JOURNAL OF BACTERIOLOGY, Oct. 1988, p. 4808–4815
0021-9193/88/104808-08$02.00/0

Vol. 170, No. 10

# Changes in Activities of Several Enzymes Involved in Carbohydrate Metabolism during the Cell Cycle of *Saccharomyces cerevisiae*

J. VAN DOORN,[1] J. A. C. VALKENBURG,[2] M. E. SCHOLTE,[1] L. J. W. M. OEHLEN,[1] R. VAN DRIEL,[1] P. W. POSTMA,[1] N. NANNINGA,[2] AND K. VAN DAM[1]*

*Laboratory of Biochemistry and Biotechnology Centre[1] and Department of Electron Microscopy and Molecular Cytology,[2] University of Amsterdam, P. O. Box 20151, 1000 HD Amsterdam, The Netherlands*

Received 20 January 1988/Accepted 11 July 1988

**Activity changes of a number of enzymes involved in carbohydrate metabolism were determined in cell extracts of fractionated exponential-phase populations of *Saccharomyces cerevisiae* grown under excess glucose. Cell-size fractionation was achieved by an improved centrifugal elutriation procedure. Evidence that the yeast populations had been fractionated according to age in the cell cycle was obtained by examining the various cell fractions for their volume distribution and their microscopic appearance and by flow cytometric analysis of the distribution patterns of cellular DNA and protein contents. Trehalase, hexokinase, pyruvate kinase, phosphofructokinase 1, and fructose-1,6-diphosphatase showed changes in specific activities throughout the cell cycle, whereas the specific activities of alcohol dehydrogenase and glucose-6-phosphate dehydrogenase remained constant. The basal trehalase activity increased substantially (about 20-fold) with bud emergence and decreased again in binucleated cells. However, when the enzyme was activated by pretreatment of the cell extracts with cyclic AMP-dependent protein kinase, no significant fluctuations in activity were seen. These observations strongly favor posttranslational modification through phosphorylation-dephosphorylation as the mechanism underlying the periodic changes in trehalase activity during the cell cycle. As observed for trehalase, the specific activities of hexokinase and phosphofructokinase 1 rose from the beginning of bud formation onward, finally leading to more than eightfold higher values at the end of the S phase. Subsequently, the enzyme activities dropped markedly at later stages of the cycle. Pyruvate kinase activity was relatively low during the G1 phase and the S phase, but increased dramatically (more than 50-fold) during G2. In contrast to the three glycolytic enzymes investigated, the highest specific activity of the gluconeogenic enzyme fructose-1,6-diphosphatase 1 was found in fractions enriched in either unbudded cells with a single nucleus or binucleated cells. The observed changes in enzyme activities most likely underlie pronounced alterations in carbohydrate metabolism during the cell cycle.**

Biochemical conversions which occur in bioreactors are the combined contributions of cells of different ages, i.e., cells at different stages of the cell cycle. Knowledge of possible variations in enzyme activities during the cell cycle is necessary to understand fully the metabolic behavior of the entire microbial culture. This requires methods to obtain subpopulations at defined stages of the cell cycle.

In the past, the activity patterns of a variety of enzymes have been determined in synchronously dividing yeast cultures (10, 27, 28). However, as discussed by other investigators, many of these earlier results may have been flawed by technical artifacts, since the synchronization techniques that were used could cause serious perturbations in enzyme activity (6, 7, 10, 29). Creanor and Mitchison (7) developed a method of producing synchronous cultures of *Saccharomyces cerevisiae* by selection of small unbudded cells from an elutriator rotor, a method which appeared to reduce metabolic perturbations markedly. Creanor et al. (6) measured the activity changes of acid phosphatase and α-glucosidase (previously considered to be step enzymes) in these synchronous cultures. Neither acid phosphatase nor α-glucosidase showed periodic changes during the cell cycle. Similar results were obtained by us for invertase and glucose-6-phosphate dehydrogenase by using synchronous cultures of *S. cerevisiae* also prepared by centrifugal elutriation (35a). On the other hand, in these same synchronous cultures the trehalase activity showed a characteristic peak pattern in which the enzyme activity rapidly and transiently dropped around the period of cell division. Such changes in enzyme activity were absent in asynchronous (elutriated) control cultures (35a). Thus, the latter results suggest that genuine peak enzymes do exist, although they may be relatively rare.

An important disadvantage of selection synchrony by centrifugal elutriation, however, is the low yield of cells per fraction, which makes it difficult to measure the activities of a number of enzymes in the same culture and limits the possibilities for further metabolic studies related to the cell cycle (e.g., determination of metabolic fluxes). In contrast, cell-size fractionation of a yeast culture in the exponential phase (after blocking cell-cycle progress by, for example, chilling) in an elutriator rotor offers a much higher yield of cells with minimal metabolic perturbations.

In the present study, we used this technique, as modified from that originally described by Gordon and Elliott (13), to determine the specific activities of several enzymes involved in carbohydrate metabolism during the mitotic cycle. These determinations form part of our project on cell-cycle-dependent control of intermediary metabolism in *S. cerevisiae*. Separation of cells by centrifugal elutriation according to successive stages of the cell cycle was verified by examining the fractions for their size distribution and their microscopic

* Corresponding author.

appearance and by flow cytometric determinations of the distribution patterns of cellular DNA and protein contents. It was found that the specific activities of trehalase, hexokinase, phosphofructokinase 1, pyruvate kinase, and fructose-1,6-diphosphatase 1 varied considerably during the cell cycle, whereas those of alcohol dehydrogenase and glucose-6-phosphate dehydrogenase remained virtually unchanged.

## MATERIALS AND METHODS

**Yeast strain and growth conditions.** The yeast *S. cerevisiae* DL1 (α *leu2-3 leu2-112 his3-11 his3-15 ura3-251 ura3-372 ura3-328*) was used. The cells were grown in a liquid medium containing glucose (2%), yeast extract (Difco Laboratories, Detroit, Mich.) (1%), and Bacto-Peptone (Difco) (2%). The cultures were incubated at 28°C to a cell density of $1 \times 10^7$ to $2 \times 10^7$ cells per ml. Growth was retarded by chilling to 0°C, and the cells were centrifuged and washed once with distilled water at 4°C. The cells ($4 \times 10^9$ to $6 \times 10^9$) were suspended in 12 ml of ice-cold distilled water and lightly sonicated. Small portions of the cell suspension were taken and stored at −20°C until analysis (see below). The remainder was then loaded into the elutriator rotor.

