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Intracellular Accumulation of AMP as a Cause for the Decline in Rate of Ethanol Production by *Saccharomyces cerevisiae* during Batch Fermentation†

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A general hypothesis is presented for the decline in the rate of ethanol production (per unit of cell protein) during batch fermentation. Inhibition of ethanol production is proposed to result from the intracellular accumulation of AMP during the transition from growth to the stationary phase. AMP acts as a competitive inhibitor of hexokinase with respect to ATP. When assayed in vitro in the presence of ATP and AMP concentrations equivalent to those within cells at different stages of fermentation, hexokinase activity declined in parallel with the in vivo decline in the rate of ethanol production. The coupling of glycolytic flux and fermentation to cell growth via degradation products of RNA may be of evolutionary advantage for *Saccharomyces cerevisiae*. Such a coupling would reduce the exposure of nongrowing cells to potentially harmful concentrations of waste products from metabolism and would conserve nutrients for future growth under more favorable conditions.

The commercial production of ethanolic beverages by *Saccharomyces cerevisiae* is one of the oldest and most economically successful applications of biotechnology. Concerns over heavy metal pollution and the availability of petroleum fuels in recent years have led to an expansion of this general process for the production of ethanol as a liquid fuel and as a nontoxic octane enhancer. Wider application of this biological conversion process has fueled extensive research into the nature of the physiological constraints which limit the rate and extent of ethanol production (8, 22, 26, 31, 39).

In batch fermentations, the high initial rates of glycolysis and ethanol production (per unit of cell protein) decline progressively as ethanol accumulates in the surrounding broth (18, 31, 39). These rate declines begin at ethanol concentrations of 1% (wt/vol), and 50% of the metabolic activity is lost after the accumulation of 5% (wt/vol) ethanol (18). Removal of ethanol from these cells by washing and suspending the cells in fresh medium does not restore fermentative activity (17). Growth of cells for five generations in the presence of 5% (wt/vol) ethanol (added initially) causes only a small decline in glycolytic flux and ethanol production (17). Although added ethanol causes a dose-dependent inhibition of glycolytic flux, the potency of this added ethanol as an inhibitor of fermentation is considerably less than that which is indicated by the observed decline in fermentative activity as ethanol accumulates in batch culture (18, 31, 39). Nutrient limitations for unsaturated membrane lipids (1, 8) or magnesium (16) further accelerate the loss of fermentative activity during batch fermentation. These results have led to the hypothesis that factors other than ethanol concentration also contribute to the decline in metabolic activity during the batch fermentation of sugars to ethanol (18).

The onset of the decline in fermentative activity coincides with the transition of yeast growth from the exponential to the stationary phase (18). Numerous physiological changes occur in cells during this time as the cellular machinery is converted from an active biosynthetic mode to that for survival and cell maintenance (6). In attempting to delineate which of these changes may cause fermentative activity to decline, we considered the essential requirements for fermentation. These include functional glycolytic enzymes and ethanologenic enzymes, appropriate concentrations of cofactors and coenzymes, the maintenance of internal pH near neutrality (despite acidification of the surrounding broth), a functional sugar uptake system, an ADP regeneration system, and a semipermeable membrane which allows the maintenance of intracellular metabolites and the escape of fermentation products.

Results of previous studies (16, 18) (under conditions of nutrient sufficiency) demonstrated that the decline in metabolic activity during batch fermentations with *S. cerevisiae* does not result from a decline in the levels of functional glycolytic or alcohologenic enzymes, nor from a decline in intracellular magnesium ions, which are important cofactors for many of these enzymes. The internal pH of cells remains high throughout fermentation, and cells retain the ability to exclude methylene blue, indicating that membrane integrity is preserved (18). In this study, we investigated the intracellular levels of glycolytic intermediates, glucose uptake, and the availability of appropriate levels of nucleotides during the conversion of 20% (wt/vol) glucose to 10% (wt/vol) ethanol in a batch fermentation.

MATERIALS AND METHODS

Organism and growth conditions. *S. cerevisiae* KD2 (petite strain) was used in this study (15). Fermentation broth contained the following, per liter: 5 g of yeast extract, 10 g of peptone, 200 g of glucose, and 0.5 mM magnesium sulfate (18). Batch fermentations were carried out in spinner bottles at 30°C with a 1% inoculum, by volume (initial optical density at 550 nm of 0.035; 0.01 mg of cell protein per ml), as described previously (18).

Analyses of fermentation broth. Cell protein was determined by the method described by Lowry et al. (28).

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Glucose, ethanol, and cell mass were measured as described previously (18).

Respirometry measurements. The rate of fermentation was estimated by measuring carbon dioxide evolution with a differential respirometer (Gilson, Middleton, Wis.). Results are expressed as micromoles of carbon dioxide evolved per hour per milligram of cell protein (18). Since each mole of glucose is converted to 2 mol of ethanol and 2 mol of carbon dioxide with 95% efficiency, this rate of fermentative activity can be regarded as being equal to that for ethanol production and approximately equal to twice the rate of glycolytic flux.

