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# A Kinetic Study of Glycolytic Enzyme Synthesis in Yeast

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## SUMMARY

**The kinetics of induced synthesis of glycolytic enzymes of the hybrid yeast *Saccharomyces fragilis* × *Saccharomyces dobzhanskii* in response to the addition of glucose or galactose has been studied in a basal medium containing peptone, acetate, and yeast extract.**

**Glucose and galactose bring about a 3- to 100-fold increase in specific activity of various glycolytic enzymes during a 7-hour period. The smallest increase is observed in the case of P-glucoisomerase (EC 5.3.1.9) and the largest with glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12). In the stationary state of cells grown in the presence or absence of glucose, the glycolytic enzymes display a coordinate relationship to one another. The time course of enzyme synthesis by glucose and galactose, as well as of enzyme disappearance on removal of the sugars, however, suggests a kinetic heterogeneity. With galactose as the inducing carbohydrate, the enzymes increase in specific activity in the following sequence: pyruvate kinase (EC 2.7.1.40), within 20 min after addition of galactose; P-glucomutase (EC 2.7.5.1), P-fructokinase (EC 2.7.1.11), and glyceraldehyde-3-P dehydrogenase, within 1 hour; P-glucoisomerase, P-glycerate kinase (EC 2.7.2.3), P-glycerate mutase (EC 2.7.5.3), and enolase (EC 4.2.1.11), between 1 and 3 hours; hexokinase (EC 2.7.1.1), aldolase (EC 4.1.2.7), triose-P isomerase (EC 5.3.1.1), and pyruvate decarboxylase (EC 4.1.1.1), between 2 and 5 hours. Alcohol dehydrogenase (EC 1.1.1.1) falls outside any of these groups; after an initial period of repression, galactose causes increased synthesis of this enzyme during later periods. The increase of glycolytic enzyme activity elicited by sugars seems to be due to increased production of the same enzyme species already existing in uninduced cultures. The glucose-induced increase in glycolytic enzyme activity in *Saccharomyces cerevisiae* is completely prevented by cycloheximide, an inhibitor of protein synthesis in this yeast. P-Fructokinase and pyruvate decarboxylase decay rapidly inside the cells in the presence of this antibiotic.**

Although the regulation of glucose utilization is one of the earliest described phenomena in cellular control systems, very little is known about the control of synthesis of glycolytic enzymes. One of the reasons for this, perhaps, is our lack of understanding of the genetic determinants of the glycolytic system. Another factor that might have been responsible for this apparent lack of interest in glycolytic enzyme induction is the so-called constitutive nature of such enzymes. The relatively large amounts of glycolytic enzymes present in cells and tissues irrespective of growth conditions permit little modulation of enzyme activity to be seen in response to a specific environmental stimulus. As a result, the glycolytic system has been considered to be a case in which modulation of enzyme synthesis is not the preferred mode of regulation (1).

The fact that glycolytic enzymes do indeed change in response to dietary influence, hormone administration, or developmental stimulus is now well documented, however (2, 3). Hommes has observed that growth of a strain of *Candida* in varying concentrations of glucose produces large changes in the activity of glycolytic enzymes (4). The change of the various enzymes was such as to suggest five coordinate groups of induction. Without any kinetic data, the steady state nature of most such studies, however, allows one to draw only restrictive conclusions regarding such grouping. In view of this, we have sought to investigate the kinetics of enzyme synthesis in a strain of hybrid yeast that displays considerable inducibility by glucose and permits a fairly rapid rate of synthesis. The question of coordination of glycolytic enzymes has been examined in the stationary state of this culture, as well as during its exponential growth on a number of sugars, with the conclusion that the glycolytic system constitutes a kinetically inhomogeneous group of enzymes. The terms enzyme induction and synthesis, which have been used here interchangeably, refer simply to the increase in specific activity of the enzymes. No inference is made as to the mechanism mediating such increase.

## METHODS

*Growth of Yeast*—A hybrid strain of *Saccharomyces fragilis* × *Saccharomyces dobzhanskii*, given to us by Dr. H. O. Halvorson, a haploid stock of *Saccharomyces cerevisiae*, and a strain of *Candida albicans* were used in these studies. In most of the experiments, however, we used the hybrid yeast, inasmuch as the ratio of glycolytic enzyme activity of cultures grown in the presence of glucose to that in its absence is much higher for this yeast than for the other two. Cultures were grown overnight in a basal medium containing peptone (1 g/100 ml), yeast extract (0.3 g/100 ml), and sodium acetate (50 mM) at 30° with shaking. The culture was resuspended in fresh medium and, when exponential growth was achieved, the appropriate sugar was added and the culture was allowed to grow. At suitable intervals, each flask was removed and chilled, the yeast was washed by centrifugation once in 150 mM cold KCl and then in a medium containing 2 mM 2-mercaptoethanol and 2 mM EDTA in 50 mM

potassium phosphate buffer, pH 7.4, and stored at −25°. The initial density of yeast in the induction medium was adjusted to $2 \times 10^6$ to $2 \times 10^7$ cells per ml so that, at the moment the cells were harvested, the culture was growing exponentially. Generally, each 500-ml Erlenmeyer flask contained no more than 150 ml of medium. Since the total amount of cells processed for each set of assays was 300 to 600 mg, wet weight, cells pooled from more than one identical flask had to be used in some cases. When the initial inoculum culture came from more than one flask, cells were pooled before dispensing. In experiments in which the effect of sugar withdrawal was studied, the bulk of the fluid was decanted after allowing the cells to settle for 1 min or so. The last traces of sugar were removed by washing three times in sugar-free medium in a centrifuge at room temperature, the whole process requiring about 10 min. Time was counted from the moment the washed cells were suspended in yeast extract-peptone medium containing acetate, as described above. Aseptic procedures were used throughout. The doubling time of the hybrid yeast in yeast extract-peptone-acetate was 120 min, whereas supplementation of this medium with galactose or glucose brought this time down to 110 and 60 min, respectively.

Cell count was made on a hemocytometer after a brief treatment with 5 mM galactose, which was found to produce a uniformly dispersed suspension. One gram of wet yeast contained $1.42 \times 10^{10}$ cells.

Although for most experiments the yeast was grown aerobically in the yeast extract-peptone medium, we used a more defined medium in some cases. This consisted of: $(NH_4)_2SO_4$, 38 mM; potassium phosphate, pH 7.4, 20 mM; NaCl, 8 mM; $MgSO_4$, 3 mM; $FeSO_4$, 18 μM; $ZnSO_4$, 3.5 μM; $CuSO_4$, 0.4 μM; inositol, 55 μM; pyridoxine, 10 μM; thiamine, 6 μM; pantothenic acid, 4 μM; and yeast extract, 0.03 g/100 ml, supplemented with either 40 mM sodium acetate or 50 mM glucose. For anaerobic experiments, a slow stream of a water-saturated nitrogen (95%)-$CO_2$ (5%) mixture was allowed to bubble through the medium.

*Preparation of Yeast Extract*—In experiments in which the kinetics of enzyme changes following sugar addition or withdrawal was studied, cells were frozen overnight. For all other experiments, freshly washed cells were used. There was no appreciable difference between these two sets of experiments except for alcohol dehydrogenase which was found to be unstable to EDTA. The washed cells were suspended in phosphate buffer containing mercaptoethanol and EDTA (described above) so as to contain about 200 mg of wet yeast per ml, and the suspension was crushed in a chilled French pressure cell at 20,000 p.s.i. After a preliminary clarification at low speed, the suspension was finally centrifuged at 20,000 × *g* for 10 min, and the supernatant served as the enzyme solution in all of the experiments. Enzyme assays were made on the crude extract.

*Assay of Enzymes*—All enzymes were assayed by coupling the particular step to the appropriate $NAD^+$- or $NADP^+$-linked reaction, with the use of commercially available crystalline enzymes in a $(NH_4)_2SO_4$ suspension as coupling enzymes (5). The rate of production or disappearance of reduced nucleotides was followed continuously on a fluorimeter with a 10 mv servo recorder (6). One full scale deflection of the recorder corresponded to a span of 5 to 25 mμmoles of NADH or NADPH in a total volume of 2.0 ml at room temperature (24–26°). The concentration of the test enzyme was chosen so as to require 5 to 15 min for a full scale traverse. In some experiments, conventional spectrophotometry at 340 mμ was employed with a Gilford absorbance recording system (Gilford Instrument Laboratories, Inc., Oberlin, Ohio); the enzyme activities obtained by either of the methods were comparable. That the rate was first order with respect to the amount of enzyme solution was ensured for at least 2- and generally 3-fold or higher amounts of the aliquots. Except for a few enzymes (described below), rates were estimated during the first 4 min of reaction. In most cases, enzymes were diluted in cold phosphate buffer containing EDTA and mercaptoethanol as described above; diluted enzymes were discarded within 100 min. Evaluation of various samples for a particular enzyme was made from the same series of dilution. Assays were generally completed within 9 hours of the preparation of the extract. In cases in which we used enzyme solutions stored overnight in an ice bath, the data always referred to the same set of enzymes so that relative activities between samples were comparable. With the exception of alcohol and aldehyde dehydrogenases (EC 1.1.1.1 and EC 1.2.1.4), the enzyme activities were fairly stable. Alcohol dehydrogenase was assayed in 100 mM glycine-40 mM hydrazine buffer, pH 8.5. For all others, a buffer containing 50 mM triethanolamine hydrochloride (neutralized with KOH) and 10 mM $MgCl_2$, pH 7.4, was used. The yeast extract contained a NADH oxidase activity which was insensitive to antimycin A. Reactions were therefore started with the substrate, the rate of NADH oxidation prior to substrate addition serving as the control. Except for alcohol dehydrogenase and triose-P isomerase, all assays were corrected for this control rate. Cultures grown in the absence of sugar had activities of endogenous NADH oxidation rate higher than those of cultures that were adapted to the sugars. This made the assays of some of the enzyme activities of the uninduced culture subject to a variation of 20%. Enzyme activities are expressed as international units, *i.e.* as micromoles of substrate per min at 25°, or as milliunits ($10^{-3}$ i.u.). For aldolase (EC 4.1.2.7), the values refer to fructose-1,6-$P_2$ cleavage.