**Fractionation by centrifugal elutriation.** A Beckman JE-6B elutriator rotor was used, driven in a centrifuge (J2-21) which had been modified for elutriation and equipped with a stroboscope assembly by the manufacturer (Beckman Instruments, Inc., Palo Alto, Calif.). The flow system was modified from that described by Figdor et al. (11). Instead of a peristaltic pump, in our system the counterflow was generated by air pressure on the water reservoir. The resulting liquid flow could be adjusted accurately by a valve which was interposed between the reservoir outlet tubing and a bubble trap (capacity, 20 ml), the latter being inserted in the inflow line of the elutriator rotor. The flow rate was measured with a manostat flow meter.

In this system, pulsations in the liquid flow are avoided, which allows a greater precision of the elutriation procedure. One outlet of the bubble chamber was connected with a three-way bypass valve and Silastic tubing. At a continuous counterflow, manipulation of the bypass valve allowed direct flow from the water reservoir into the spinning rotor or, alternatively, loading of the cell suspension into the rotor with a syringe.

Centrifugal elutriation was performed at a centrifuge well temperature of 4°C, while the water reservoir, bubble chamber, and collection flasks were jacketed in ice baths. Water flow through the rotor was started, and the elutriator rotor was brought to a speed of 3,750 rpm. Care was taken to remove all air bubbles from the system before the cells were loaded. The chilled and sonically treated cell suspension was placed in the loading syringe, and subsequently the flow rate was adjusted to 12 ml/min. The cells were loaded at this flow rate and then flushed with 150 ml of water. Next, 100-ml fractions of the effluent were collected. The first fraction was collected at 14 ml/min (in a previous study, this cell population was used to obtain synchronously growing cultures) (35a), and successive fractions were obtained with incremental increases in the flow rate of 1 ml/min. When the flow rate had reached 22 ml/min, subsequent fractions were collected with increments of 2 ml/min. At a flow rate of 40 ml/min, by far most (i.e., more than 99.5%) of the cells were removed from the rotor. Remaining in the rotor were predominantly large binucleated cells. The cells in each fraction were centrifuged at 0°C.

During centrifugal elutriation, 0.5-ml samples of the successive fractions were withdrawn to determine cell numbers and the relative changes in cell-size distribution (after fixation of the cells in 0.25% formaldehyde), using a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) equipped with a multichannel analyzer and a 30-μm aperture. Relative cell volume was expressed in terms of unit mean Coulter Counter volume of cells obtained at a flow rate of 14 ml/min. Another series of samples (1.5 ml) of the various elutriated fractions was prepared for microscopic evaluation by fluorescent staining of cell nuclei with 4′,6-diamidino-2-phenylindole (DAPI) (39). Cells from a third series of samples (1.5 ml) were centrifuged, suspended in 70% (vol/vol) ethanol, and stored at 4°C until analysis for DNA and protein distribution patterns by flow cytometry.

**Flow cytometry.** Cells were fixed in 70% ice-cold ethanol and stored at 4°C. Before staining, ethanol was removed by centrifugation of the cell suspension. For DNA measurements, cells were washed in 0.01 M Tris hydrochloride–0.01 M $MgCl_2$ (pH 7.4) and stained in 2-μg/ml bisbenzimide trihydrochloride (Hoechst 33342; Sigma Chemical Co., St. Louis, Mo.) in the same buffer for 2 h. For protein measurements, cells were suspended overnight in 1-mg/ml 5-dimethylamino-1-naphthalenesulfonylchloride (DANS; Serva, Heidelberg, Federal Republic of Germany) in 96% ethanol in saturated $NaHCO_3$. After the cells were washed with Tris buffer, measurements were done with a modified version of the Partec Pass II flow cytometer system based on a microscope (Partec AG, Bottmingen, Switzerland). A 100-W mercury lamp was used as the light source. Both fluorochromes were excited below 420 nm. Hoechst 33342 emission was measured above 435 nm, and DANS emission was measured above 515 nm. In our instrument, a VME processor board (CPU2VC; Force Computers, Inc., Los Gatos, Calif.) handles data acquisition. A Macintosh SD personal computer (Apple Computers, Inc., Cupertino, Calif.) is used for controlling the instrument and displaying histograms.

**Enzyme assays.** Samples taken from the original cell suspension used for centrifugal elutriation and the cell pellets of the various fractions were suspended in 5 mM sodium phosphate buffer (pH 7.0)–1 mM phenylmethylsulfonyl fluoride (PMSF)–20 mM NaF in a final volume of 100 to 200 μl. Glass beads (0.45 mm) were added (0.1 to 0.2 g), and the cells were disrupted by mixing on a Vortex mixer (The Vortex Manufacturing Co., Cleveland, Ohio) at 4°C. The lysates were then clarified by centrifugation for 10 min at 4°C in an Eppendorf microcentrifuge, and the cell extracts were divided into portions of 30 μl.

Total hexokinase activity in the various cell extracts was measured in an incubation mixture (1 ml) that had a pH of 7.6 and contained 50 mM triethanolamine, 1 mM PMSF, 1 mM ATP, 0.1 mM NADP, 10 mM fructose, 0.14 U of phosphoglucose isomerase, 0.55 U of glucose-6-phosphate dehydrogenase, and 10 mM $MgCl_2$.

Assays for phosphofructokinase 1 were performed in 1 ml of 100 mM Tris hydrochloride buffer (pH 7.2) which contained 1 mM PMSF, 5 mM $MgSO_4$, 5 mM $(NH_4)_2SO_4$, 0.6 mM ATP, 0.2 mM NADH, and 3 mM fructose-6-phosphate, with aldolase (14 U), triosephosphate isomerase (136 U), and glycerol-3-phosphate dehydrogenase (12 U) as auxiliary enzymes (17). Activation of phosphofructokinase was accomplished by addition of either 5′-AMP (1 mM) or fructose-2,6-diphosphate (20 μM), or both (3, 30).

The activity of pyruvate kinase was measured by the method described by Hess and Wieker (16). The reaction mixture (1 ml) consisted of the following reagents: 100 mM potassium phosphate buffer (pH 6.0), 1 mM PMSF, 30 mM $MgSO_4$, 10 mM ADP, 0.25 mM NADH, 5 mM phosphoenol-

pyruvate, 2 mM fructose-1,6-diphosphate, and 36 U of lactate dehydrogenase.

Glucose-6-phosphate dehydrogenase activity was determined at pH 7.6 in the presence of 86 mM triethanolamine, 7 mM $MgCl_2$, 0.1 mM NADP, and 1 mM glucose-6-phosphate in a final volume of 2.5 ml.

Alcohol dehydrogenase (total) was assayed by the method of Bergmeyer et al. (4).