Analysis of glycolytic intermediates. Fermentations were carried out in medium supplemented with 20 μCi of $^{32}P_i$ per ml. Samples (0.5 ml) were removed at 6-h intervals and inactivated by pipetting them directly into an equal volume of precooled (4°C), 82% (vol/vol) ethanol containing 7.7 mM EDTA, as described by Holmsen et al. (21). The ethanol-EDTA extraction solution was freshly prepared before each use to avoid the precipitation of EDTA which occurs during storage. Samples were mixed vigorously for 10 s and extracted for 30 min at 4°C with periodic agitation. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min at 4°C, and the supernatant was stored at −70°C prior to analysis. Samples were removed from a parallel batch fermentation that lacked radioactive phosphate and were analyzed for cell protein, glucose, and ethanol.

Extracts were analyzed by two-dimensional thin-layer chromatography with the solvent systems described by Holmsen et al. (21). Portions of extract containing a total of 70,000 cpm were spotted onto plates (20 by 20 cm; Analtech Cellulose MN300; Fisher Scientific Co., Orlando, Fla.). Spots were visualized by autoradiography and marked. Combined regions of triose phosphates (without phosphoenolpyruvate) and hexose phosphates were scraped into scintillation vials. Radioactivity was measured with a scintillation counter. Values were corrected for the presence of phosphorylated intermediates in the broth. These were converted to counts per minute per milligram of cell protein to correct for the different cell densities during batch fermentation and are plotted as a percentage of the value at 12 h, the time at which the highest rate of fermentation occurs (18).

Nucleotides. Samples were removed from batch fermentations and inactivated as described above for the glycolytic intermediates. Adenine nucleotides were analyzed as described by Chapman et al. (10). Nicotinamide nucleotides were analyzed as described by Karp et al. (24). Extracts were stored at −70°C, thawed once, and assayed without further storage. Values were corrected for the nucleotides that were present in the broth. Assay kits for adenine and nicotinamide nucleotides were obtained from LKB Instruments, Inc. (Gaithersburg, Md.). Luminescence was measured by sequential counting at 3-s intervals with a scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.) with the coincidence switched off. Standards were prepared in the same ethanolic buffers that were used for extraction. Cell volumes were determined as described previously (15) and were used to calculate the intracellular concentrations of nucleotides.

Glucose transport. The uptake of 2-deoxy-D-glucose was measured in cells from different stages of fermentation by using a modification of the method described by Bisson and co-workers (4, 5). Two variations of this method were used. In the first, cells were harvested by centrifugation in a centrifuge (7,000 × *g*, 5 min, 4°C; Ivan Sorvall, Inc., Norwalk, Conn.), washed in an equal volume of fresh medium lacking glucose, suspended in cold medium lacking glucose to a final concentration of 20 to 30 mg of cell protein per ml, and held on ice until they were used. In the second method, 1.7-ml samples of cells were harvested from batch fermentations by centrifugation in a microfuge (10,000 × *g*, room temperature), washed once in fresh medium lacking glucose (room temperature), suspended in 170 μl of medium lacking glucose, and used immediately for the assay of glucose uptake without storage. Cells and the 2-deoxy-D-glucose solution (0.1 μCi, 20 μmol in 100 μl of medium lacking glucose) were equilibrated at 30°C for 2 min, and the assay was begun by adding 100 μl of cell suspension to the sugar solution. Uptake was stopped by adding 10 ml of cold distilled water (4°C). Cells were harvested and washed, and the radioactivity was measured as described previously (4). Uptake was measured after 5, 10, and 15 s. At time zero, the control was measured by adding cells directly to cold water containing labeled 2-deoxy-D-glucose. Uptake was estimated graphically and is expressed as micromoles of sugar per minute per milligram of cell protein.

Assay of glucose-phosphorylating enzymes. Glucose-phosphorylating activity was measured in disrupted cell preparations as described previously (18, 29). Assays were conducted with a constant level of ATP (1 mM) and different concentrations of AMP by using crude enzyme preparations from cells that were removed after 12 h from batch fermentations. Activities were also measured in enzyme preparations from cells removed from batch fermentations after 12 and 36 h under conditions in which both the ATP and AMP levels were varied to correspond with the measured values at different times during the batch fermentation.

Materials. Yeast extract, peptone, and agar were obtained from Difco Laboratories (Detroit, Mich.). Nonradioactive biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). Absolute ethanol was supplied by AAPER Alcohol and Chemical Co. (Shelbyville, Ky.). All radioactive compounds were purchased from New England Nuclear Corp. (Boston, Mass.), except for $^{32}P_i$, which was obtained from Amersham Corp. (Arlington Heights, Ill.).

RESULTS

Glycolytic intermediates. The effectiveness of the ethanol-EDTA extraction method (21) was compared with that of the perchloric acid method (10) by using parallel samples. By the ethanol-EDTA procedure, 60% more radioactivity was extracted from cells than by the perchloric acid method. This higher level of extracted products with ethanol-EDTA may reflect the partial solubilization of lipidic compounds, in addition to nucleotides and intermediates. The ethanol-EDTA extraction procedure was used for subsequent experiments with *S. cerevisiae*.