The reaction mixture for P-glucomutase (EC 2.7.5.1) contained 1 mM glucose-1-P, 0.05 mM $NADP^+$, 5 mM cysteine, 0.1 unit of glucose-6-P dehydrogenase (EC 1.1.1.49), and 0.05 unit of gluconate-6-P dehydrogenase (EC 1.1.1.44), and the rates were halved. Proportionality between the rate and the amount of enzyme was achieved only when relatively large amounts of the extract (0.1 mg of protein and more) were used. Hexokinase (EC 2.7.1.1) assay was done with 5 mM glucose, 1 mM ATP, 0.05 mM $NADP^+$, and 0.3 unit of glucose-6-P dehydrogenase. For glucose-6-P dehydrogenase, 1 mM glucose-6-P and 0.05 mM $NADP^+$ were used, and for gluconate-6-P dehydrogenase, 0.5 mM gluconate-6-P and 0.05 mM $NADP^+$ were used. For both of these assays, the rate in the very 1st minute was taken as the activity in order to minimize the contribution of gluconate-6-P dehydrogenase and of NADPH, respectively. The latter seems to inhibit the gluconate-6-P dehydrogenase activity of the extract. For the assay of P-glucoisomerase (EC 5.3.1.9), the reaction mixture contained 1 mM fructose-6-P free of glucose-6-P, 0.05 mM $NADP^+$, and 0.3 unit of glucose-6-P dehydrogenase. Since the extracts contained gluconate-6-P dehydrogenase, low levels of enzymes that were assayed with glucose-6-P dehydrogenase as the coupling enzyme, *e.g.* hexokinase activity of the uninduced culture, were always checked by adding an excess of crystalline gluconate-6-P dehydrogenase and ensuring that the observed rates were

doubled. In other cases, however, this was not of much consequence, as the average maximal velocity of gluconate-6-P dehydrogenase in the extract was 0.08 unit per mg of protein. Since most enzyme assays were completed within a full scale traverse of the recorder pen, a maximum of 10 $\mu$M concentration of gluconate-6-P could have been produced. By intrapolation on the velocity *versus* gluconate-6-P concentration curve, it was observed that this concentration could have contributed no more than 0.008 unit per mg of protein.

The reaction mixture for P-fructokinase (EC 2.7.1.11) assay consisted of 5 mM glucose-6-P, 2 units of P-glucoisomerase, 0.03 mM NADH, 1 mM ATP, 0.3 mM ADP, 1 unit each of aldolase and $\alpha$-glycero-P dehydrogenase (EC 1.1.1.8), and 10 units of triose-P isomerase (EC 5.3.1.1), the reaction being started with ATP. Aldolase was assayed in 0.03 mM NADH, 1 mM fructose 1,6-$P_2$, 1 unit of $\alpha$-glycero-P dehydrogenase, and 10 units of triose-P isomerase. For both P-fructokinase and aldolase, the reaction velocity was divided by 2. Triose-P isomerase was estimated by following the rate of oxidation of NADH in a reaction mixture containing 0.03 mM NADH, 0.4 mM glyceraldehyde-3-P, and 1 unit of $\alpha$-glycero-P dehydrogenase.

Glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12) activity was measured in a mixture containing 0.03 mM NADH, 5 mM cysteine, 1 mM 3-P-glycerate, 1 mM ATP, and 1 unit of P-glycerate kinase (EC 2.7.2.3). For P-glycerate kinase assay, the reaction mixture was the same except that it contained 1 unit of glyceraldehyde-3-P dehydrogenase in place of the kinase. Both of these reactions were started with ATP.

P-Glycerate mutase (EC 2.7.5.3) was assayed in a mixture containing 0.03 mM NADH, 1 mM 3-P-glycerate, 0.5 unit of enolase (EC 4.2.1.11), 1 mM ADP, and 1 unit each of pyruvate kinase (EC 2.7.1.40) and muscle lactate dehydrogenase. Generally, the reaction was started with 3-P-glycerate, except for certain samples of this substrate which had 2-P-glycerate as an impurity; the substrate in this case was incubated with the reaction mixture before adding the enzyme extract, the blank rate being determined separately in a reaction mixture lacking P-glycerate. Enolase assay was done in the same way as for P-glycerate mutase, 0.5 unit of P-glycerate mutase substituting for the crystalline enolase. Pyruvate kinase was determined in a system with 1 mM of each of P-enolpyruvate, ADP, fructose-1,6-$P_2$ (7), 0.03 mM NADH, and 1 unit of muscle lactate dehydrogenase.

Pyruvate decarboxylase (EC 4.1.1.1) was assayed with 50 mM pyruvate, 5 mM cysteine, 0.03 mM NADH, 0.25 mM thiamine pyrophosphate, and 1 unit of alcohol dehydrogenase. The reaction was initiated with pyruvate.

Alcohol dehydrogenase was assayed with 150 mM ethyl alcohol and 1 mM $NAD^+$ in an alkaline glycine-hydrazine buffer. Since there was no detectable $NADP^+$-linked alcohol dehydrogenase in the extract, aldehyde dehydrogenase was assayed by monitoring $NADP^+$ reduction with 0.5 mM acetaldehyde and 0.05 mM $NADP^+$, measuring the rate obtained within the first 2 min. Higher concentrations of acetaldehyde inhibited the enzyme.

Galactokinase (EC 2.7.1.6) was assayed by following the rate of ADP generation in a system containing 5 mM galactose, 1 mM ATP, 0.05 mM NADH, 1 mM P-enolpyruvate, and 1 unit each of pyruvate kinase and muscle lactate dehydrogenase. Since the blank rate of NADH oxidation was high because of ATPase, the reaction was started by adding galactose. Hexokinase was also measured occasionally in this manner, glucose being substituted for galactose; the activity was the same as found by glucose-6-P assay.

*Presentation of Enzyme Activity*—In most of the results presented here, the enzyme activities have been expressed in units per mg of extract protein, thereby normalizing with respect to the increase in cell mass and variable extraction of cells. The assumption that growth on sugars does not change the amount of protein per cell mass has been verified in our experimental system. One gram of wet yeast has been found to contain, on an average, 60 mg of protein. The more conventional method of plotting enzyme activity against cell mass has not been followed as the breakage of cells by the present method led to variable extraction, necessitating further normalization with respect to degree of extraction.

In the later phase of these studies, it was found that breakage of cells by toluene treatment at 37° is just as effective in releasing enzymes from the cells. A 2- to 20-ml portion of growing culture was chilled, centrifuged, and broken by shaking the cell pellet in 1 ml of phosphate-mercaptoethanol-EDTA buffer in the presence of 2 drops of toluene for 25 min at 37°. Growth was followed by measuring extinction at 650 m$\mu$ in a 1-cm cuvette. For this purpose, an aliquot of growing cells of the hybrid yeast was diluted in 150 mM KCl containing 5 mM galactose. For *S. cerevisiae* and *C. albicans*, galactose treatment was omitted. Results are expressed either directly as in Fig. 9, or by plotting the observed specific activity against time (Fig. 5).

*Assay of Substrates*—Glucose was determined by glucose oxidase (EC 1.1.3.4)-peroxidase (EC 1.11.1.7) assay (8), and galactose was determined by galactose oxidase-peroxidase assay (9). Glycolytic intermediates and low concentrations of glucose were estimated fluorometrically (10). Protein was estimated by the method of Lowry *et al.* (11), with crystalline bovine serum albumin as standard.

*Enzymes and Substrates*—Glucose oxidase, galactose oxidase, and peroxidase were obtained from Worthington. Galactose and 2-deoxyglucose were obtained from Sigma; galactose contained 0.02% glucose and was used without further purification. Sugars used were of D configuration. All other substrates and enzymes were obtained from Boehringer Mannheim. Fructose-6-P solution containing nearly 3% glucose-6-P was rendered free of this substance by treating with catalytic amounts of $NADP^+$, glucose-6-P dehydrogenase, and gluconate-6-P dehydrogenase in the presence of glutathione reductase (EC 1.6.4.2) and oxidized glutathione (250% of molar excess over glucose-6-P) and incubating for 1 hour at 37°. The solution was then adjusted to pH 3, heated for 15 min at 65°, chilled, centrifuged, and neutralized to pH 7. Yeast extract and peptone were from Difco.

## RESULTS

*Coordinate Synthesis of Glycolytic Enzymes*—Growth of the hybrid yeast *S. fragilis* × *S. dobzhanskii* in the presence of glucose in either salt-vitamin medium or in yeast extract-peptone was accompanied by increased levels of all glycolytic enzymes except alcohol dehydrogenase and aldehyde dehydrogenase. Replacement of glucose by acetate, ethanol, pyruvate, or glycerol led to reduced enzyme levels for all except alcohol and aldehyde dehydrogenases. When extracts of uninduced and induced cultures were mixed and incubated, the mixture always gave a resultant rate that was a sum of the individual rates. The