For the enzyme assays described above, the appropriate NAD(H)- or NADP-linked reactions were measured fluorometrically at 25°C. In all cases, the control incubation mixtures lacked substrate or cell extract. The amounts of cell extracts used in the various assays were equivalent to 5 to 10 μg of protein. The specific enzyme activities are expressed as micromoles of substrate converted per minute per milligram of protein.

Activation of trehalase was performed as follows: Suitable dilutions (in 5 mM sodium phosphate buffer [pH 7.0], 1 mM PMSF, 50 mM NaF) of the cell extracts, corresponding to 50 to 100 μg of protein (final volume, 150 μl), were mixed with an equal volume of a mixture containing 1 mM PMSF, 4 mM ATP, 9 mM $MgSO_4$, 50 mM NaF, 5 mM theophylline, 50 μM cyclic AMP (cAMP), and 30 pU of cAMP-dependent protein kinase per ml (from rabbit muscle) in 5 mM sodium phosphate buffer (pH 7.0). The reaction mixtures were incubated at 30°C for 30 min and used immediately thereafter for determination of trehalase activity (see below). The degree of activation was maximal at that time, and prolonged incubation did not lead to significant increases in enzyme activity. Another series of samples from the cell extracts was treated as described above, except that cAMP and cAMP-dependent protein kinase were omitted from the incubation mixtures. These samples were used to assess basal trehalase activities. Subsequently, enzyme activities in the various incubation mixtures were determined by measuring the amount of glucose derived from the cleavage of trehalose. Standard enzyme assays (final volume, 1.0 ml) contained 1 mM PMSF, 50 mM NaF, 25 mM trehalose, 75 mM sodium phosphate buffer (pH 5.6), and a particular preparation of cell extract. The samples were incubated for 1 h (30°C), and the reaction was stopped by heating in a water bath at 100°C for 3 min. In all cases, parallel controls were run to correct for potential spontaneous hydrolysis of the substrate. The amount of glucose liberated was proportional to the time of incubation and was determined enzymatically with hexokinase and glucose-6-phosphate dehydrogenase (21). The amount of NADPH produced in this reaction was measured fluorometrically. Specific activities of trehalase are expressed as nanomoles of substrate hydrolyzed per minute per milligram of protein.

Fructose-1,6-diphosphatase 1 activity was assessed by determining the amount of fructose-6-phosphate released by hydrolysis of fructose-1,6-diphosphate. Reaction mixtures (final volume, 660 μl) containing 100 mM Tris hydrochloride buffer (pH 7.2), 1 mM PMSF, 1 mM EDTA, 10 mM $MgCl_2$, 100 mM KCl, 50 mM NaF, 1 mM fructose-1,6-diphosphate, and 200 to 300 μg of protein of cell extract were incubated for several hours at 30°C. The reaction was stopped by adding 1 ml of ice-cold 2 N $HClO_4$. Subsequently, the samples were adjusted to pH 7.0 by the addition of 320 μl of 5 M $K_2CO_3$. Precipitated $KClO_4$ was removed by centrifugation (10,000 × *g*, 10 min). The amount of fructose-6-phosphate in the supernatant was determined enzymatically by the method of Lang and Michal (22). For all samples investigated, parallel control incubations were performed to correct for any side reactions or autohydrolysis of the substrate. The reaction rate appeared to be constant during the incubation. Fructose-1,6-diphosphatase activity is expressed as nanomoles of substrate converted per hour per milligram of protein.

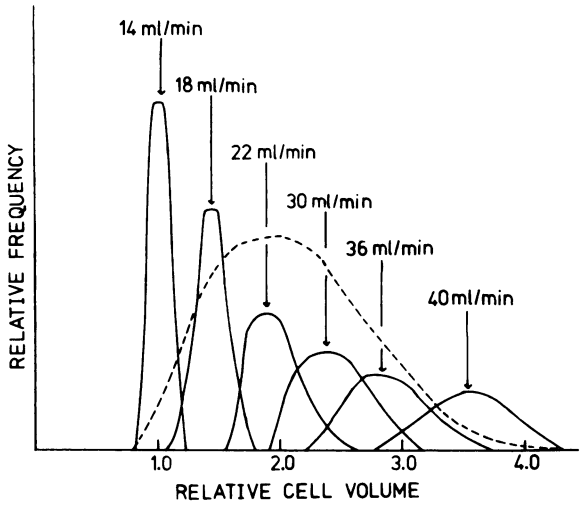


FIG. 1. Mode of frequency distributions of cell volumes in several fractions isolated from an asynchronous exponential-phase culture of *S. cerevisiae* DL1 by centrifugal elutriation. Relative cell volume is given arbitrarily in terms of unit mean cell volume of unbudded cells elutriated at 14 ml/min. For each fraction, 10,000 cells were counted with a Coulter Counter equipped with a multichannel analyzer and 30-μm aperture. The dotted curve represents the cell-size distribution (40,000 cells were counted) in the original cell suspension loaded into the elutriator rotor.

Protein contents of the cell extracts were determined by the method of Lowry et al. (25) with bovine serum albumin as a standard.

**Chemicals.** ATP (disodium salt), $NAD^+$ (free acid), NADH (disodium salt), NADP (disodium salt), and the auxiliary enzymes used in the various assays were purchased from Boehringer GmbH (Mannheim, Federal Republic of Germany). PMSF, cAMP-dependent protein kinase from rabbit muscle, trehalose from yeasts, theophylline, and DAPI were obtained from Sigma Chemical Co., and cAMP was from Serva. All other chemicals from commercial sources were of the highest purity available.

## RESULTS

**Cell separation by centrifugal elutriation.** The relative size distributions of cell populations in several successive fractions obtained from the elutriator rotor are illustrated in Fig. 1. It is evident that there was an increase in the Coulter Counter volume of cells elutriated with increasing flow rate. Over the range of flow rates used, 14 to 40 ml/min, the mean cell volume increased by a factor of 3.5. Evidence that the centrifugal elutriation procedure used effected a separation of cells according to different stages of the cell cycle was obtained from microscopic analysis of the fractions after fluorescent staining of the cellular DNA with DAPI. In these preparations, four types of cells could be discriminated, as shown schematically in the inset to Fig. 2. At lower flow rates (14 to 16 ml/min), the population consisted predominantly of unbudded (G1-phase) cells (Fig. 2). Budded cells, in which the amount of DNA had been doubled (see Fig. 3) and the nucleus had not yet migrated into the bud neck (i.e., representing the G2 phase), reached a peak in the fractions