The identities of the spots on the chromatogram (Fig. 1) were established in three ways. (i) A comparison with the published chromatograms of Holmsen et al. (21) was used to make a preliminary identification. (ii) Authentic standards of glycolytic intermediates and nucleotides were chromatographed under the same conditions that were used for cell extracts and visualized with sulfosalicylic acid (38). (iii) Enzymatic digestions were performed on cellular extracts (dried to remove ethanol). These were treated with pyruvate kinase or with fructose-1,6-diphosphate aldolase under the conditions described by Clifton et al. (12) and were chromatographed in parallel with undigested samples. Aldolase caused the disappearance of the spot labeled glucose-1,6-bisphosphate and the appearance of triose phosphates. Pyruvate kinase caused an increase in nucleotide phosphate

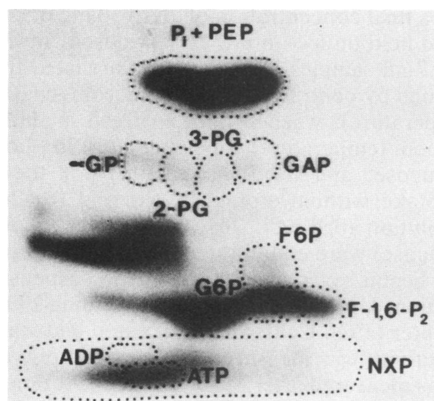


FIG. 1. Autoradiogram of ^{32}P-labeled phosphorylated compounds in ethanolic extracts of *S. cerevisiae* from batch fermentation after 24 h. Compounds were separated by two-dimensional thin-layer chromatography. The chromatogram was developed from bottom to top as the first dimension and from right to left as the second dimension. Abbreviations: P_i + PEP, P_i plus phosphoenolpyruvate; GP, glycerolphosphate; 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; GAP, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F-1,6-P_2, fructose-1,6,-bisphosphate; NXP, region in which most of the cellular nucleotides, including ATP and ADP, migrated.

(ATP). Phosphoenolpyruvate was poorly resolved from P_i and is not included in estimates of triose phosphate or in the total level of intermediates reported.

The changes in the levels of phosphorylated intermediates of glycolysis on a per milligram of cell protein basis during batch fermentation are summarized in Fig. 2. These results are plotted relative to the value for cells removed from batch fermentations after 12 h, the time at which fermentation was most active. Plots of fermentation rates per milligram of cell protein and the concentrations of accumulated ethanol in the broth are included in Fig. 2A for comparison. The level of total glycolytic intermediates (phosphoenolpyruvate not included) decreased by 50% between 12 and 18 h and then gradually increased to that present in cells at 12 h. This initial decline was more pronounced in the hexose fraction but also occurred in the triose phosphate fraction (phosphoenolpyruvate not included) (Fig. 2B). Triose phosphates accumulated between 18 and 36 h, while hexose phosphates remained at approximately half the level in cells at 12 h. The triose

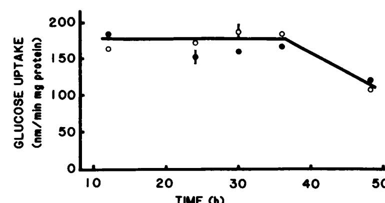


FIG. 3. Uptake of 2-deoxy-D-glucose by cells from various stages of fermentation. Experiments were conducted in two ways. Uptake was measured immediately following rapid washing at room temperature (●). Cells were harvested, washed, and held at 4°C before they were assayed (○).

phosphate levels declined, and there was a corresponding increase in the hexose phosphate levels during the final 12 h of fermentation. None of the pools of intermediates exhibited more than a twofold fluctuation in intracellular concentration during the initial 42 h of fermentation. Glycolytic flux, which was measured as fermentative activity per milligram of cell protein, progressively declined during the course of fermentation (Fig. 2A). The observed decline in glycolytic flux in the absence of a large increase in glycolytic intermediates indicates that none of the enzymes which convert glucose-6-phosphate to ethanol cause a major restriction in the flow of intermediates.

Glucose uptake during batch fermentation. The ability of cells to take up 2-deoxy-D-glucose was used as a measure of glucose transport in cells from different stages of fermentation (Fig. 3). Although glycolytic flux declined during the course of fermentation, the capacity of cells to transport 2-deoxy-D-glucose remained constant for the first 36 h, with only a 30% decline after 48 h. Thus, the capacity of cells for glucose uptake, as measured by this method, does not appear to be responsible for the progressive decline in glycolytic flux during batch fermentation.

Intracellular concentrations of NAD^+ and NADH during batch fermentation. The intracellular concentration of NADH remained low (approximately 0.15 mM) during batch fermentation. The intracellular concentration of NAD^+ varied in a cyclic fashion between 1 and 2 mM during the first 42 h of batch fermentation, followed by a large increase to over 5 mM at the end of fermentation (Fig. 4). The ratio of NAD^+/NADH remained high throughout the fermentation, a condition which should promote glycolytic flux (19).