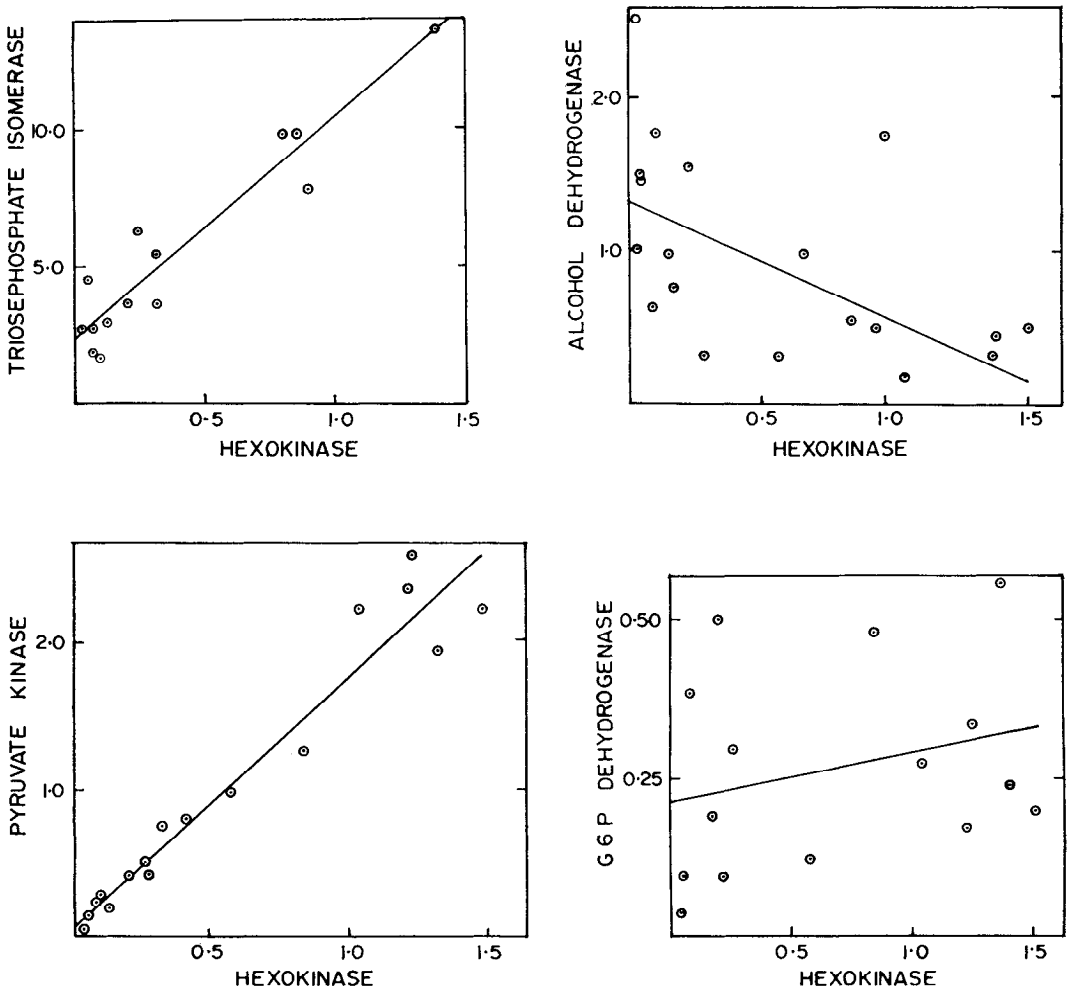


FIG. 1. Plots of activity of triose-P isomerase, pyruvate kinase, alcohol dehydrogenase, and glucose-6-P (*G 6 P*) dehydrogenase against hexokinase activity in extracts of *S. fragilis* × *S. dobzhanskii*. Cultures were grown 16 to 20 hours in minimal medium containing acetate or glucose, or in yeast extract-peptone medium supplemented with acetate, pyruvate, ethanol, glycerol, or glucose. Points indicating relatively low levels of hexokinase were obtained from cells grown in the absence of glucose, and those with relatively high levels were grown in the presence of glucose. Each *point* represents data for a particular batch of cells. Both of the coordinate axes indicate enzyme activity measured in units per mg of protein. The *lines* through the data describe the best linear fit by the least square method. Details are given in the text.

difference in the two cultures was therefore not due to any activator present in the extract from induced cells or any inhibitor or a proteolytic activity in the uninduced extract. The level of enzymes was always high whenever glucose was present, either alone or in combination with any of these 2-carbon or 3-carbon compounds. In a typical experiment, the hexokinase activity of culture grown for 6 hours in yeast extract-peptone medium containing acetate, glucose, and acetate plus glucose were, respectively, 0.14, 1.14, and 1.22 units per mg of protein. This suggested either that glucose was acting as an inducer, rather than that acetate, ethanol, glycerol, or pyruvate was acting as the repressor of the glycolytic enzymes, or else that glucose was removing an internal repressor. The absolute level of the enzymes expressed as specific activity per unit of protein, however, showed considerable variation from one experiment to another. To examine the relationship of the enzymes against this variation, we have plotted the activity of each enzyme against that of another obtained in each experiment; a family of such points was established from data pooled from several independent experiments. Results in Fig. 1 illustrate the relationship of hexokinase to triose-P isomerase, pyruvate kinase, alcohol dehydrogenase, and glucose-6-P dehydrogenase. The straight line represents the best fit by the method of least squares as calculated by a digital computer (CDC 3600).[1] The relationship of hexokinase with a few other enzymes of glycolysis is shown in Table I. Despite variations in the absolute activity, the glycolytic enzymes, except for P-glucomutase and alcohol dehydrogenase, show a strong correlation among themselves; glucose-6-P dehydrogenase, however, seems to fall outside this group. Alcohol dehydrogenase shows a negative correlation with hexokinase, being repressed by glucose. The scatter of the experimental points around the probable straight line makes the standard deviation for the intercept rather high, although all of the $c$ values turn out positive. We have not included the measurements of P-fructokinase and pyruvate decarboxylase in these analyses, as these activities were not measured as often as the others were. Kinetic data

[1] We are grateful to Dr. S. Ramani and Mr. R. P. Sinha of this Institute for assisting us with the computer program.

TABLE I

*Summary of certain coordination parameters for glycolytic enzymes in hybrid yeast*

The activity of the enzymes in question for different cultures of hybrid yeast was plotted against that of hexokinase for that particular extract as illustrated in Fig. 1. The data were subjected to statistical analysis by the least square method as described in the text. Enzyme activities are expressed as units per mg of protein. The quantity $m$ refers to the slope of the line describing the best linear fit between hexokinase and the enzyme in question, and the intercept $c$ refers to the value of that enzyme at hexokinase = 0. The standard deviations $\sigma_m$ and $\sigma_c$ for the slope and intercept, respectively, and the correlation coefficients were calculated with the aid of a computer program.

| Enzyme | No. samples | $m$ | $\sigma_m$ | $c$ | $\sigma_c$ | Correlation coefficient |
|---|---|---|---|---|---|---|
| PGLUMUT[a] | 17 | 0.02 | 0.00 | 0.00 | 0.00 | 0.65 |
| PHISO | 24 | 1.37 | 0.31 | 0.15 | 0.23 | 0.95 |
| ALD | 18 | 0.41 | 0.12 | 0.08 | 0.10 | 0.81 |
| TPISO | 14 | 8.07 | 2.45 | 2.38 | 1.39 | 0.95 |
| GAPDH | 16 | 1.91 | 0.52 | 0.08 | 0.42 | 0.98 |
| PGK | 17 | 2.13 | 0.58 | 0.57 | 0.47 | 0.94 |
| PGLYMUT | 22 | 2.12 | 0.50 | 0.52 | 0.37 | 0.92 |
| ENOL | 22 | 1.42 | 0.34 | 0.22 | 0.25 | 0.92 |
| PK | 17 | 1.69 | 0.45 | 0.07 | 0.34 | 0.97 |
| ALCDH | 19 | −0.76 | 0.29 | 1.33 | 0.22 | 0.65 |
| G6PDH | 15 | 0.07 | 0.07 | 0.22 | 0.07 | 0.27 |

[a] The abbreviations used are: PGLUMUT, P-glucomutase; PHISO, P-glucoisomerase; ALD, aldolase; TPISO, triose-P isomerase; GAPDH, glyceraldehyde-3-P dehydrogenase; PGK, P-glycerate kinase; PGLYMUT, P-glycerate mutase; ENOL, enolase; PK, pyruvate kinase; ALCDH, alcohol dehydrogenase; G6PDH, glucose-6-P dehydrogenase.

presented below, however, indicate that strong correlation exists between these enzymes and hexokinase.

*Effect of Anaerobiosis*—Results in Table II provide a comparison of the anaerobic and aerobic induction of a number of glycolytic enzymes brought about by glucose in the hybrid yeast. The levels of the four enzymes, hexokinase, P-glucoisomerase, P-fructokinase, and pyruvate kinase did not show any marked effect of aerobiosis. In the present study, all other experiments were performed under aerobic conditions in yeast extract-peptone-acetate medium. A number of experiments was done in synthetic medium in which similar induction pattern was observed, but the rate of enzyme synthesis was considerably slower with this medium.

*Kinetics of Enzyme Induction with Glucose and Galactose*—The time course of changes of glycolytic enzymes following the addition of glucose to a growing culture of the hybrid yeast is shown in Fig. 2. Control experiments, not shown here, indicated that the yeast extract-peptone-acetate medium supported exponential growth during the interval of the experiment with no significant changes in the enzyme specific activity, except for alcohol and aldehyde dehydrogenases. The basal rate of synthesis of the other glycolytic enzymes in the absence of glucose was at a constant low value such that the enzyme activity at 0 hour (Fig. 2) was a measure of the steady state of endogenous synthesis. Addition of glucose increased the rate of synthesis of all of the enzymes shown in Fig. 2, *A*, *B*, and *C*, while that of the two dehydrogenases of the hexose monophosphate pathway remained constant. As already mentioned, alcohol and aldehyde dehydrogenases were repressed by glucose addition. Among enzymes whose synthesis was stimulated by glucose, those reported in Fig. 2*C* show a delayed induction, the rate till the 2nd hour after glucose addition being slower than that attained afterwards. The scatter of the experimental points requires a mention here. Thus it is difficult to say whether P-glucoisomerase was being synthesized from the moment of glucose addition or after a lag of 30 min. The same comment applies in the case of P-glycerate kinase, enolase, and pyruvate decarboxylase. In other experiments, enolase synthesis was found to take place within 30 min of glucose addition. P-Glycerate mutase, although not shown here, was synthesized without any lag. We have not plotted P-glucomutase in Fig. 2; it did not appear to increase after glucose addition, although the scatter of experimental points does not warrant any definite conclusion. The analysis of a number of steady state samples, however, seemed to indicate that P-glucomutase has some correlation with other glycolytic enzymes (correlation coefficient with hexokinase, 0.65, Table I). As seen below, it is synthesized along with other glycolytic enzymes in response to galactose. The specific activity of alcohol dehydrogenase decreased rapidly and tended to attain a steady value. Its total amount increased despite the fall of specific activity, suggesting synthesis of a minor component of the enzyme. Aldehyde dehydrogenase continued to fall even after alcohol dehydrogenase had become steady.