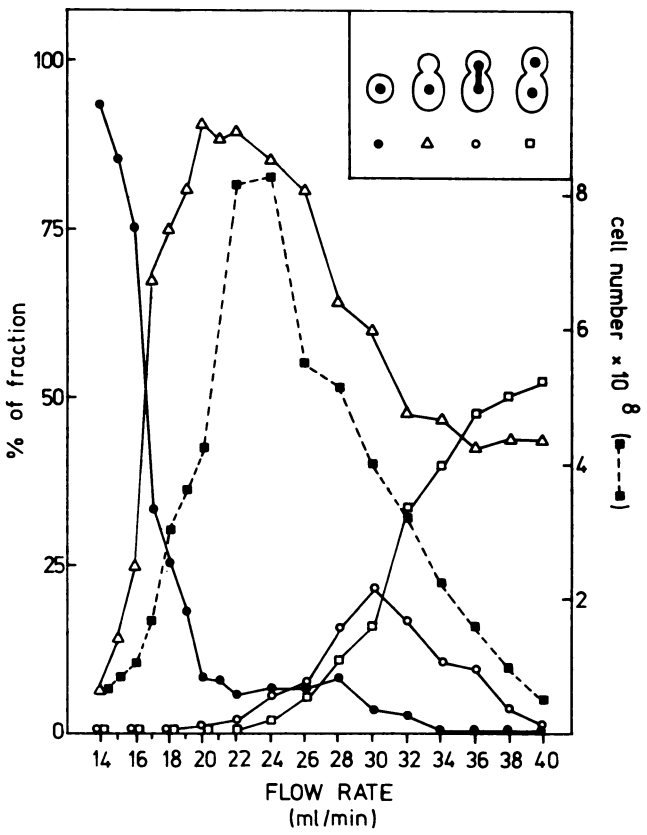


FIG. 2. Separation of cell markers by centrifugal elutriation. The inset shows four different cell types examined in fractions obtained at the various flow rates. Symbols: ●, unbudded cells; △, budded cells with a single nucleus; ○, cells with migrating nuclei; and □, binucleated cells. Cells were stained with DAPI, and the proportion of each cell type in a particular fraction was estimated microscopically. At least 100 cells were scored per fraction. Total cell numbers (---) in the various fractions were determined with a Coulter Counter.

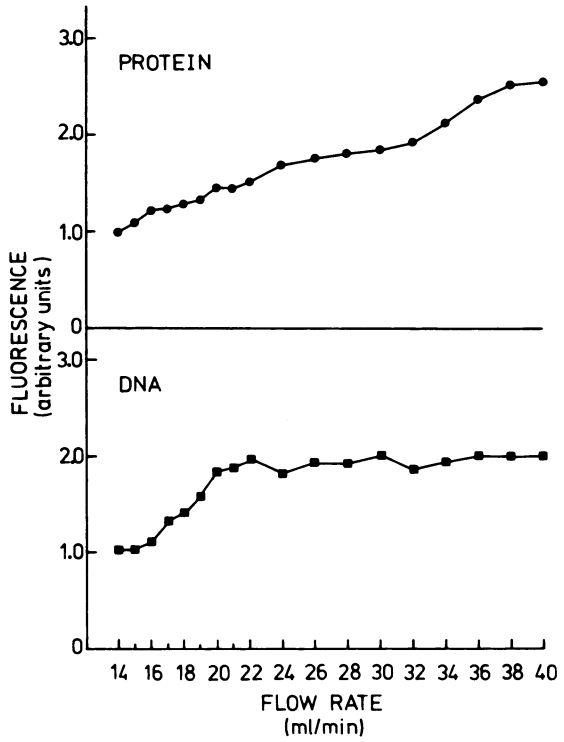


FIG. 3. Average protein and DNA contents of cells elutriated at counterflow rates between 14 and 40 ml/min as determined by flow cytometry after fluorescent labeling with DANS and Hoechst 33342, respectively. Both fluorochromes were excited below 420 nm. DANS emission was measured above 515 nm, and Hoechst 33342 emission was measured above 435 nm.

elutriated at 21 to 26 ml/min. Cells with migrating nuclei (M-phase cells) peaked in the fraction collected at 30 ml/min, whereas the fractions obtained at higher flow rates were enriched in binucleated cells. Under most conditions, in *S. cerevisiae* the G1 phase begins immediately following mitosis, before cytokinesis and abscission (2). For this reason, a substantial part of G1-phase cells may be present in binucleate (postmitotic) fractions, elutriated at the highest flow rates. The percentages of contribution of the various cell types to the total number of cells loaded into the rotor were the same as those determined for DAPI-stained preparations of the original suspension of exponential-phase cells. The average protein and DNA contents of the cells in the elutriated fractions, as measured by flow cytometry after fluorescent labeling with DANS and Hoechst 33342, respectively, are presented in Fig. 3. Like the mean cell volume, cellular protein levels increased with increasing flow rate. The protein content of cells elutriated at 40 ml/min was about 2.5 times higher than that of cells collected at 14 ml/min. As can be deduced from Fig. 3, DNA synthesis (S phase) occurred in cells elutriated from 16 ml/min onward. The cellular DNA content was doubled in fractions obtained at flow rates higher than 20 ml/min. The DNA distribution patterns of the various fractions (data not shown) were narrow (with a coefficient of variation around 10%) compared with those of asynchronous control cultures (25%).

**Patterns of enzyme activity during the cell cycle.** Figure 4 shows the trehalase activity in an age-fractionated yeast culture representing four independent experiments. The basal enzyme activity increased rapidly and substantially (i.e., about 20-fold) with the emergence of buds and appeared to decrease again in fractions enriched in binucleated cells. Treatment of the cell extracts with cAMP and cAMP-dependent protein kinase prior to trehalase assay resulted in a 1.3-fold enhancement of the enzyme activity in budded cells elutriated between 18 and 30 ml/min. In the other cell fractions, the trehalase activity was stimulated by a higher factor, being maximal (12- to 20-fold) in cells collected at both the lowest and highest flow rates used. As a result, the activated enzyme did not show large fluctuations in activity.

Typical results (from five experiments) for total hexokinase and pyruvate kinase are presented in Fig. 5. As observed for trehalase, the specific activity of hexokinase rose markedly (about eightfold) with the beginning of bud formation, reaching a maximum value in cell fractions obtained at flow rates between 19 and 34 ml/min. Thereafter, the enzyme activity dropped again to lower levels. Pyruvate kinase activity was relatively low in cell fractions collected at 14 to 21 ml/min. However, the specific activity increased dramatically in cells with outgrowing buds that had completed DNA synthesis, reaching a peak (more than a 50-fold increase) in the fractions from 24 to 28 ml/min (i.e., G2 phase). The subsequent decrease in pyruvate kinase activity appeared to be associated with an increasing proportion of binucleated cells.

