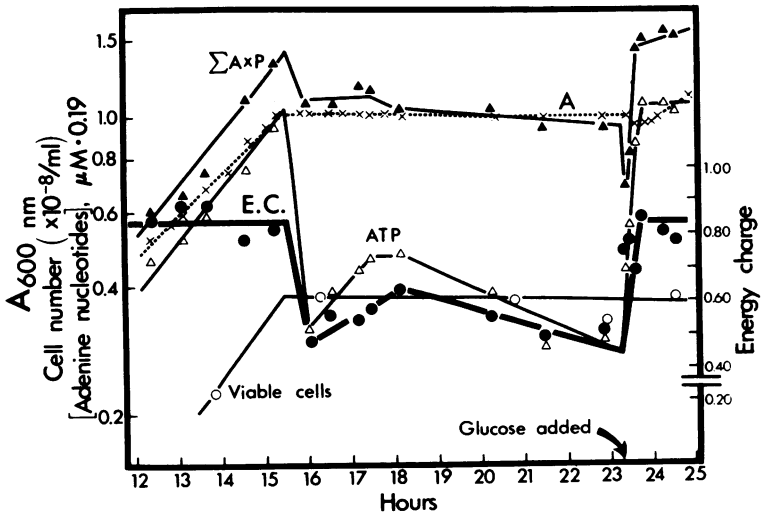

FIG. 5. *Changes in the energy charge in S. cerevisiae grown anaerobically on limiting glucose and resupplied with glucose after starvation. Cells were grown anaerobically in medium containing 55 mM glucose. The glucose concentration was restored to 55 mM 7.5 h after the cessation of growth. Identifications of curves are as in Figs. 1 and 2.*

dropped from 0.86 to 0.66 and stabilized at this level. Again there was no change in the extracellular adenine nucleotide level either during centrifugation or during the 1-h starvation period.

When log phase cells were centrifuged and suspended in a starvation salts medium and aerated, the suspended cells showed a steady decrease in the energy charge value, which dropped to 0.39 after 24 h of starvation (Fig. 6). Throughout the starvation period there was no drop in cell viability.

Aerobic stationary-phase cells responded differently to starvation. When cells from the stationary phase of an aerobic culture (36 h old) were centrifuged and suspended in medium lacking an energy source, the energy charge was unaffected (Fig. 7). The energy charge value of cells resuspended in a minimal salts buffer without NH_4^+ was also unaffected by either centrifugation or starvation, and little change occurred until after 36 h of starvation. Cell viability remained high for at least 140 h. These results demonstrate a remarkable ability of aerobic stationary-phase yeast cells to maintain high energy charge values in spite of various changes in their environment.

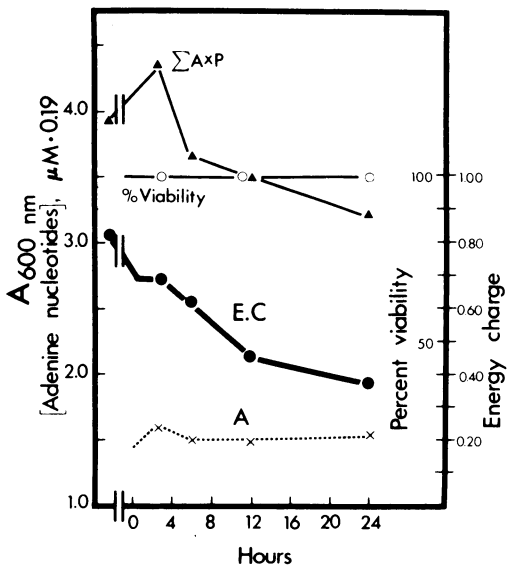

FIG. 6. *Decline in energy charge in S. cerevisiae cells after transfer to starvation medium. Mid-exponential phase cells growing aerobically in medium containing 0.11 M glucose were centrifuged and resuspended in the starvation salts medium and incubated aerobically for 24 h. The pH of the culture remained at 6.0 throughout the experiment. Identifications of curves are as in Figs. 1 and 2; % Viability, colony-forming cells as percent of the values at time zero.*