Fructose and mannose, like glucose, brought about increased specific activity of glycolytic enzymes without any appreciable lag. The following compounds, however, were ineffective: sorbitol, mannitol, arabinose, *N*-acetyl glucosamine, and glycerol. Galactose, whose metabolism requires, besides glycolysis, a separate pathway leading it to glucose-1-P, behaved differently. Overnight cultures of the hybrid yeast grown in yeast extract-peptone medium containing galactose were always found to have high levels of hexokinase and alcohol dehydrogenase; cultures grown for periods up to 6 hours, however, had low

TABLE II

*Aerobic and anaerobic induction of some glycolytic enzymes in S. fragilis × S. dobzhanskii*

An overnight culture of the hybrid yeast grown aerobically on yeast extract-peptone-acetate medium was resuspended in the same medium in the presence and in the absence of 100 mM glucose and grown for 6.5 hours at 30° in a shaking bath. A wet stream of nitrogen gas containing 5% $CO_2$ was passed through the anaerobic culture, a similar mixture of 5% $CO_2$ in oxygen being passed through the aerobic cultures. After growth, the cultures were treated as described under "Methods." The abbreviations used are; HK, hexokinase; PHISO, P-glucoisomerase; PFK, P-fructokinase; PK, pyruvate kinase.

| Culture | Generation time | Enzyme activity | | | |
|---|---|---|---|---|---|
| | | HK | PHISO | PFK | PK |
| | *min* | *units/mg protein* | | | |
| Acetate, aerobic | 120 | 0.23 | 0.82 | 0.03 | 0.38 |
| Acetate + glucose, aerobic | 60 | 1.03 | 2.26 | 0.28 | 1.43 |
| Acetate + glucose, anaerobic | 90 | 0.93 | 2.82 | 0.34 | 1.30 |

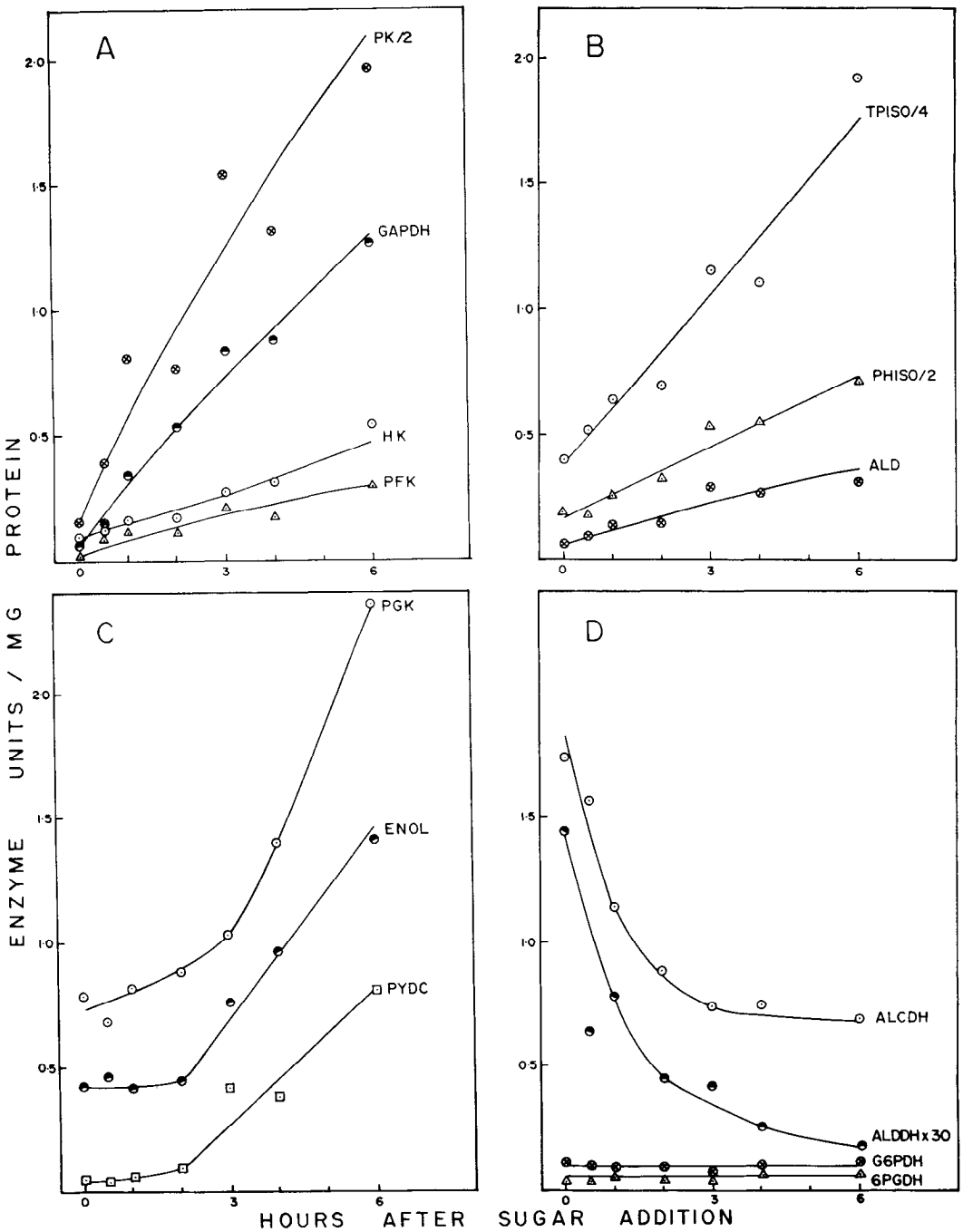


FIG. 2. Kinetics of glycolytic enzyme changes following glucose addition to a growing culture of the hybrid yeast. At time 0, 100 mM glucose was added to all of the flasks containing yeast suspension growing exponentially in yeast extract-peptone-acetate medium. To avoid overlap of the curves, constant factors were incorporated in the case of some enzymes. During the 6 hours of induction, the culture went through four and one-half generations. Hexokinase (*HK*), glucose-6-P dehydrogenase (*G6PDH*), and gluconate-6-P dehydrogenase (*6PGDH*) were assayed after overnight storage at 0°. *PGK*, P-glycerate kinase; *PFK*, P-fructokinase; *PYDC*, pyruvate decarboxylase; *ALDDH*, aldehyde dehydrogenase; *ALCDH*, alcohol dehydrogenase; *GAPDH*, glyceraldehyde-3-P dehydrogenase; *ALD*, aldolase; *ENOL*, enolase; *PK*, pyruvate kinase; *TPISO*, triose-P isomerase; *PHISO*, P-glucoisomerase.

levels of these two enzymes. Fig. 3 depicts an experiment in which the kinetics of changes of these two enzymes and of galactokinase was studied. Before addition of galactose, the specific activity of hexokinase and galactokinase was constant, while that of alcohol dehydrogenase was rising slowly. Immediately after galactose addition, galactokinase started rising linearly and alcohol dehydrogenase activity decreased, as with glucose. After the 6th hour following galactose addition, there ensued a sudden synthesis of alcohol dehydrogenase. Hexokinase activity remained unchanged until it started rising after 4 hours (2 doublings). The growth rate on galactose was constant with a doubling time of 110 min.

In order to resolve the temporal relationship between various glycolytic enzymes during their induction with galactose, ex-

periments similar to those described in Fig. 3 were carried out for the entire complement of glycolytic enzymes. Results in Fig. 4 illustrate a typical experiment. P-Glucomutase, P-fructokinase, glyceraldehyde-3-P dehydrogenase, and pyruvate kinase seemed to increase in relative activity from the moment the first sample was withdrawn at 30 min, and they continued to increase until the end; glyceraldehyde-3-P dehydrogenase, however, seemed to increase at an accelerating rate. There was a definite rise of activity within the 1st hour in this as well as in other experiments. P-Glucoisomerase, P-glycerate mutase, and enolase increased after the 2nd hour, hexokinase, aldolase, and P-glycerate kinase increased after the 3rd hour, and triose P-isomerase and pyruvate decarboxylase increased after the 4th hour. Aldehyde dehydrogenase was repressed monotonically, while alcohol dehydrogenase behaved as shown in Fig. 3. Glucose-6-P dehydrogenase and gluconate-6-P dehydrogenase curves have been drawn parallel to the time axis, although we cannot rule out the possibility that these activities do not oscillate with time. In any case, they behaved very differently compared with the other enzymes shown in Fig. 4. We should point out that the rate of synthesis of enzymes in this particular experiment was relatively sluggish compared with a few others performed under very similar conditions; the other element of variability in these experiments is the time of increased synthesis of the various enzymes. The following is a summary of the intervals since galactose addition during which time no increased enzyme synthesis took place.

This is based on the results of five experiments with galactose with a maximal sampling interval of 1 hour. Less than 0.5 hour, galactokinase and pyruvate kinase; 1 hour or less, P-glucomutase, P-fructokinase, and glyceraldehyde-3-P dehydrogenase; between 1 and 3 hours, P-glucoisomerase, P-glycerate kinase, P-glycerate mutase, and enolase; between 2 and 5 hours, hexokinase, aldolase, triose-P isomerase, and pyruvate decarboxylase. Continued presence of galactose is necessary for the increases in specific activity to take place. Thus, when galactose was removed from a culture that had grown for only 2 hours on this sugar, the specific activity of hexokinase did not increase over the basal level during subsequent growth for 6 hours.

In the experiments described above, enzyme activity had been measured in cell-free extracts prepared by a French press. Since this required handling of a relatively large mass of cells, each point on the curves shown in Figs. 2 through 4 represents a population of cells from separate flasks. Later experiments with toluene-disrupted cells allowed examination of a larger number of samples from the same flask. Such experiments yielded results showing the same pattern of enzyme appearance as reported in Figs. 2 through 4. In one such experiment with galactose as the inducer, the increase in the differential rate of pyruvate kinase synthesis took place within 20 min of galactose addition, while that for P-glucoisomerase and pyruvate decarboxylase took place after the culture underwent 1.4 and 3 doublings, respectively. The lag period was also found to be independent of the density of the culture.

*Kinetics of Glycolytic Enzyme Changes upon Removal of Sugar*—The effect of removing the sugar from a cell suspension actively synthesizing glycolytic enzymes was investigated next. Results shown in Fig. 5 are those of one such experiment. A culture of the hybrid yeast growing on yeast extract-peptone-acetate medium was divided in two parts, to one of which fructose was added. The differential rate of formation of pyruvate

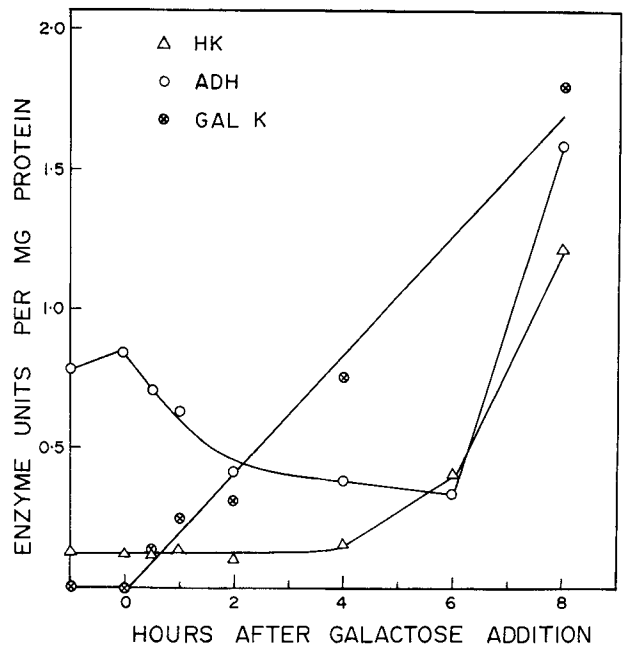


FIG. 3. Time course of changes of galactokinase (*GAL K*), hexokinase (*HK*), and alcohol dehydrogenase (*ADH*) of *S. fragilis* × *S. dobzhanskii* during growth on galactose. To a culture growing exponentially on yeast extract-peptone-acetate medium, 50 mM galactose was added at the time marked 0. A flask representing each point on the time axis was taken out at intervals, and the cells were processed for enzyme assay as described under "Methods." Note that the curves start with a sample taken 1 hour before the addition of galactose. The culture went through 4.3 doublings during the 8 hours since galactose addition.

kinase measured on toluenized cells was estimated by dividing the observed enzyme activity per ml of culture by its extinction ($E_{650}$). A plot of this quantity against time is shown in Fig. 5. Fructose addition increased the specific activity of pyruvate kinase within 10 min, and this increase continued until fructose was removed. Soon after fructose removal, the increase of enzyme specific activity was halted and, as the culture resumed exponential growth in the new medium, the specific activity of pyruvate kinase started decreasing. The drop in pyruvate kinase specific activity at the end of the experiment could be nearly accounted for by the dilution resulting from growth. The discrepancy perhaps reflects the basal synthesis in the absence of fructose. The control culture that did not receive fructose continued to grow with constant specific activity.