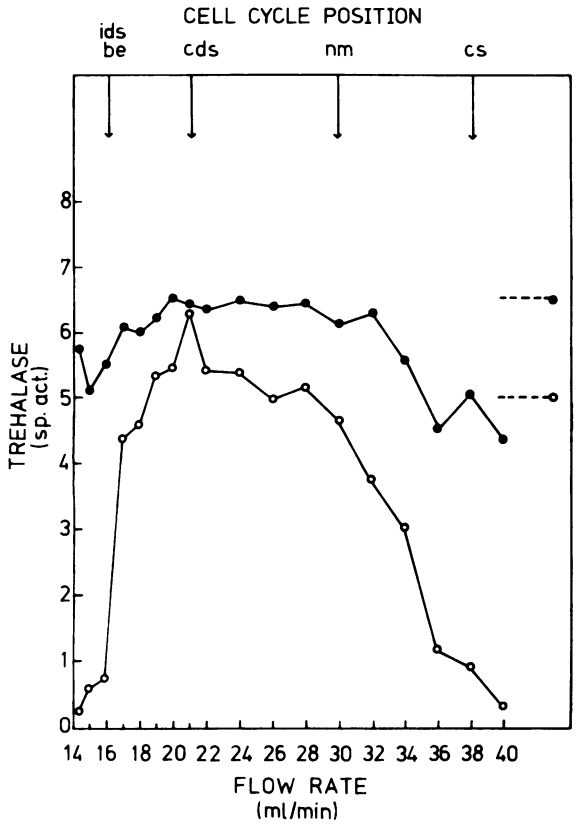


FIG. 4. Specific activity of trehalase (nanomoles of substrate hydrolyzed per minute per milligram of protein) as determined in various cell fractions obtained after cell-size fractionation of an exponentially growing culture of *S. cerevisiae* DL1 by centrifugal elutriation. Symbols: ○, basal enzyme activity; ●, enzyme activity after treatment of the cell extracts with cAMP and cAMP-dependent protein kinase. Dotted lines represent the specific enzyme activities determined in a sample of the original cell suspension loaded into the elutriator rotor. The various cell cycle markers were estimated from the peaks in cell type (Fig. 2) and measurements of cellular DNA contents by flow cytometry (Fig. 3). Abbreviations: ids, initiation of DNA synthesis; be, bud emergence; cds, completion of DNA synthesis; nm, nuclear migration; cs, cell separation.

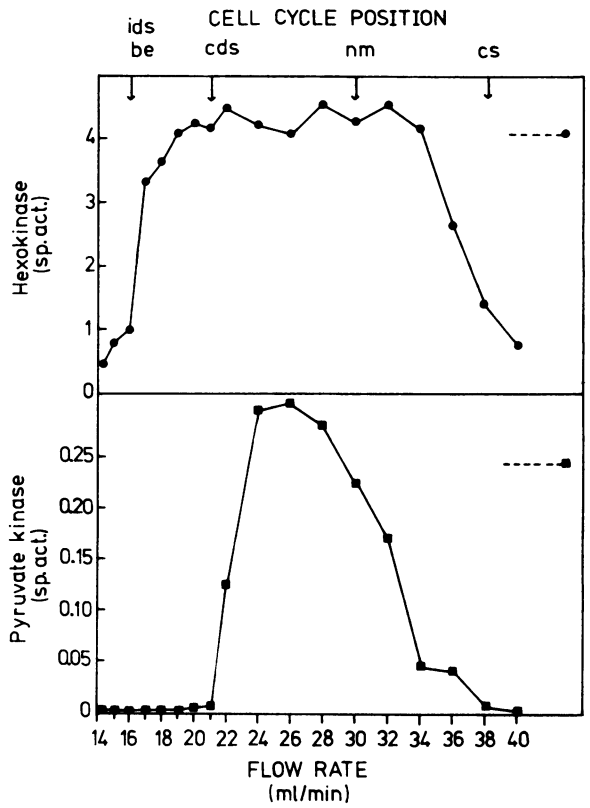


FIG. 5. Specific activities of (total) hexokinase and pyruvate kinase (micromoles of substrate converted per minute per milligram of protein) through the cell cycle of *S. cerevisiae* DL1. The various cell fractions were separated by centrifugal elutriation at counterflow rates between 14 and 40 ml/min. Dotted lines represent the specific enzyme activities determined in samples of the original cell suspension loaded into the elutriator rotor. For an explanation of the assignment of cell cycle parameters, see the legend to Fig. 4.

Figure 6 shows examples (of three independent experiments) of the patterns found for phosphofructokinase 1 and fructose-1,6-diphosphatase 1, as measured in the same fractionated culture. The specific activity of phosphofructokinase increased rapidly at the beginning of the budding process and DNA synthesis, finally leading to more than 10-fold-higher values in cell fractions elutriated at flow rates between 21 and 32 ml/min. Again, cells collected at higher flow rates exhibited lower enzyme activities. When phosphofructokinase 1 was assayed in the presence of either 5′-AMP or fructose-2,6-diphosphate, in all cell fractions the enzyme activity was stimulated 1.5- to 2.0-fold. The activating effects of 5′-AMP and fructose-2,6-diphosphate under the assay conditions used were similar and found to be not synergistic. Evidently, the changes in activity seen during the cell cycle were not due to changes in the endogenous concentrations of fructose-2,6-diphosphate or 5′-AMP. In contrast to phosphofructokinase 1 and the other two glycolytic enzymes investigated, the highest specific activity of the gluconeogenic enzyme fructose-1,6-diphosphatase 1 (being repressed under the growth conditions applied in this study) was found in fractions enriched in either unbudded cells with a single nucleus or in binucleated cells. The enzyme activity reached a minimum value (about five times lower) in fractions containing predominantly budded cells with a single nucleus.

As can be seen in Fig. 7 (representing four experiments), the specific activities of two other enzymes, glucose-6-phosphate dehydrogenase and alcohol dehydrogenase, remained virtually unchanged during the cell cycle.

For all enzymes assayed, the specific activities determined in samples withdrawn from asynchronous exponential-phase yeast cultures were the same as those found for samples of the cell suspension loaded into the elutriator rotor. As one should expect, the specific activities found in the fractions elutriated at flow rates between 22 and 28 ml/min (representing the largest part of the total cell population) appeared to be close to those measured in the cell suspension used for centrifugal elutriation (Fig. 4 to 7).