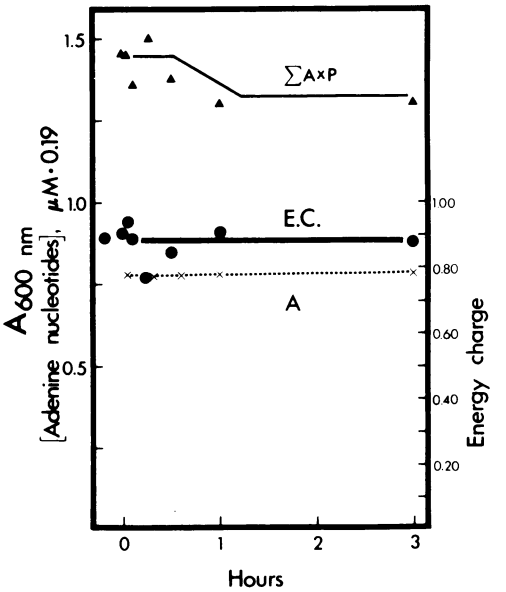

FIG. 7. *Stability of energy charge in stationary phase S. cerevisiae cells centrifuged and resuspended in starvation salts medium. A 50-ml sample of stationary phase cells grown aerobically in medium containing 55 mM glucose was centrifuged. The cells were then suspended in 250 ml of the starvation salts medium and incubated with aeration. Identifications of curves are as in Figs. 1 and 2.*

DISCUSSION

Kopperschläger et al. (14) reported determinations of the adenine nucleotides in growing yeast cells under aerobic and anaerobic conditions. The energy charge values derived from their data, 0.85 for cells growing aerobically and 0.81 for cells growing anaerobically, are almost identical with our values. Somlo (23), using a wild strain of yeast, reported an energy charge of 0.80 for cells growing on glucose. A high energy charge level appears to be associated with growth under a variety of conditions, and presumably is necessary for cell growth. Other values reported for the concentrations of adenine nucleotides in yeast are in general agreement with this conclusion (3-6, 16), except for two papers, from the same laboratory, from which very low energy charge values may be calculated (9, 19).

The finding that the energy charge in yeast cells can drop to as low as 0.15 with no loss of viability may be contrasted with observations on starving *E. coli* cells. In that organism, cell viability is severely impaired when the energy charge drops below about 0.5 (8). With yeast cells, however, cell viability is maintained over a wide range of energy charge values, and when

adequate growth conditions are restored the charge rises rapidly to about 0.8. The lowest values observed here are well below those previously seen in viable vegetative cells, and approach those reported by Setlow and Kornberg (21) for spores of *Bacillus megaterium*, *B. cereus*, and *B. subtilis*.

Somlo (23) compared energy charge values in a normal wild strain of *S. cerevisiae* with those in strain op_1. This mutant strain cannot grow on oxidizable, nonfermentable carbon sources even though it has normal amounts of cytochromes and is capable of normal respiration. The two yeast strains differed in several properties. Stationary phase normal cells, starved for 2 to 6 h, showed no drop in ATP level, and the energy charge remained at about 0.8. In contrast, there was substantial decrease in the ATP level in the mutant cells, and the energy charge fell to about 0.4. When ethanol was added to the normal cells, the energy charge rose to 0.86, the value obtained for cells growing on ethanol. In the mutant, within 2 min the energy charge stabilized at 0.67, a level apparently too low to maintain cell growth. The total ATP levels in the two yeasts were almost identical, but an unusually large amount of AMP was present in the mutant cells. This high level of AMP seems to be the cause of the inability of the mutant strain to grow on ethanol. This conclusion is consistent with the generalization that the ratios of concentrations of the adenine nucleotides are more significant than the absolute concentrations over a considerable range.

Comparison of the ability of cells from different cultures to maintain a high value of energy charge during starvation presents an interesting pattern. Cells grown anaerobically, and cells growing aerobically on glucose during the initial period of log phase growth, are unable to maintain an adenylate charge value above 0.7 when starved for an energy source. In contrast, cells grown on ethanol and cells grown aerobically on glucose but allowed to pass through the normal adaptation to use of ethanol after depletion of glucose maintain a high energy charge very effectively. Cells of the latter type presumably differ from those of the former type in the possession of functional mitochondria and the derepression of tricarboxylic acid cycle, electron transport, oxidative phosphorylation, and glyoxylate pathway enzymes. Thus, it seems likely that some or all of these systems are required for maintenance of high energy charge during starvation in *S. cerevisiae*.

ACKNOWLEDGMENTS

This research was supported by U. S. Public Health Service grant AM09863 from the National Institute of Arthritis, Metabolism, and Digestive Diseases, and by grant GB28458X from the National Science Foundation. W. J. Ball was a predoctoral trainee supported by Biochemistry Research Training grant GM0463 from the National Institute of General Medical Sciences.

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