A similar experiment in which enzyme activity was determined following removal of galactose from a culture growing exponentially on this sugar is indicated in Fig. 6. To ensure induction of all of the enzymes, the culture was grown overnight on galactose, following which it was regrown in fresh galactose medium for 2 doublings before galactose was removed. The specific activity of most of the enzymes started falling from the moment the cells began to grow in the new medium. Only P-glycerate kinase and pyruvate kinase suffered exponential dilution with half-times of 3.5 and 3.3 hours, respectively, while the doubling time in this medium was nearly 2 hours. This is presumably due to the semiconstitutive nature of the glycolytic system. For all of the enzymes shown in the upper half of Fig. 6, however, the dilution rate was still lower than the growth rate. On the other hand, hexokinase, pyruvate decarboxylase, and

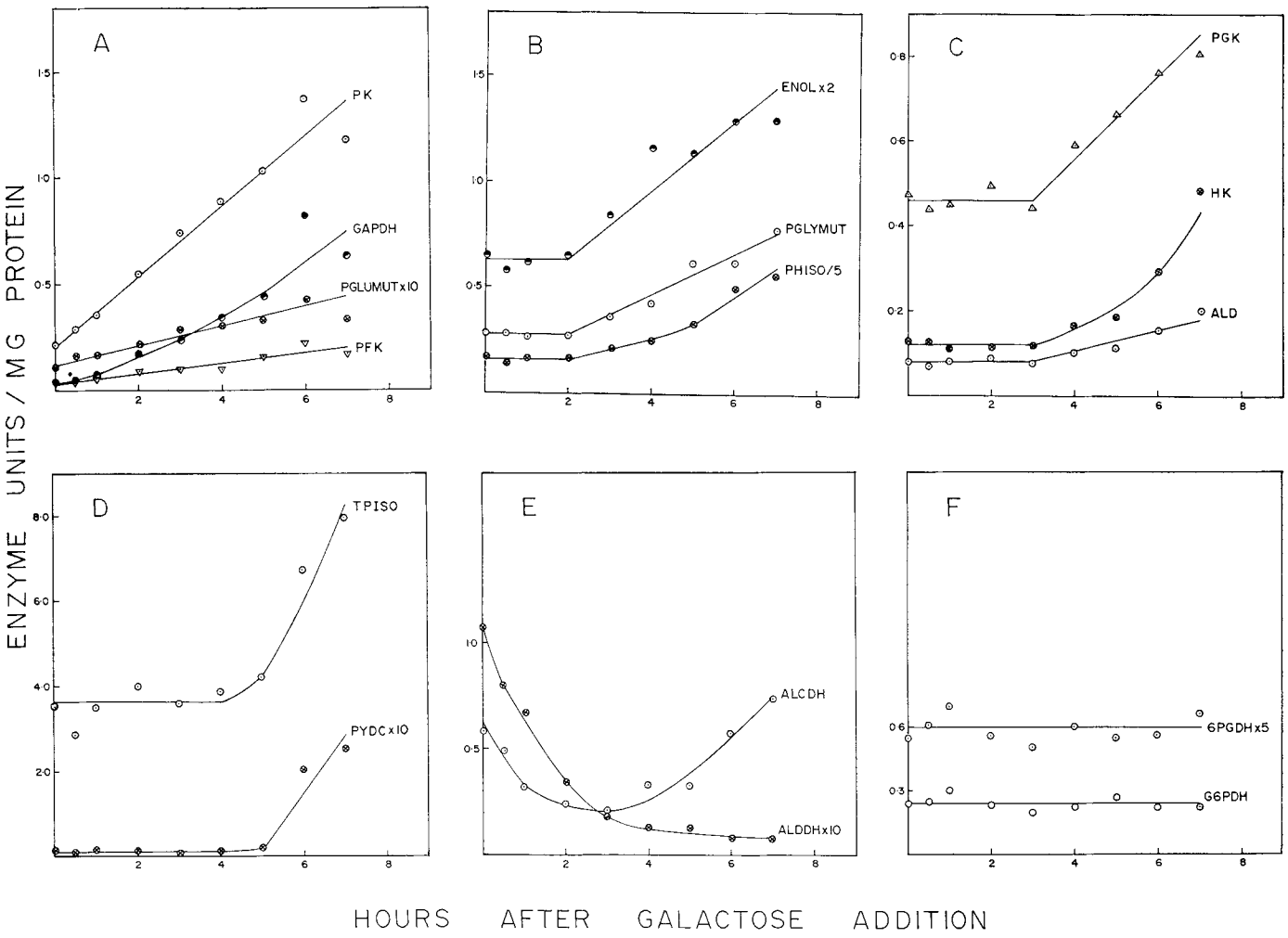


FIG. 4. Kinetics of glycolytic enzyme synthesis in the hybrid yeast during induction by galactose. Each *point* refers to analysis of cell extract from a single set of flasks. Galactose (50 mM) was added at time marked 0. The initial concentration of glucose in the flasks was 0.01 mM carried over as a contaminant in galactose; the number of yeast cells present was such that this should have been consumed within 4 min. The culture underwent 4 doublings during the course of the experiment. Data for some enzymes have been multiplied by factors to minimize overlap of curves. Alcohol dehydrogenase and aldehyde dehydrogenase were assayed in samples stored overnight at 0°. At least two-thirds of the added galactose remained unutilized at the end of the experiment. *PK*, pyruvate kinase; *HK*, hexokinase; *PFK*, P-fructokinase; *PGK*, P-glycerate kinase; *PYDC*, pyruvate decarboxylase; *PGLUMUT*, P-glucomutase; *PGLYMUT*, P-glycerate mutase; *PHISO*, P-glucoisomerase; *TPISO*, triose-P isomerase; *ALD*, aldolase; *ALCDH*, alcohol dehydrogenase; *ALDDH*, aldehyde dehydrogenase; *GAPDH*, glyceraldehyde-3-P dehydrogenase; *G6PDH*, glucose-6-P dehydrogenase; *6PGDH*, gluconate-6-P dehydrogenase; *ENOL*, enolase.

P-fructokinase required nearly a generation before being subject to exponential dilution by growth. A very similar decay pattern in specific activity of all glycolytic enzymes was observed when glucose was removed from an exponentially growing culture of the hybrid yeast. On removal of glucose following 5-hour growth on this sugar, there was a 90-min delay before exponential decay of enzyme specific activity set in. Cell counts during this period indicated that the culture went through 0.8 doubling. Obviously some residual synthesis in excess of the endogenous rate was continuing even in the absence of glucose.

*Pattern of Glycolytic Intermediates during Induction*—The fast chemical transformations undergone in the yeast by sugars raise questions regarding the identity of the inducing metabolite or metabolites. The inducibility of glycolytic enzymes by sugar analogues, as reported in the accompanying paper (12), throws some light in this direction. Here we have attempted to measure some metabolites during growth of the hybrid yeast on glucose and galactose. The delayed appearance of the enzymes during growth on galactose makes such a study particularly relevant.

There are two sources of difficulty in such measurements. The small concentration of cells in the growing medium and the high background fluorescence of this medium render estimates of glycolytic intermediates unreliable. On the other hand, the rapid metabolic turnover of these compounds does not permit concentration of the cells by centrifugation prior to acid extraction. We have therefore chosen to examine the capability of the cell suspension at various stages of growth to maintain levels of glycolytic intermediates, rather than trying to estimate their steady state levels during induction. To this end, cells of the hybrid yeast concentrated from the induction media were treated with glucose or galactose for a fixed period of time and then extracted with acid. Assay of a number of glycolytic intermediates in the acid extract of cells growing on glucose or galactose is shown in Fig. 7. The results indicate a profile of

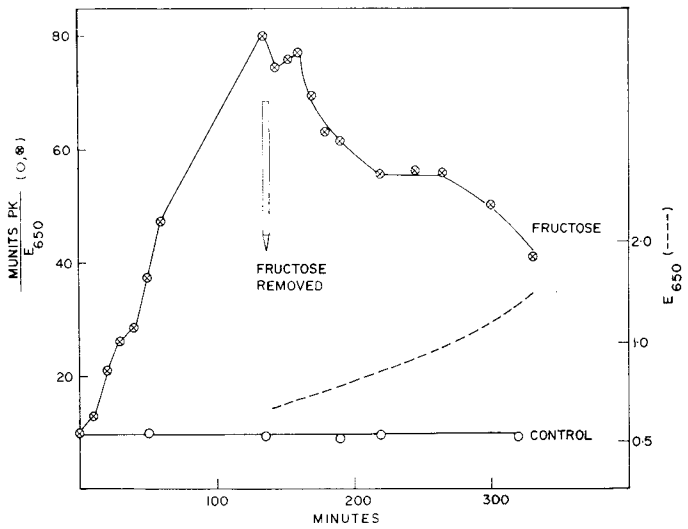


FIG. 5. Induction of pyruvate kinase (*PK*) of the hybrid yeast by fructose and its disappearance on removal of the sugar. Fructose was added at time 0 and removed as shown by the *arrow* by membrane filtration of the cells and subsequent washing and resuspension in yeast extract-peptone-acetate medium. The curve marked *control* indicates a parallel culture not containing fructose. – – –, extinction of the culture at 650 mμ ($E_{650}$). At each instant indicated, an aliquot was removed, its $E_{650}$ was measured, and the centrifuged cell pellet was toluenized as described under "Methods." *Munits*, milliunits.