## DISCUSSION

Our results indicate that the centrifugal elutriation procedure used here is an effective method to separate a yeast population in the exponential phase into fractions representing successive stages of the cell cycle. The technical improvements described in Materials and Methods reduced the frequently observed tendency of the fractions containing the

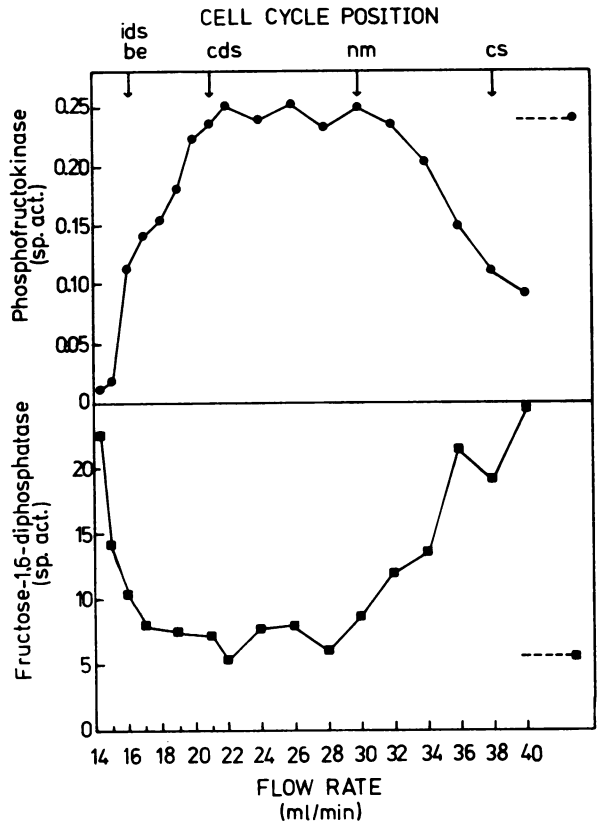


FIG. 6. Specific activities of phosphofructokinase 1 (micromoles of substrate converted per minute per milligram of protein) and fructose-1,6-diphosphatase 1 (nanomoles of substrate hydrolyzed per hour per milligram of protein) as determined in cell extracts of various cell fractions separated by centrifugal elutriation. Dotted lines represent the specific enzyme activities determined in samples of the original cell suspension loaded into the elutriator rotor. For an explanation of the assignment of cell cycle position, see the legend to Fig. 4.

larger cells to be contaminated with small cells (10, 13). As discussed by others (1, 6, 10), certain limitations inherent in the method prevent strict separation of the cells on the basis of size alone, i.e., to some extent the separation may have been the result of differences in cell shape and density. When the various elutriated fractions are assigned to particular stages of the cell cycle, other factors need to be considered, such as the variation in cell size at any given stage of the cycle (which is in part due to the increasing size of the mother cells with every generation), and the unequal size of mother and daughter cells (5, 15). Such phenomena presumably explain why the mean cell volume and total protein content of the successive elutriated cell populations had increased from an ideal twofold to higher values. Whereas the mean volume of cells elutriated at the maximum flow rate of 40 ml/min was about 3.5 times higher than that of cells collected at 14 ml/min, the average cellular protein levels increased by only a factor of 2.5. This finding may point to a decrease in cell density at later stages of the cell cycle. Indeed, Hartwell (14) has found that, in budding yeasts, there are density fluctuations during the cell cycle, with a maximum density after DNA replication and a minimum at the time of cell division. Microscopic examination and evaluation of the DNA distribution patterns of the various cell fractions separated revealed that DNA synthesis in *S. cerevisiae* is initiated at or near the time of bud emergence, which is in accordance with earlier reports (18, 32).

Despite a certain degree of discrepancy between cell size and position in the cell cycle, centrifugal elutriation seems to be the method of choice for simultaneous determination of the activities of a number of enzymes as a function of the cell cycle, with minimal metabolic perturbations. In contrast to the other enzymes investigated, no cell-cycle-dependent changes in the specific activities of (total) alcohol dehydrogenase and glucose-6-phosphate dehydrogenase could be demonstrated, suggesting that these enzymes are synthesized continuously throughout the cell cycle. The activity of glucose-6-phosphate dehydrogenase was also measured by us previously in synchronously dividing yeast populations. In these experiments, there were also no indications of periodic changes in enzyme activity during the cell cycle (Van Doorn et al., in press). Moreover, the results for alcohol dehydrogenase and glucose-6-phosphate dehydrogenase provide additional evidence that the fluctuations in the specific activities of the other enzymes investigated (as determined in the same cultures fractionated according to cell size) represent changes genuinely related to the cell cycle and are not due to general effects induced by the technical procedures used.

The results of measurements of trehalase activity in the cultures fractionated according to cell size indicate that the

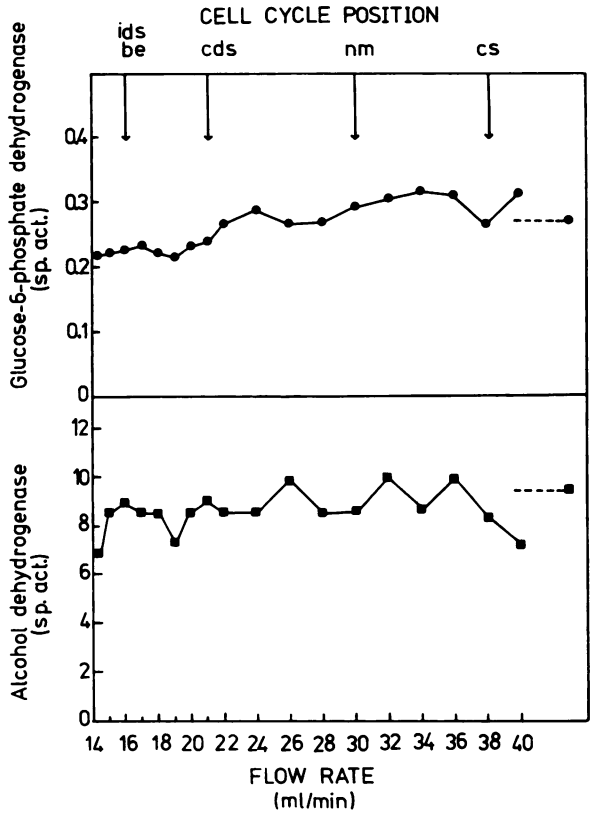


FIG. 7. Specific activities of glucose-6-phosphatase and (total) alcohol dehydrogenase (micromoles of substrate converted per minute per milligram of protein) as determined in cell extracts of various cell fractions separated by centrifugal elutriation. Dotted lines represent the specific enzyme activities determined in samples of the original cell suspension loaded into the elutriator rotor. For an explanation of the assignment of cell cycle position, see the legend to Fig. 4.