Intracellular concentrations of adenine nucleotides during

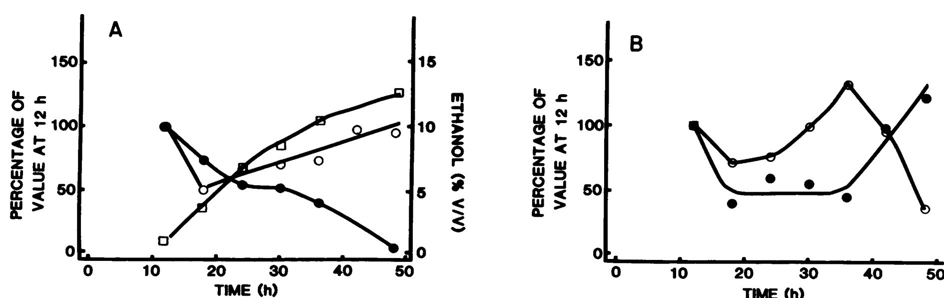


FIG. 2. Changes in ^{32}P-labeled cellular metabolites during batch fermentation. Regions corresponding to triose phosphates without phosphoenolpyruvate (Fig. 1) and hexose phosphates (Fig. 1) were scraped and counted. Values for intermediates were calculated per milligram of cell protein and are expressed as a percentage of that present after 12 h, the period during which fermentation was most active. For comparison, the levels of ethanol present in the broth and the fermentative activities of the cells were also plotted. (A) Symbols: ○, total intermediates (without phosphoenolpyruvate); ●, fermentative activity of cells at various times during batch fermentation; □, ethanol concentration in the broth at various times during batch fermentation. (B) Symbols: ●, total hexose phosphates; ○, total triose phosphates (without phosphoenolpyruvate).

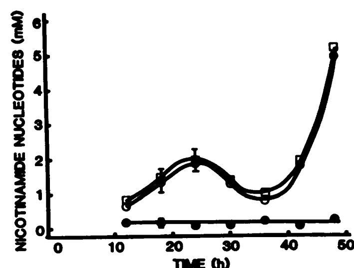


FIG. 4. Intracellular concentrations of nicotinamide nucleotides during batch fermentation. Symbols: ●, NADH; ○, NAD^+; □, total nicotinamide nucleotides.

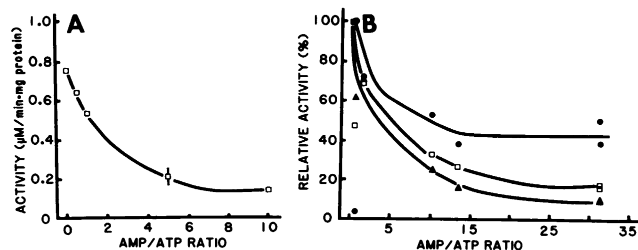


FIG. 6. Effects of AMP on glucose phosphorylation by crude enzyme extracts prepared from cells removed from batch fermentations after 12 and 36 h. Results are plotted as activity with 1 mM ATP lacking AMP (A) or as a percentage of the activity observed with the nucleotide levels in cells during batch fermentation after 12 h (B). Phosphorylating activities are plotted as a function of the AMP/ATP ratio. For comparison, the rate of glycolytic flux is plotted as a percentage of the maximum rate (cells removed from batch fermentations at 12 h) versus intracellular AMP/ATP ratios at various stages of fermentation. (A) Effects of increasing amounts of AMP on glucose phosphorylation by extracts from cells removed from batch fermentations at 12 h with 1 mM ATP (□). (B) Effects of AMP/ATP ratios which were measured in cells at different times during fermentation on glucose phosphorylation. (B) Symbols: ▲, enzyme preparation from cells removed from batch fermentations at 12 h; □, enzyme preparation from cells removed from batch fermentations at 36 h; ●, relative rates of fermentation in vivo.

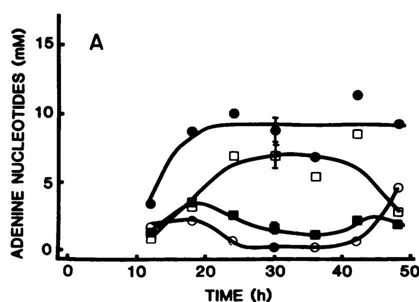
batch fermentation. Total intracellular adenine nucleotides increased between 12 and 24 h and remained constant (approximately 10 mM) during the remainder of the batch fermentation (Fig. 5A). Intracellular concentrations of ADP increased and then fell slowly, reaching a minimum of 1.1 mM after 36 h. This was followed by a small increase at the end of the fermentation. Intracellular concentrations of ATP increased between 12 and 18 h of 1.7 mM and then fell, reaching a minimum of 0.2 mM after 36 h. Intracellular ATP increased sharply between 42 and 48 h and reached a final concentration of 4 mM. AMP levels rose sharply between 12 and 30 h, reaching an intracellular concentration of approximately 7 mM. AMP increased most dramatically during the time at which the initial decline in fermentative activity was observed. At the end of fermentation, the intracellular concentration of AMP declined coordinately with the rise in ATP.

The adenylate energy charge fell rapidly between 12 and 30 h, remained low, and rose to near the initial level after 48 h (Fig. 5B). The changes in energy charge were primarily due to the initial increase in total nucleotides, most of which appeared as AMP. The changes in energy charge were primarily caused by the changes in the AMP/ATP ratio rather than a variation in the levels of ADP. The fall in energy charge was mirrored by the increase in the AMP/ATP ratio (Fig. 5B).