the levels of the early glycolytic intermediates when cell suspensions harvested under conditions of induction as in Figs. 2 and 4 are exposed to glucose or galactose for a period of 10 min. Perhaps because of the basal level of the enzymes in uninduced cultures, the concentrations of the aldolase metabolites and also of glucose-6-P are quite high at the 30-min point for both the cultures, although the induction of enzymes is far from maximal. Nevertheless, the relative difference between the two sugars is indicated by these data. The levels of glucose-1-P are distinctly higher in the presence of galactose than in the presence of glucose at all periods. For glucose-6-P and fructose-1,6-$P_2$ plus triose phosphates, the rates of appearance are slower in the galactose medium, although the final levels are no less than in glucose. The other feature of these data relates to the equilibration between glucose-6-P and glucose-1-P. In the galactose culture, the ratio of their concentrations is closer to equilibrium (13) than in the glucose culture. This is consistent with the observed induction of P-glucomutase by galactose. When similar experiments were performed by incubating cells in the yeast extract-peptone medium instead of in buffered sugar solutions, the same trend was observed; the levels of the intermediates, however, were lower by a factor of 2. When cells growing in the absence of sugar were incubated for similar periods in yeast extract-peptone-acetate, they were found to contain 0.7 to 0.9 μmole of glucose-6-P per g of wet yeast. When the level of glucose in the growth media was examined during growth on 50 mM galactose, a slow rise was observed during a 10-hour period. In a particular experiment, the level of glucose rose from an initial level of 4 to 38 μM at the end of 8 hours and then fell abruptly. The fall in the concentration of glucose coincided with the onset of a significant rise of activity of hexokinase in the cells. The control medium lacking galactose showed a constant level of 4 μM glucose during this time.

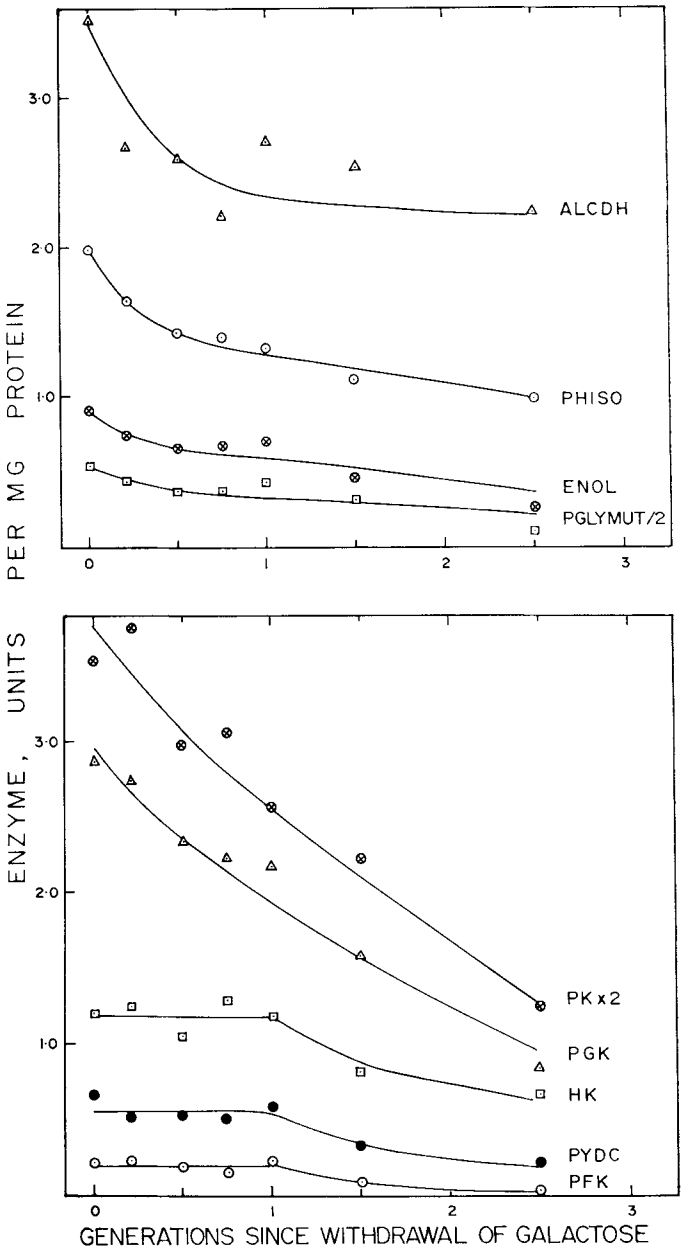


FIG. 6. Effect of galactose removal on the specific activity of several glycolytic enzymes of the hybrid yeast. An overnight culture grown in the presence of 50 mM galactose was washed and freshly diluted in the same medium containing galactose. After nearly 2 doublings, galactose was removed as described under "Methods," and the culture was allowed to grow in yeast extract-peptone-acetate medium. Flasks were withdrawn at the indicated periods. The doubling time in the absence of galactose was 120 min. Hexokinase, P-glucoisomerase, P-fructokinase, and P-glycerate kinase were estimated in samples stored overnight in an ice bath; all others were assayed on the fresh extracts. *ALCDH*, alcohol dehydrogenase; *PHISO*, P-glucoisomerase; *ENOL*, enolase; *PGLYMUT*, P-glycerate mutase; *PK*, pyruvate kinase; *PGK*, P-glycerate kinase; *HK*, hexokinase; *PYDC*, pyruvate decarboxylase; *PFK*, P-fructokinase.

*Isozyme Analysis of Constitutive and Induced Glycolytic Enzymes*—Two distinct modes of regulation of a semiconstitutive enzyme system are possible. (*a*) One involves a single protein amenable to low levels of internal induction in the absence of added inducer and high levels in its presence. (*b*) The other

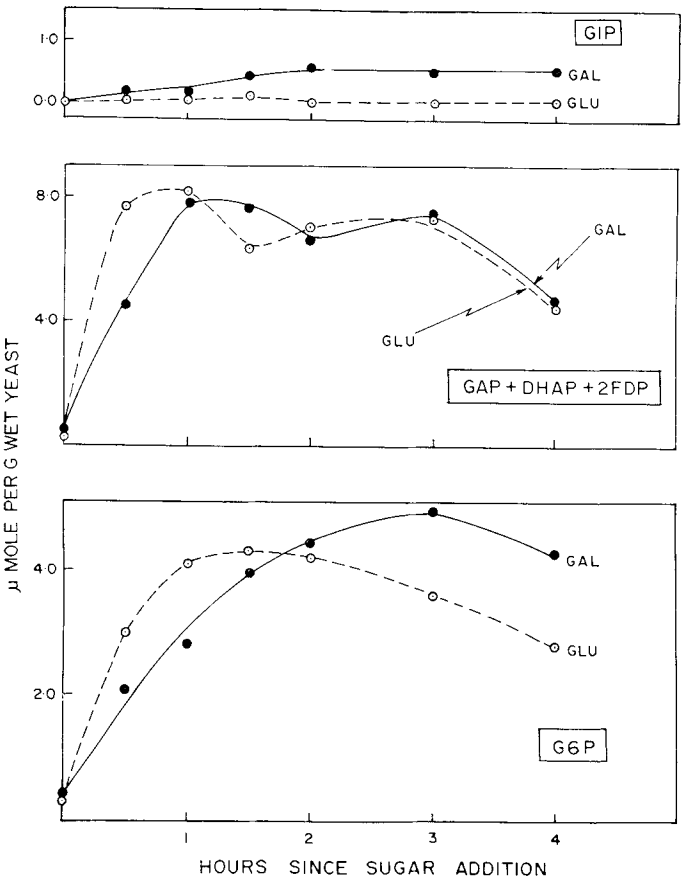


FIG. 7. Time course of certain glycolytic intermediates in hybrid yeast cells grown on glucose or galactose. One part of a culture growing exponentially on yeast extract-peptone-acetate was treated with 50 mM glucose (*GLU*) and the other part with 50 mM galactose (*GAL*), and the suspension was allowed to grow. At various instants indicated by the *experimental points*, 10 to 30 ml of the suspension were removed, centrifuged quickly at room temperature, and suspended in 2 ml of a mixture containing 25 mM triethanolamine, 20 mM potassium phosphate, pH 7.4, and 50 mM either glucose or galactose. The sample at 0 hour was incubated without added sugars. The entire process took about 3 min. A stream of water-saturated oxygen containing 5% $CO_2$ was passed slowly for 10 min, after which dilute perchloric acid was added to acidify the suspension to 0.7 N. The tubes were allowed to stand for 10 min before being chilled in ice. Estimation of glycolytic intermediates was made in the neutralized supernatant by fluorometry. Cell count was made on each of the suspensions in order to estimate the cell mass. *G1P*, glucose-1-P; *G6P*, glucose-6-P; *GAP + DHAP + 2FDP*, the sum of aldolase metabolites, measured as triose.

mode may involve a double set of enzymes, one of which is constitutive and not subject to quantitative modulation, the second of which is exclusively inducible and present only in induced culture. To distinguish between these two possibilities, a semiquantitative analysis of the isozymes of a few glycolytic enzymes of the hybrid yeast was carried out. Results shown in Fig. 8 are those of such an experiment. Here we show the relative proportions of the isozymes of hexokinase and triose-P isomerase on electrophoresis in a polyacrylamide gel. The photograph in the inset shows a typical pattern of hexokinase isozymes present in the crude extracts of the hybrid yeast grown in the absence and in the presence of glucose. When the extract was treated with the proteolytic inhibitor, phenylmethanesulfonyl fluoride (14), three bands containing hexokinase activity appeared; two of these comprised the bulk of the enzyme, while the third, a faster moving band, was a minor component. Fig. 8, *A* and *B*, shows their relative amounts obtained from fluorometric assay, the area bounded by each fraction being measured with a planimeter. The results show that the two major bands of hexokinase, in the order of their increasing mobility, were in the proportion of 9 and 8 milliunits in the uninduced extract (*A*) and 44 and 39 milliunits in the extract from the culture induced with glucose (*B*). Although the third minor band of hexokinase could not be measured with sufficient accuracy in the uninduced extract, it seemed to constitute no more than 5% of the total hexokinase activity in both the induced and in the uninduced cultures. In other words, the extent of induction of all of the hexokinase isozymes was the same. On the other hand, the results shown in the lower half of Fig. 8 (*C* and *D*) indicate that, of the two isozymes of triose-P isomerase, the one with a lower mobility constituted a third of the total activity in the uninduced culture (*C*), their amounts being 110 and 220 milliunits for the slower and faster bands, respectively. In the induced culture grown with glucose (*D*), however, the slowly moving isozyme of triose-P isomerase was 480 milliunits and that of the faster moving band 220 milliunits, indicating thereby a preferential induction of the slowly moving isozyme by glucose. Since the amount of the protein used was the same for both the uninduced and induced extracts, it appears that the faster moving band of triose-P isomerase was not induced at all by glucose. Similar experiments, not shown here, indicated that P-glucoisomerase and P-glycerate kinase behave as does hexokinase; two bands were observed in each case and both of these seemed to be induced equally by glucose. When hexokinase of cultures induced with galactose was examined, the band pattern was found to be indistinguishable from that of glucose-grown cultures. These results suggest that induction of glycolytic enzymes by sugars does not involve synthesis of a species of enzyme that is absent in uninduced culture, and to this extent these results eliminate possibility *b* outlined above.