enzyme activity changes dramatically through the cell cycle. Such changes largely disappeared after activation of the enzyme by phosphorylation in vitro. In a previous study, we used synchronous cultures of *S. cerevisiae* (prepared by selection of small, unbudded cells from an elutriator rotor) to measure the activity of trehalase during the cell cycle (35a). In the synchronized yeast populations, the trehalase activity appeared to increase from the beginning of the budding process onward. However, around the period of cell division, the enzyme activity rapidly and transiently dropped more than fivefold. Treatment of the cell lysates with cAMP-dependent protein kinase (in the presence of cAMP) largely abolished the oscillatory pattern of enzyme activity originally observed. Thus, these results are in agreement with the observations summarized in the present report and strongly favor posttranslational modification of a constitutively synthesized enzyme through phosphorylation-dephosphorylation as the mechanism underlying the periodic changes in trehalase activity during the cell cycle. In view of several studies in vitro, cAMP is probably involved in the activation of trehalase during part of the budding cycle by activating a cAMP-dependent protein kinase resulting in the subsequent phosphorylation of the enzyme (8, 33, 35, 36). Changes in the phosphorylation state of the trehalase protein pool during the cell cycle could in principle also be due to periodic changes in the activity of a protein phosphatase. The observed periodicity in trehalase activity is also compatible with findings reported earlier by Küenzie and Fiechter (20). They observed that in partially synchronous cultures of *S. cerevisiae* (induced by feeding then starving) the degradation of trehalose and glucogen was initiated with the formation of buds, whereas accumulation of reserve carbohydrates largely took place during the G1 period. The liberated glucose may serve as an important endogenous energy source for bud formation (and as a substrate for the synthesis of structural carbohydrates), which would allow the cell to proceed through the S and M phases of the mitotic cycle within a relatively constant time interval, independent of possible changes in the concentrations of nutrients in the surrounding medium (32, 37). On the basis of this assumption, one may also explain the observed rapid increase in glycolytic activity of the cells with bud emergence through the activation of hexokinase and phosphofructokinase 1. In cells with outgrowing buds, the specific activities of the three glycolytic enzymes investigated appeared to be maximal. Conversely, during this part of the cell cycle the fructose-1,6-diphosphatase 1 activity had reached very low values. Presumably, gluconeogenesis is effectively inhibited, and hence the utilization of glucose as the preferred energy source is guaranteed. Apparently, there is an adaptation of the metabolism from glycolysis to gluconeogenesis around the period of cell division. Von Meyenburg (37) and, more recently, Käppeli et al. (19) determined the gas exchange of populations of *S. cerevisiae* synchronized at different growth rates under glucose limitation. The specific $O_2$ uptake and $CO_2$ formation rate, and presumably ATP generation, were shown to increase with the initiation of budding. Thus, our present findings on cell-cycle-dependent changes in enzyme activities would complement the apparent oscillations in glycolytic activity throughout the cell cycle reported by others.

It has been demonstrated that inactivation of fructose-1,6-diphosphatase 1 (and several other enzymes participating in gluconeogenesis) occurs upon the addition of glucose or other fermentable sugars to yeast cells grown on acetate or ethanol (12, 23). Evidence has been provided that the rapid, reversible loss of about 60% of the fructose-1,6-diphosphatase 1 activity is mediated by phosphorylation of the enzyme catalyzed by a cAMP-dependent protein kinase (31, 34). Considering this finding, it may be that the frucose-1,6-diphosphatase 1 activity changes during the cell cycle are regulated (at least in part) by a similar phosphorylation-dephosphorylation mechanism as encountered previously for trehalase.

At present, it cannot be excluded that the periodicity in the specific activities of hexokinase, phosphofructokinase 1, pyruvate kinase (and perhaps fructose-1,6-diphosphatase 1) reflects cell-cycle-dependent alterations in protein synthesis (i.e., regulation at the level of transcription) or protein degradation. On the other hand, the results for trehalase illustrate that enzyme activity and enzyme synthesis or degradation are not necessarily coupled throughout the cell cycle. In fact, in budding yeasts, total protein and mRNA seem to increase continuously during the mitotic cycle (10). In addition, two-dimensional gel electrophoresis (after dual pulse-labeling of protein) on size-fractionated cultures of *S. cerevisiae* revealed that only a few of the abundant cellular proteins display periodic changes in either synthesis or proteolytic degradation (9). All other proteins, including alcohol dehydrogenase, hexokinase isoenzymes, and pyruvate kinase, seem to be synthesized continuously during the cell cycle (9, 26). Recently, a survey of 900 proteins identified only 9 (besides histones) that may be periodically synthesized or degraded (24). It would appear, therefore, that our observations of periodic activity during the cell cycle are due to (as yet unknown) regulatory mechanisms that affect enzyme activity and not the rate of protein turnover. These cell-cycle-controlled mechanisms may involve posttranslational modifications, alterations in noncovalent binding of regulatory molecules to the enzyme, or monomer-dimer changes. Such changes are not likely to be detected by two-dimensional electrophoresis procedures, as used in the studies referred to above. The metabolic consequences as well as the molecular basis underlying the apparent changes in enzyme activities during the cell cycle are subjects for current further investigations.

## ACKNOWLEDGMENT

The excellent technical assistance of N. Vinker is gratefully acknowledged.

## LITERATURE CITED

1. **Barford, J. P.** 1986. A mathematical model for cell separation technique of centrifugal elutriation. Biotechnol. Bioeng. **28**:570–577.
2. **Barford, J. P., and R. J. Hall.** 1976. Estimation of the length of cell cycle phases from asynchronous cultures of *Saccharomyces cerevisiae*. Exp. Cell Res. **102**:276–284.
3. **Bartrons, R., E. Van. Schaftingen, S. Vissers, and H. G. Hers.** 1982. The stimulation of yeast phsophofructokinase by fructose-2,6-bisphosphate. FEBS Lett. **143**:137–140.
4. **Bergmeyer, H. U., K. Gawehn, and M. Crassl.** 1974. Enzymes as biochemical reagents, p. 428–429. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, vol. 1. Academic Press, Inc., New York.
5. **Carter, B. L. A., and M. N. Jagadish.** 1978. The relationship between cell size and cell division in the yeast *Saccharomyces cerevisiae*. Exp. Cell Res. **112**:15–24.
6. **Creanor, J., S. G. Elliott, Y. C. Bisset, and J. M. Mitchison.** 1983. Absence of step changes in activity of certain enzymes during the cell cycle of budding and fission yeasts in synchronous cultures. J. Cell Sci. **61**:339–349.
7. **Creanor, J., and J. M. Mitchison.** 1979. Reduction of perturbations in leucine incorporation in synchronous cultures of *Schiz-*