Effects of AMP and AMP/ATP ratio on glucose-phosphorylating activity. Although it is generally regarded that a low energy charge favors glycolytic flux (9, 19), high levels of AMP and high AMP/ATP ratios similar to those observed during fermentation (Fig. 5) have been reported to inhibit glucose phosphorylation by hexokinase in *S. cerevisiae* (20, 34). Inhibition by AMP of total glucose-phosphorylating activity in crude enzyme preparations from cells removed from batch fermentations at 12 h was readily demonstrated (Fig. 6A) by increasing the AMP levels from 0 to 10 mM in the presence of a constant amount of ATP (1 mM). Under these conditions, enzyme activity was progressively inhibited, with only 15% of the original activity remaining in the presence of 10 mM AMP.

Glucose-phosphorylating activities in crude enzyme preparations from cells removed from batch fermentations at 12 and 36 h were measured in the presence of AMP and ATP concentrations which corresponded to those observed at different times during the batch fermentation. These activities were computed as a percentage of the maximal activity and are plotted as a function of the AMP/ATP ratio (Fig. 6B). The activity was highest with the nucleotide levels that corresponded to these that were present in cells removed from batch fermentations at 12 h, the point at which the in vivo rate of fermentation was highest. The results for cells removed from batch fermentations at 12 and 36 h were the same, within experimental error, and indicated a strong inhibition of glucose-phosphorylating activity by increasing AMP/ATP ratios. Only the values for the cells assayed at the ATP and AMP concentration observed at the end of fermentation deviated from the trend defined by the other points.

Under conditions in which both ATP and AMP levels were varied (Fig. 6B) and under conditions in which AMP was



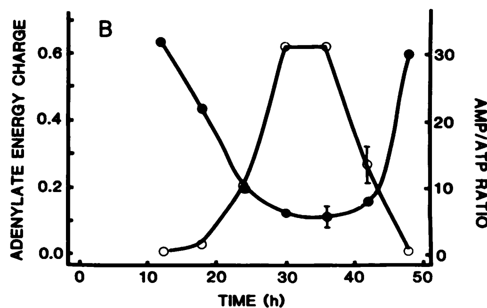


FIG. 5. Intracellular concentrations of adenine nucleotides. (A) Symbols: ○, ATP; ■, ADP; □, AMP; ●, total adenine nucleotides. (B) Symbols: ●, adenylate energy charge; ○, AMP/ATP ratio.

increased with ATP remaining constant (Fig. 6A), the extent of inhibition increased most rapidly at AMP/ATP ratios below 10 to 15. During batch fermentation, the rapid initial decline in fermentation was accompanied by a rapid initial increase in the intracellular AMP/ATP ratio of 0.4 to 30 between 12 and 30 h (Fig. 5B). Figure 6B includes a plot of the relative rate of glycolytic flux as a function of the AMP/ATP ratios. Although glycolytic flux in vivo was not as strongly inhibited as glucose phosphorylation in vitro, the shapes of the curves were quite similar, with the most rapid decline in glycolytic flux (fermentative activity) being observed at AMP/ATP ratios below 10 to 15.

DISCUSSION

The decline in glycolytic flux during batch fermentation has been widely investigated (15–18, 31, 39). Recent studies in our laboratory have focused on the cellular requirements for fermentation and the activities of individual enzymes which convert glucose to ethanol (18). Although the in vitro activities of these enzymes did not decline during batch fermentation (18), in vitro activities cannot be assumed to represent true in vivo catalytic rates. The extent to which these enzymes function in vivo can be inferred, however, from the pools of glycolytic intermediates. Previous researchers (13, 14) have used this approach to investigate the control of glycolysis in *S. cerevisiae* during shifts between anaerobic and aerobic growth conditions. These studies identified three probable sites for control: (i) hexose entry into the pathway (glucose uptake, initial phosphorylation, or both), (ii) phosphofructokinase, and (iii) pyruvate kinase.

Our measurements of phosphorylated triose and hexose pools at different times during batch fermentation revealed a lack of accumulation of high levels of glycolytic intermediates, despite a marked decline in the rate of glycolytic flux and ethanol production. Since it has been shown previously (11, 32) that mutational blocks in glycolytic and ethanologenic enzymes cause major shifts in metabolic pools with increases in phosphorylated hexose and triose pools of 2- to 14-fold, we conclude that the decline in glycolytic flux is not due to inactivity of the enzymes between glucose-6-phosphate and ethanol in vivo. Indeed, the initial decline in fermentative activity was accompanied by a decline in the pool size of glycolytic intermediates, which is indicative of glucose starvation. These results are consistent with the hypothesis that the decline in the rate of glycolysis in cells during batch fermentation is caused by a reduction in the rate of entry of glucose into the Embden-Meyerhof-Parnas pathway, i.e., glucose uptake, its initial phosphorylation, or both.