*Effect of Inhibitors of Protein Synthesis on Induction of Glycolytic Enzymes in Yeasts*—No specific inhibitor of protein synthesis could be found that acts on intact cells of the hybrid yeast. Among a number of compounds tried, only L-ethionine at 50 μg per ml inhibited glucose-induced increase of glycolytic enzymes nearly 60%. Actinomycin D (25 μg per ml), rifampin (50 μg per ml), 5-bromouracil (50 μg per ml), *p*-fluorophenylalanine (50 μg per ml), and cycloheximide (50 μg per ml) were found inactive. Although cycloheximide inhibits protein synthesis in *S. cerevisiae* (17), it fails to do so in *S. fragilis* (18). We therefore examined the effect of cycloheximide on the glucose-linked induction of glycolytic enzymes in *S. cerevisiae*. Exponentially growing cultures of a haploid strain of this organism were treated with cycloheximide, and the synthesis of enzymes was followed as a function of increase in yeast mass. This compound promptly arrested both the glucose-induced and the constitutive synthesis of glycolytic enzymes. For most of the enzymes, there was a fall in the total activity, suggesting metabolic turnover of the enzyme proteins.

Results shown in Fig. 9 illustrate the effect of cycloheximide on the differential synthesis of glycolytic enzymes. All of the glycolytic enzymes from hexokinase to pyruvate decarboxylase were assayed in this particular experiment, although we show

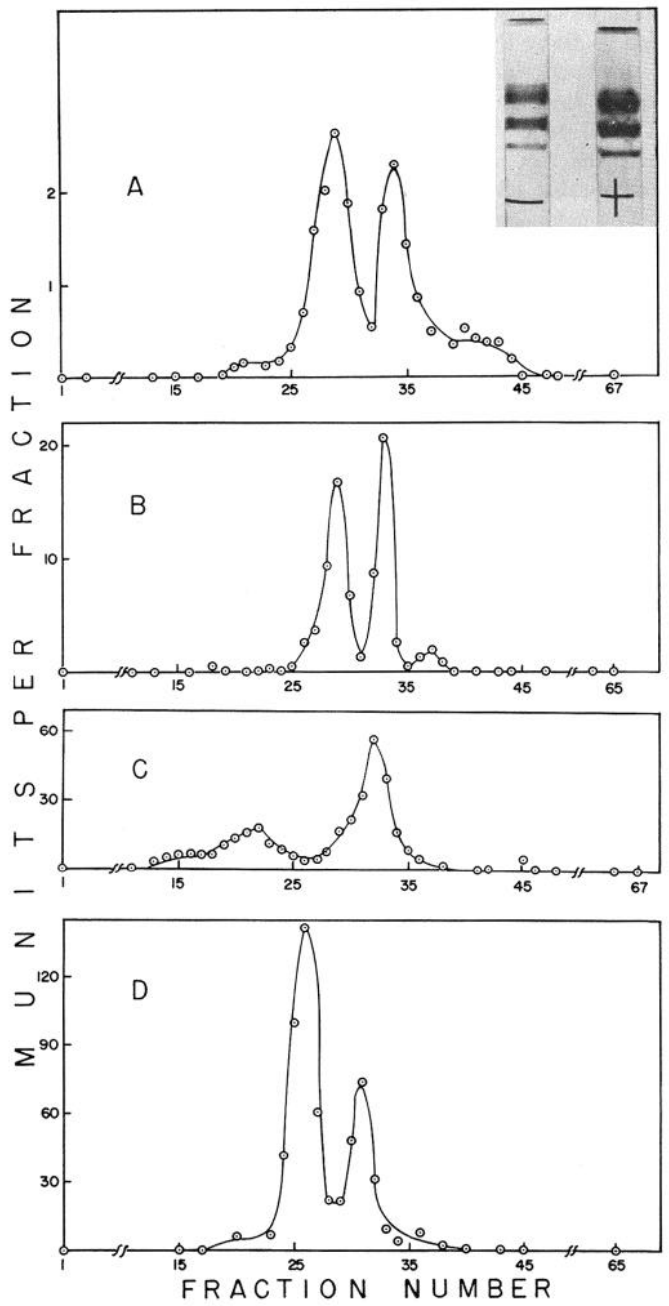


FIG. 8. Relative proportions of isozymes of hexokinase and triose-P isomerase of the hybrid yeast grown in the absence and in the presence of glucose. Exponentially growing cultures of *S. fragilis* × *S. dobzhanskii* from yeast extract-peptone-acetate medium with and without glucose were extracted by a French press as described under "Methods." The extracting buffer contained in addition 2 mM phenylmethanesulfonyl fluoride to prevent proteolysis (14). The centrifuged extracts were subjected to disc electrophoresis in a bed of 7.5% polyacrylamide in Tris-glycine buffer at pH 8.8 (15), with approximately 150 μg of protein per each gel. The buffers for making the gels contained 2 mM β-mercaptoethanol and 1 mM EDTA. At the end of electrophoresis, the gels were frozen and cut into slices of 1 mm in thickness by a razor blade assembly. Each slice was shaken up in 0.5 ml of 50 mM triethanolamine buffer, pH 7.4, containing 10 mM glucose, and left at 24° for 30 min. Aliquots (25 to 100 μl) were used for assay as described under "Methods" in a total volume of 0.8 ml. In *A*, assays were made by incubating 100 μl of the enzyme extract in 0.5 ml of the assay mixture and measuring the fluorescence after 30 min. *A*, hexokinase of uninduced cells; *B*, hexokinase of induced cells; *C*, triose-P isomerase of uninduced culture; *D*, triose-P isomerase of induced cells. Fractions are numbered from the cathodal (*1*) to the anodal (*65* and *67*) ends. *Inset*, a photograph of gels developed for hexokinase activity by nitroblue tetrazolium (16); −, extract of cells grown without glucose; +, that with glucose. The cathodal end is at the *top* and the anodal at the *bottom*, migration being in the direction from cathode to anode.

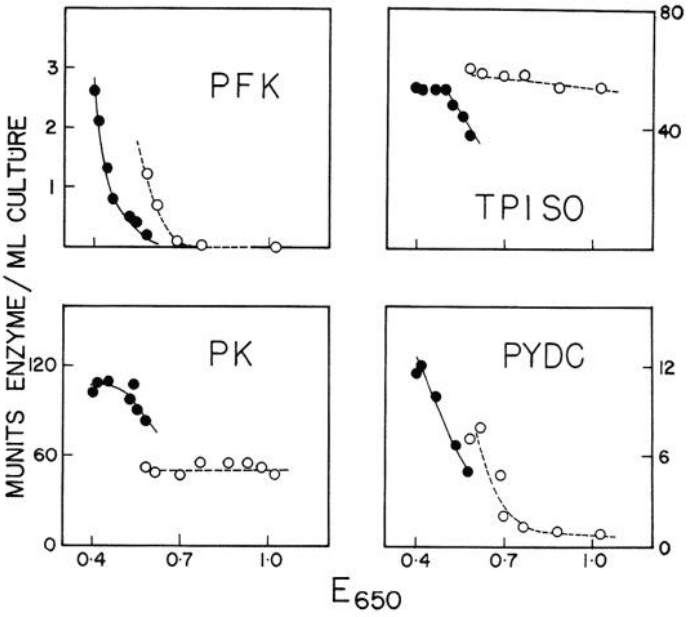


FIG. 9. Effect of cycloheximide on the synthesis of several glycolytic enzymes in *S. cerevisiae* during growth in the presence and absence of glucose. O- - -O, culture without glucose; ●——●, that with glucose. Overnight cultures from yeast extract-peptone-acetate medium growing in the absence and presence of glucose were diluted freshly in the respective media. Upon resumption of exponential growth, cycloheximide (10 μg per ml) was added to both. Aliquots withdrawn periodically were assayed for enzymes in the toluenized cells as described under "Methods." Enzyme activities per ml of the culture medium were plotted against growth, measured by extinction at 650 mμ ($E_{650}$). The total span of growth for both the cultures covered 7 hours after addition of the antibiotic. Pyruvate kinase was assayed 24 hours after toluenization, and the rest were assayed after 48 hours. Samples were stored in crushed ice during the 2 days. *Munits*, milliunits; *PFK*, P-fructokinase; *TPISO*, triose-P isomerase; *PK*, pyruvate kinase; *PYDC*, pyruvate decarboxylase.

here the results of only triose-P isomerase, P-fructokinase, pyruvate kinase, and pyruvate decarboxylase as representatives of the various patterns of actidione-induced changes. At the concentration of cycloheximide used, growth was not completely arrested, although amino acid incorporation in proteins of this yeast is inhibited 95% in 3 min by 5 μg of cycloheximide per ml (17). While control cultures were in a steady state of enzyme synthesis as shown by linear differential plots, addition of cycloheximide prevented any further rise of enzyme activity. Most of the enzymes, however, showed a decay in their total activity. The decay was most marked in the case of P-fructokinase, and least in the case of constitutive synthesis of pyruvate kinase. In case of triose-P isomerase, the glucose-treated culture indicated decay of enzyme activity only after an initial lag. P-Glycerate kinase behavior was similar to that of triose-P isomerase, although its decay in absence of glucose was more pronounced, the half-time of decay of total activity ($t_{\frac{1}{2}}$) being

5.5 hours. The $t_{\frac{1}{2}}$ for glyceraldehyde-3-P dehydrogenase decay was 3 hours in the presence of glucose and 2 hours in its absence. For hexokinase, P-glucoisomerase, aldolase, P-glycerate mutase, and enolase, the decay was slower, with $t_{\frac{1}{2}}$ greater than 7 hours. Pyruvate decarboxylase decay, like that of P-fructokinase, appeared faster in the absence of glucose than in its presence. The doubling time of the yeast in the absence of cycloheximide in this medium was 1.7 and 3.8 hours, respectively, in the presence and in the absence of glucose. The decay of most of these enzymes is therefore slow compared with their generation time. Cycloheximide was also found to bring about 60% inhibition of glucose-linked increase of pyruvate kinase in *C. albicans*.