*osaccharomyces pombe* made by elutriation. J. Gen. Microbiol. **112**:385–388.
8. **Dellamora-Ortiz, G. M., C. H. D. Ortiz, J. C. C. Maia, and A. D. Panek.** 1986. Partial purification and characterization of the interconvertible forms of trehalase from *Saccharomyces cerevisiae*. Arch. Biochem. Biophys. **251**:205–214.
9. **Elliott, S. G., and C. S. McLaughlin.** 1978. Rate of macromolecular synthesis through the cell cycle of the yeast *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **75**:4384–4388.
10. **Elliott, S. G., and C. S. McLaughlin.** 1983. The yeast cell cycle: coordination of growth and division rates. Prog. Nucleic Acid Res. Mol. Biol. **28**:143–175.
11. **Figdor, C. G., A. J. M. Olijhoek, S. Klencke, N. Nanninga, and W. S. Bont.** 1981. Isolation of small cells from an exponential growing culture of *E. coli* by centrifugal elutriation. FEMS Microbiol. Lett. **10**:349–352.
12. **Gancedo, C.** 1971. Inactivation of fructose-1,6-diphosphatase by glucose in yeast. J. Bacteriol. **107**:401–405.
13. **Gordon, C. N., and S. G. Elliott.** 1977. Fractionation of *Saccharomyces cerevisiae* cell populations by centrifugal elutriation. J. Bacteriol. **129**:97–100.
14. **Hartwell, L. H.** 1970. Periodic density fluctuations during the yeast cell cycle and the selection of synchronous cultures. J. Bacteriol. **104**:1280–1285.
15. **Hartwell, L. H., and M. W. Unger.** 1977. Unequal division in *Saccharomyces cerevisiae* and its implications for the control of cell division. J. Cell Biol. **75**:422–435.
16. **Hess, B., and H. J. Wieker.** 1974. Pyruvate kinase from yeast, p. 778–783. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, vol. 2. Academic Press, Inc., New York.
17. **Hofmann, E., and G. Kopperslager.** 1982. Phosphofructokinase from yeast. Methods Enzymol. **90**:49–60.
18. **Johnston, G. C., R. A. Singer, S. O. Sharrow, and M. L. Slater.** 1980. Cell division in the yeast *Saccharomyces cerevisiae* growing at different rates. J. Gen. Microbiol. **118**:479–484.
19. **Käppeli, O., M. Arreguin, and M. Rieger.** 1985. The respirative breakdown of glucose by *Saccharomyces cerevisiae*: an assessment of a physiological state. J. Gen. Microbiol. **131**:1411–1416.
20. **Küenzie, M. T., and A. Fiechter.** 1969. Changes in carbohydrate composition and trehalase activity during the budding cycle of *Saccharomyces cerevisiae*. Arch. Microbiol. **64**:396–407.
21. **Lachenicht, R., and E. Bernt.** 1974. D-Glucose. Fluorometric determination in blood automatic analysis, p. 1201–1205. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, vol. 3. Academic Press, Inc., New York.
22. **Lang, G., and G. Michal.** 1974. D-Glucose-6-phosphate and D-fructose-6-phosphate, p. 1238–1242. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, vol. 3. Academic Press, Inc., New York.
23. **Lenz, A. G., and H. Holzer.** 1980. Rapid reversible inactivation of fructose-1,6-bisphosphatase in *Saccharomyces cerevisiae* by glucose. FEBS Lett. **109**:271–274.
24. **Lörincz, A. T., M. J. Miller, N. H. Xuong, and E. P. Geiduschek.** 1982. Identification of proteins whose synthesis is modulated during the cell cycle of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **2**:1532–1549.
25. **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.
26. **Ludwig, J. R., J. J. Foy, S. G. Elliott, and C. S. McLaughlin.** 1982. Synthesis of specific identified, phosphorylated, heat shock, and heat stroke proteins through the cell cycle of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **2**:117–126.
27. **Mitchison, J. M.** 1969. Enzyme synthesis in synchronous cultures. Science **165**:657–663.
28. **Mitchison, J. M.** 1977. Enzyme synthesis during the cell cycle, p. 377–401. *In* L. Nover and K. Mothes (ed.), Cell differentiation in microorganisms, plants and animals. Gustav Fischer Verlag, Jena, German Democratic Republic.
29. **Mitchison, J. M., and B. L. A. Carter.** 1975. Cell cycle analysis. Methods Cell Biol. **9**:201–219.
30. **Nissler, K., A. Otto, W. Scheelenberger, and E. Hofman.** Similarity of activation of yeast phosphofructokinase by AMP fructose-2,6-bisphosphate. Biochem. Biophys. Res. Commun. **111**: 294–300.
31. **Purwin, C., F. Leiding, and H. Holzer.** 1982. Cyclic AMP-dependent phosphorylation of fructose-1,6-bisphosphatase in yeast. Biochem. Biophys. Res. Commun. **107**:1482–1489.
32. **Slater, M. L., S. O. Sharrow, and J. J. Gart.** 1977. Cell cycle of *Saccharomyces cerevisiae* in populations growing at different rates. Proc. Natl. Acad. Sci. USA **74**:3850–3854.
33. **Thevelein, J. M.** 1984. Regulation of trehalose mobilization in fungi. Microbiol. Rev. **48**:42–59.
34. **Toyoda, Y., and J. Sy.** 1984. Purification and phosphorylation of fructose-1,6-bisphosphatase from *Kluyveromyces fragilis*. J. Biol. Chem. **259**:8718–8723.

35a. **Van Doorn, J., M. E. Scholte, P. W. Postma, R. van Driel, and K. van Dam.** 1988. Regulation of trehalose activity during the cell cycle of *Saccharomyces cerevisiae*. J. Gen. Microbiol. **134**: 785–790.

35. **Uno, I., K. Matsumo, K. Adachi, and T. Ishikana.** 1983. Genetic and biochemical evidence that trehalase is a substrate of cAMP-dependent protein kinase in yeast. J. Biol. Chem. **258**:10867–10872.
36. **Van Solingen, P., and J. B. Van Der Plaat.** 1975. Partial purification of the protein system controlling the breakdown of trehalose in baker's yeast. Biochem. Biophys. Res. Commun. **62**:553–560.
37. **Von Meyenburg, H. K.** 1969. Energetics of the budding cycle of *Saccharomyces cerevisiae* during glucose limited aerobic growth. Arch. Microbiol. **66**:289–303.
38. **Williamson, D. M., and D. J. Fennell.** 1975. The use of fluorescent DNA-binding agent for detecting and separating yeast mitochondrial DNA. Methods Cell Biol. **12**:335–351.