The glucose uptake capacity of cells from different stages of fermentation was measured by using the analog 2-deoxy-D-glucose. Cells retained full uptake capacity for the first 36 h, with a modest decrease in uptake capacity near the end of fermentation. Thus, the entry of glucose into the glycolytic pathway does not appear to be limited by glucose uptake, leaving the initial phosphorylation as the most likely control point for glycolytic flux during batch fermentation.

Further support for control at the level of glucose phosphorylation was provided by measurements of intracellular nucleotides and their effects on hexokinase activity in vitro. Although the allosteric effects of the high $NAD^+/NADH$ ratios and the low ATP/ADP ratios within cells during batch fermentation would be expected to promote glycolysis (19), inhibition of hexokinase by the high intracellular concentrations of AMP (up to 7 mM) appears to prevent the entry of glucose into this pathway. AMP is known to be a competitive inhibitor of hexokinase with respect to ATP (20, 34). The rise in AMP concentrations within cells and the increase in the AMP/ATP ratio during the transition from the exponential to the stationary phase correlated well with the decline of fermentative activity.

The addition of AMP was shown to inhibit glucose phosphorylation in disrupted cell preparations. This activity was measured in vitro in nucleotide concentrations that corresponded to those that are present within cells at different stages of fermentation. The highest rate of glucose phosphorylation was observed by using the concentrations of ATP and AMP present in cells removed from batch fermentations at 12 h, which is the most active stage of fermentation. A plot of phosphorylating activity in vitro versus the AMP/ATP ratio was very similar to the analogous plot of the fermentative activity in vivo versus the AMP/ATP ratio. Since the low-affinity (high-mass) transport system for glucose uptake in *S. cerevisiae* is coupled to phosphorylation in *S. cerevisiae* (5, 25, 35), the inhibition of glucose phosphorylation may also reduce transport efficiency. Such a change may not have been detected by our uptake experiments with 100 mM 2-deoxy-D-glucose.

The onset of the decline in fermentative activity coincided with a decline in adenylate energy charge. The decline in adenylate energy charge was caused by both a decline in ATP and an increase in AMP. This decline corresponded to the transition from growth to the stationary phase, with glucose levels remaining high. In previous studies (2, 33), a similar decline in energy charge and increase in AMP at the end of anaerobic growth in glucose-limited medium has been demonstrated. Thus, the changes in adenylate nucleotide levels appear to be related to the end of growth, but independent of glucose levels.

The early fall in adenylate energy charge and ATP concentrations in *S. cerevisiae* during batch fermentation was somewhat surprising since glucose remained in excess. Results of a previous study in our laboratory (18) demonstrated that intracellular pH is maintained near neutrality throughout fermentation, despite the low pH of the surrounding medium. The addition of ethanol has been shown to cause an increase in proton leakage across the membrane of *S. cerevisiae* (7, 23, 27), and it is likely that proton leakage is also increased by the accumulation of ethanol during batch fermentation. Thus, the decline in the ATP concentration may result from increased ATP consumption by the membrane-bound ATPase for the maintenance of intracellular pH.

It is interesting that yeast cells exhibit increased resistance to membrane disruption by ethanol (18) and to ethanol-induced leakage (23) during the later stages of fermentation. This adaptation appears to be due to changes in membrane composition (3, 37), and includes an increase in the proportion of longer-chain unsaturated fatty acids in phospholipids. The increase in acyl chain length and the accompanying increase in membrane thickness would be expected to decrease proton leakage (22) and may be responsible for the recovery of intracellular levels of ATP near the end of fermentation.

A general model for the decline in glycolytic flux during batch fermentation is given in Fig. 7. The onset of the decline in activity occurred at the end of growth and coincided with the accumulation of intracellular AMP and the decline in intracellular ATP. The resulting high AMP/ATP ratio inhibited the phosphorylation of glucose and its entry into the glycolytic pathway. The increase in AMP could result from

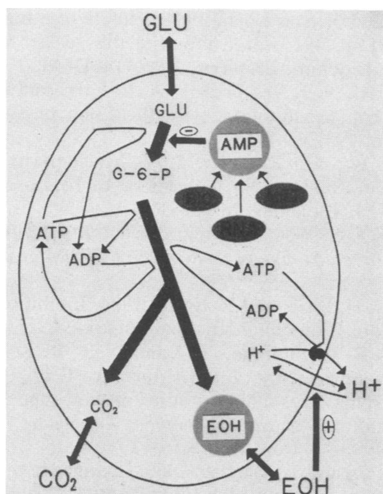


FIG. 7. Model for the role of AMP and ethanol in the decline in glycolytic flux during batch fermentation. The plus sign adjacent to the bold arrow connecting ethanol (EOH) and the transmembrane diffusion of H^+ denotes a stimulation with a net increase in the rate of entry due to the higher acidity of the extracellular environment. The minus sign adjacent to the bold arrow connecting AMP to the phosphoryation of glucose (GLU) indicates inhibition of this reaction by AMP. Abbreviations: GLU, Glucose; G-6-P, glucose-6-phosphate; BIO, de novo biosynthesis; MET, unspecified reactions in intermediary metabolism; EOH, ethanol.

continued biosynthesis; a variety of reactions in intermediary metabolism, including increased ATP consumption and the regeneration of ATP by adenylate kinse; or the degradation of RNA. RNA represents the largest intracellular pool of adenylate residues (9), and the degradation of both mRNA and rRNA is activated during the transition from growth to the stationary phase (6, 30, 36). The accumulation of ethanol is proposed to increase proton leakage as well, which increases the energy requirement for the maintenance of intracellular pH and reduces the intracellular concentration of ATP that is available for glucose phosphorylation. Thus, the increase in the AMP/ATP ratio within the cell is proposed as the primary factor which causes the progressive decline in ethanol production during batch fermentation.