## DISCUSSION

The glycolytic pathway is one of those instances in which a biochemical sequence is driven either way. When hexoses are the sole source of carbon, the direction of metabolic flow is from glucose-6-P to pyruvate, whereas growth in absence of added sugar requires a reversed flow, leading to the synthesis of precursors of certain amino acids, structural carbohydrates, glycerides, nucleic acids, or aromatic skeletons. The difference in the metabolic rates in the two directions is matched by the relative difference between the activity of, for example, fructose diphosphatase and P-glucoisomerase of tissues (19). Questions have been raised regarding the role of glyceraldehyde-3-P dehydrogenase in gluconeogenesis in *Pseudomonas putida* because of the very low activity found in cultures grown on succinate, lactate, or acetate (20). Cultures of the hybrid yeast behave similarly. In neither of these cases, however, has it been determined whether the low activity is a result of inhibition of the enzyme by 1,3-$P_2$ glycerate (21) maintained at high concentrations during the assay. Results in this paper bear out the expected quantitative mode of regulation of glycolytic enzymes, depending on whether carbohydrates or gluconeogenic compounds serve as the major source of carbon. A 3- to 100-fold increase in specific activity of various glycolytic enzymes resulted from the addition of glucose or galactose to a culture of *S. fragilis* × *S. dobzhanskii* growing in the absence of added sugars. A minimal average value by which the specific activity of most of the enzymes increases lies between 3- and 7-fold the basal level, while for pyruvate decarboxylase and glyceraldehyde-3-P dehydrogenase, the increase is 50- and 70-fold, respectively, of the basal specific activity. With the exception of P-glucomutase, which was not induced by glucose, the increase in specific activity of all of the glycolytic enzymes in the stationary state was the same with glucose as with galactose.

Alcohol dehydrogenase and aldehyde dehydrogenase behaved differently. The bulk of their activity was repressed by sugars. Alcohol dehydrogenase synthesis, however, continued even in the presence of glucose, although at a reduced rate, as shown by calculating the total enzyme activity in experiments such as the one shown in Fig. 2. This is evident also from experiments shown in Fig. 3, in which galactose was found to bring about, after an initial period of repression, an increase in its specific activity. Whether this galactose-induced enzyme is the same as the fermentative alcohol dehydrogenase I as described by Schimpfessel (22) for *S. cerevisiae* has not been determined.

The parallel relationship between hexokinase and the other glycolytic enzymes and the low limiting value of the majority of these at the point at which hexokinase extrapolates to zero, points to the coordinate nature of their regulation. The exception is triose-P isomerase. Although its parallel relationship with other enzymes is indicated by the strong correlation observed, the significantly positive value of the $y$ axis intercept (Fig. 1) suggests multiplicity of its regulation elements. In fact, the results of gel electrophoresis experiments (Fig. 8) indicate that triose-P isomerase of the hybrid yeast is composed of two components, only one of which is inducible by glucose, while the other is not. The noninducible component is the major fraction in the uninduced cells. This is unlike hexokinase, P-glucoisomerase, or P-glycerate kinase, all of whose isozymes seem to be induced by glucose to the same extent. Even for the others, the coordinacy is true only in the stationary state, as the later results in Fig. 2 indicate a breakdown of this parallel relationship when the early kinetics of their induction is examined. Clearly, P-glycerate kinase, enolase, and pyruvate decarboxylase are induced by glucose with a greater delay than, for example, pyruvate kinase. This noncoordinate relationship is particularly evident in the experiments with galactose, in which at least four induction groups can be resolved by time course alone. This delayed induction pattern permits one to draw only a restrictive conclusion regarding the coordinate nature of glycolytic enzymes. Either the enzymes are all induced by the same metabolite or, if the inducing metabolites are different, they are derived from the same precursor compound. In the stationary state, all of the enzymes would appear to be coordinately linked, while in short term experiments the relative delay between enzymes will be a function of the rate at which the inducer is produced from the common precursor or will reflect some other rate-limiting step in the process of active enzyme formation.

The delayed appearance of some of the enzymes in the presence of galactose compared with that with glucose is possibly due to the slower metabolism of galactose as shown by the rate of alcohol production in separate experiments. Results in Fig. 7 may therefore be interpreted to mean that glucose-1-P, or any of its precursor metabolites in the galactose pathway, is not responsible for the synthesis of the glycolytic enzymes induced by glucose, while compounds at the level of glucose-6-P or further below in the glycolytic sequence might be mediators of the induction. The experiments shown in Fig. 6, however, indicate that the time sequence of enzyme appearance is not related to the kinetics of disappearance on removal of galactose. Thus, P-fructokinase, an "early" enzyme with galactose, follows the same decay kinetics as hexokinase or pyruvate decarboxylase, which appear late with galactose. This only suggests that, even among groups of enzymes appearing together under one condition, multiple control elements exist. In effect, the removal of the true inducing compound, particularly if it is a glycolytic intermediate or a neighbouring metabolite, is much slower than is the removal of sugars, as glycolysis may persist long after sugars may have been removed. Our results, rather than specifying the number of regulation groups in glycolytic enzymes, serve to put lower limits on this quantity, determined somewhat arbitrarily by the number of samples examined during the sugar-induced transient. It is interesting to note that the grouping of glycolytic enzymes of *Candida parapsilosis* determined by Hommes (4) bears little resemblance to the kinetic groups resolved in these studies. The experiments on the ap-

pearance of glycolytic enzymes with galactose, however, eliminate a simple sequential mode of induction, as shown by the lack of correspondence between the time sequence of enzyme synthesis and the biochemical sequence of galactose metabolism.

It has been shown by Segal and Kim (23) that the time taken by a pre-existing enzyme to rise half-way to a new steady state level is an inverse function of its degradation rate. As a result, enzymes with equal extent of stimulation of their rate of synthesis following an act of induction may differ in the time of their rise to the higher steady state level. It may appear at first sight that the delay in rise of specific activity of the various glycolytic enzymes by galactose merely reflects their different decay rates. The constancy of specific activity of the "late" enzymes (Fig. 4) until a rise occurs, however, suggests that this is not the case. That is, the observed lag in the synthesis with galactose of the late glycolytic enzymes of the hybrid yeast is a true measure of the delay in their induced synthesis. Although we ascribe the delay to the slower rate of attainment of the maximal glucose-6-P level during growth on galactose, the physiological significance of a three-generation delay between the rise of pyruvate kinase and that of pyruvate decarboxylase is far from clear. We would nevertheless like to interpret the results in Fig. 4 as a reflection of the requirement of graded concentrations of metabolites at or below the level of glucose-6-P for the induction of the various temporal groups. The ones which appear early, *viz.* pyruvate kinase, P-fructokinase, or glyceraldehyde-3-P dehydrogenase, should require smaller levels of these inducing metabolites than the ones appearing late, *e.g.* P-glycerate kinase, triose-P isomerase, or pyruvate decarboxylase. With glucose as the substrate, they all increase in specific activity from the beginning as a result of faster rise in the inducer concentration. The nonlinear response curve of the *lac* repressor-operator complex to varying concentrations of the *lac* inducer (24) makes such an interpretation appear plausible. In the uninduced culture, therefore, in which the level of glucose-6-P and later glycolytic metabolites is low (Fig. 7), the enzymes appearing early with galactose are expected in larger amounts than those induced late; no such correlation is, however, seen on calculating the number of enzyme molecules from data presented in Table I. We therefore think that the multiplicity of control parameters of glycolytic enzymes, such as their intracellular turnover, their isozymic nature, and the varying response of some of these to the inducing action of glucose (Fig. 8), might be responsible for the observed deviation. We should mention here that, although there is a direct relationship between the level of glycolytic enzymes of a yeast culture growing in the presence of glucose and galactose and the percentage of budding cells, as the data of Beck and von Meyenburg indicate (25), the parallelism breaks down when one studies the induction by 2-deoxyglucose. Over a period of 4 hours of growth of the hybrid yeast in presence of 0.3 mM 2-deoxyglucose, only 25% of the cells were budding, whereas the culture without the glucose analogue contained as much as 90% budding cells. Yet the differential rate of glycolytic enzyme synthesis was much higher in the presence of 2-deoxyglucose than in its absence (12).

We have not attempted here to determine whether the increase in level of glycolytic enzymes of the hybrid yeast effected by metabolizable sugars is a case of true induction involving synthesis of messenger RNA and its subsequent translation. Although cycloheximide failed to inhibit significantly the glucose-induced appearance of glycolytic enzymes in *S. fragilis* × *S. dobzhanskii*, the antibiotic completely prevented such enzyme increases in *S. cerevisiae* (Fig. 9). This drug is known to inhibit translation in *S. cerevisiae*, although it fails to do so in *S. fragilis* (18). At least in *S. cerevisiae*, therefore, glucose-induced increase of glycolytic enzymes presumably involves assembly of amino acids on a ribosome-bound template, *i.e.* synthesis of new protein. This is consistent with the observation that incubation of the hybrid yeast in a buffered glucose medium does not cause any increase in the level of glycolytic enzymes. Whether the primary effect of the inducing compound is at the level of transcription or translation cannot be determined from the present data. However, the decay of glycolytic enzymes when protein synthesis is inhibited by cycloheximide in *S. cerevisiae* adds another dimension to their regulation. The enzyme that suffers the fastest decay is P-fructokinase. A semilogarithmic plot of the specific activity of this enzyme against time since addition of the antibiotic indicates that this enzyme undergoes a biphasic exponential decay; the faster component has a half-life of 27 min in the culture growing in the presence of glucose and 9 min in the culture growing in the absence of glucose. The slower component decays with a half-life of approximately 4 hours. Pyruvate decarboxylase also seems to decay faster when glucose is absent. For the other enzymes, the rate of decay is not much influenced by the presence of glucose in the growth medium. Triose-P isomerase, P-glycerate kinase, and pyruvate kinase decay in the presence of glucose only after a lag period, presumably as a result of secondary degenerative processes. Since the rate of decay in the absence of glucose is no faster than in its presence, mere stabilization by glycolytic metabolites is therefore not the principal mechanism by which glucose elicits increased enzyme activity. Enzymes that behave otherwise, however, such as P-fructokinase and pyruvate decarboxylase, would possibly be subject to such a control.

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