This model is consistent with the observed changes in intracellular concentrations of AMP and changes in AMP/ATP ratios during batch fermentation and with the effects of these nucleotides on hexokinase activity in vitro. It is also consistent with the lack of intracellular accumulation of phosphorylated intermediates as fermentative activity declines and with the ineffectiveness of ethanol removal in restoring full fermentative activity. Control at the level of glucose entry into the Embden-Meyerhof-Parnas glycolytic pathway by inhibition of the initial phosphorylation reaction would serve to limit glycolytic flux, despite the presence of a functional glucose transport system and functional glycolytic enzymes, the availability of metal ions and cofactors, and the maintenance of an appropriate intracellular pH.

A reduction in both the rates of glycolysis and in the production of fermentation products as cells enter the stationary phase (18) may represent an evolutionary advantage for *S. cerevisiae*. The accumulation of fermentation products such as ethanol or organic acids would be expected to adversely affect long-term cell survival. A reduction in fermentative activity would both minimize cell exposure to these waste products and conserve nutrients for future use under more favorable conditions (provided that these are not consumed by competing organisms). Ribosome levels and mRNA are rapidly regulated in response to growth conditions and nutrient availability (6, 30, 36). The coupling of this control with that of glycolytic flux via intracellular products of RNA degradation provides a simple yet responsive mechanism to minimize the overproduction of fermentation products by *S. cerevisiae*. Analogous control systems may also occur in other microorganisms. Increases in intracellular AMP and excretion of excess AMP by *Escherichia coli* is well documented during the transition from growth to the stationary phase (10) and may represent a variation of this type of control.

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LITERATURE CITED

1. **Andreason, A. A., and T. J. B. Stier.** 1954. Anaerobic nutrition of *Saccharomyces cerevisiae*. II. Unsaturated fatty acid requirement for growth in defined medium. J. Cell. Comp. Physiol. **43**:271–281.
2. **Ball, W. J., Jr., and D. E. Atkinson.** 1975. Adenylate energy charge in *Saccharomyces cerevisiae* during starvation. J. Bacteriol. **121**:975–982.
3. **Beavan, M. J., C. Charpentier, and A. H. Rose.** 1982. Production and tolerance of ethanol in relation to phospholipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC 431. J. Gen. Microbiol. **128**:1447–1455.
4. **Bisson, L. F., and D. G. Fraenkel.** 1984. Expression of kinase-dependent glucose uptake in *Saccharomyces cerevisiae*. J. Bacteriol. **159**:1013–1017.
5. **Bisson, L. F., L. Neigeborn, M. Carlson, and D. G. Fraenkel.** 1987. The *SNF3* gene is required for high-affinity glucose transport in *Saccharomyces cerevisiae*. J. Bacteriol. **169**:1656–1662.
6. **Boucherie, H.** 1985. Protein synthesis during transition and stationary phases under glucose limitation in *Saccharomyces cerevisiae*. J. Bacteriol. **161**:385–392.
7. **Cartwright, C. P., J.-R. Juroszek, M. J. Beavan, F. M. S. Ruby, S. M. F. DeMorais, and A. H. Rose.** 1986. Ethanol dissipates the proton-motive force across the plasma membrane of *Saccharomyces cerevisiae*. J. Gen. Microbiol. **132**:369–377.
8. **Casey, G. P., and W. M. Ingledew.** 1986. Ethanol tolerance in yeasts. Crit. Rev. Microbiol. **13**:219–290.
9. **Chapman, A. G., and D. E. Atkinson.** 1977. Adenine nucleotide concentrations and turnover rates. Their correlation with biological activity in bacteria and yeast. Adv. Microb. Physiol. **15**:253–306.
10. **Chapman, A. G., L. Fall, and D. E. Atkinson.** 1971. Adenylate energy charge in *Escherichia coli* during growth and starvation. J. Bacteriol. **108**:1072–1086.
11. **Ciriacy, M., and I. Breitenbach.** 1979. Physiological effects of seven different blocks in glycolysis in *Saccharomyces cerevisiae*. J. Bacteriol. **139**:152–160.
12. **Clifton, D., S. B. Weinstock, and D. G. Fraenkel.** 1978. Glycolysis mutants in *Saccharomyces cerevisiae*. Genetics **88**:1–11.
13. **den Hollander, J. A., K. Ugurbil, T. R. Brown, M. Bednar, C. Redfield, and R. G. Shulman.** 1986. Studies on anaerobic and aerobic glycolysis in *Saccharomyces cerevisiae*. Biochemistry **25**:203–211.
14. **den Hollander, J. A., K. Ugurbil, and R. G. Shulman.** 1986. ^{31}P and ^{13}C NMR studies of intermediates of aerobic and anaerobic glycolysis in *Saccharomyces cerevisiae*. Biochemistry **25**:212–

219.
15. **Dombek, K. M., and L. O. Ingram.** 1985. Determination of intracellular concentration of ethanol in *Saccharomyces cerevisiae* during fermentation. Appl. Environ. Microbiol. **51:**197–200.
16. **Dombek, K. M., and L. O. Ingram.** 1986. Magnesium limitation and its role in the apparent toxicity of ethanol during yeast fermentation. Appl. Environ. Microbiol. **52:**975–981.
17. **Dombek, K. M., and L. O. Ingram.** 1986. Nutrient limitation as a basis for the apparent toxicity of low levels of ethanol during fermentation. J. Ind. Microbiol. **1:**219–225.
18. **Dombek, K. M., and L. O. Ingram.** 1987. Ethanol production during batch fermentation with *Saccharomyces cerevisiae*: changes in glycolytic enzymes and internal pH. Appl. Environ. Microbiol. **53:**1286–1291.
19. **Fraenkel, D. G.** 1982. Carbohydrate metabolism, p. 1–37. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Fromm, H. J.** 1969. Comments on the kinetics and mechanism of yeast hexokinase action. Is the binding sequence of substrates to the enzyme ordered or random? Eur. J. Biochem. **7:** 385–392.
21. **Holmsen, H., C. A. Dangelmaier, and J.-W. N. Akkerman.** 1983. Determination of levels of glycolytic intermediates and nucleotides in platelets by pulse-labelling with [^{32}P]orthophosphate. Anal. Biochem. **131:**266–272.
22. **Ingram, L. O., and T. M. Buttke.** 1984. Effects of ethanol on micro-organisms. Adv. Microb. Physiol. **25:**253–300.
23. **Jimenez, J., and T. Benitez.** 1987. Adaptation of yeast cell membranes to ethanol. Appl. Environ. Microbiol. **53:**1196–1198.
24. **Karp, M. T., R. P. Raunio, and T. N.-E. Lovgren.** 1983. Simultaneous extraction and combined bioluminescent assay of NAD^+ and NADH. Anal. Biochem. **128:**175–180.
25. **Lang, J. M., and V. P. Cirillo.** 1987. Glucose transport in a kinaseless *Saccharomyces cerevisiae* mutant. J. Bacteriol. **169:** 2932–2937.
26. **Leao, C., and N. van Uden.** 1982. Effects of ethanol and other alkanols on the glucose transport system of *Saccharomyces cerevisiae*. Biotechnol. Bioeng. **24:**2601–2604.
27. **Leao, C., and N. van Uden.** 1984. Effects of ethanol and other alkanols on passive proton influx in the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **774:**43–48.
28. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
29. **Maitra, P. K., and Z. Lobo.** 1977. Yeast pyruvate kinase: a mutant form catalytically insensitive to fructose 1,6-bisphosphate. Eur. J. Biochem. **78:**353–360.
30. **McFarlane, E. S.** 1980. Ribonuclease activity during G1 arrest of the yeast *Saccharomyces cerevisiae*. Arch. Microbiol. **124:**243–247.
31. **Moulin, G., H. Boze, and P. Galzy.** 1984. Inhibition of alcoholic fermentation. Biotechnol. Bioeng. **25:**365–382.
32. **Navon, G., R. G. Shulman, T. Yamane, T. R. Eccleshall, K.-B. Lam, J. J. Baronofsky, and J. Marmur.** 1979. Phosphorus-31 nuclear magnetic resonance studies of wild-type and glycolytic pathway mutants of *Saccharomyces cerevisiae*. Biochemistry **18:**4487–4499.
33. **Polakis, E. S., and W. Bartley.** 1966. Changes in the intracellular concentrations of adenosine phosphates and nicotinamide nucleotides during the aerobic growth cycle of yeast on different carbon sources. Biochem. J. **99:**521–533.
34. **Rudolph, F. B., and H. J. Fromm.** 1971. Computer simulation studies with yeast hexokinase and additional evidence for the random Bi Bi mechanism. J. Biol. Chem. **246:**6611–6619.
35. **Schuddemat, J., P. J. A. Van den Broek, and J. Van Steveninck.** 1986. Effect of xylose incubation on the glucose transport system in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **861:**489–493.
36. **Swida, U., B. Schulz-Harder, C. Kucherer, and N. Kaufer.** 1981. The occurrence of two ribosomal ribonucleases depending on growth phase in yeast. Induction of ribonuclease in glucose-starved cells. Biochim. Biophys. Acta **652:**129–138.
37. **Thomas, D. S., J. A. Hossak, and A. H. Rose.** 1978. Plasma membrane composition and ethanol tolerance in *Saccharomyces cerevisiae*. Arch. Microbiol. **117:**239–245.
38. **Thompson, J.** 1978. In vivo regulation of glycolysis and characterization of sugar: phosphotransferase systems in *Streptococcus lactis*. J. Bacteriol. **136:**465–476.
39. **van Uden, N.** 1985. Ethanol toxicity and ethanol tolerance in yeasts. Annu. Rep. Fermentat. Proc. **8:**11–58.