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Control of Glycolytic Enzyme Synthesis in Yeast by Products of the Hexokinase Reaction

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SUMMARY

Addition of 2-deoxyglucose to a culture of the hybrid yeast *Saccharomyces fragilis* × *Saccharomyces dobzhanskii* causes 2- to 200-fold increase in the differential rate of synthesis of all glycolytic enzymes except alcohol dehydrogenase (EC 1.1.1.1). For most enzymes, the stimulation in the rate of synthesis occurs with a delay. The greatest stimulation is observed in the case of glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12), *viz.* about 200-fold, while P-glycerate mutase (EC 2.7.5.3) and enolase (EC 4.2.1.11) show a 2.5-fold increase in the differential rate of synthesis. Glucosone also stimulates the rate of synthesis of hexokinase (EC 2.7.1.1) and glyceraldehyde-3-P dehydrogenase. Generally, glycolytic enzyme synthesis is elicited by all compounds studied which are substrates of yeast hexokinase, while 6-deoxyglucose fails to induce synthesis either in the hybrid yeast or in *Saccharomyces cerevisiae*. The loss of fructose-phosphorylating activity in a hexokinaseless mutant of this yeast is associated with the failure of fructose to induce any of the glycolytic enzymes that fructose induces in the wild type. The differential rates of synthesis of all of the glycolytic enzymes between aldolase (EC 4.1.2.7) and pyruvate kinase (EC 2.7.1.40) increase 6- to 150-fold over the basal rates when glucose is added to a P-glucoisomerase (EC 5.3.1.9)-deficient mutant of *S. cerevisiae*. Experiments with a P-mannoisomerase (EC 5.3.1.8)-deficient mutant of *S. cerevisiae* indicate that hexokinase and P-fructokinase (EC 2.7.1.11) synthesis is stimulated in response to the presence of mannose.

These and other data on the metabolites produced from 2-deoxyglucose have been interpreted to suggest that glucose-6-P is the inducer of the following enzymes: aldolase, triose-P isomerase (EC 5.3.1.1), glyceraldehyde-3-P dehydrogenase, P-glycerate kinase (EC 2.7.2.3), P-glycerate mutase, enolase, and pyruvate kinase. Mannose-6-P is the inducer of hexokinase and, presumably, of P-fructokinase. Pyruvate decarboxylase (EC 4.7.1.1) does not appear to be induced by glucose-6-P. These data are also consistent with P-glucomutase (EC 2.7.5.1) being induced by glucose-1-P.

The regulatory features in the synthesis of enzymes in a pathway effecting metabolic flow in one direction have turned out to be relatively straightforward. In many such cases involving inducible systems, enzyme synthesis is triggered by an early metabolite, usually the product of the first enzyme reaction (1–6). In a process such as glycolysis, however, the situation is rendered more complex, not only by the multiplicity of entry and exit points, but by the ambidirectional nature of such a pathway. That is, a cell growing on glucose as the major source of carbon glycolyzes, let us say, in the forward direction, whereas growth on 2-carbon compounds such as acetate demands a reversed metabolic flow traversing, except for a few steps, the same set of enzymes required in the forward flow. Obviously, the induction pattern in such a pathway cannot be sequential. If then the inducer or inducers are located at one end of the metabolic chain, there appears to be no obvious preference for either the glucose or the pyruvate end.

In this paper, we have addressed ourselves to the question regarding the identity of the inducer or inducers of glycolytic enzymes in two strains of yeast. The observation that all of the glycolytic enzymes except alcohol dehydrogenase (EC 1.1.1.1) can be induced by 2-deoxyglucose has led to the suggestion that either glucose-6-P or a compound metabolically close to this intermediate is the inducer of glycolytic enzymes. This has been borne out by experiments with mutants of *Saccharomyces cerevisiae* lacking P-glucoisomerase (EC 5.3.1.9) and P-mannoisomerase (EC 5.3.1.8).

METHODS

Strains and Media—Media and growth of yeasts have been described previously (7). We have used in this study two wild type yeast stocks; one was a hybrid yeast, *Saccharomyces fragilis* × *Saccharomyces dobzhanskii*, and the other a haploid strain of *S. cerevisiae*. The haploid yeast was used to obtain a hexokinase (EC 2.7.1.1)-deficient mutant, designated 7L1, that was fructose-negative but retained the ability to grow on glucose. The mutagenic treatment and procedures for isolation of the mutant have been described earlier (8). Another mutant of the haploid yeast, designated *gal-3*, isolated as a galactose-negative clone, was also used in some experiments. This mutant is unable to grow on galactose as the only source of carbon; it grows, however, as does its wild type parent, on a yeast extract-peptone-acetate medium, and the growth is not influenced by galactose. The loss of its ability to grow on galactose is not a consequence of a defect in galactose permease, as incubation of cells harvested from yeast extract-peptone-acetate-galactose medium in 20 mM galactose causes it to accumulate nearly 5 μmoles of galactose

per g of wet cells in the course of 10 min. A mutant clone of the haploid yeast, designated 9520*b*, that lacks P-glucoisomerase was isolated by mutagenesis of the wild type *S. cerevisiae* with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as a glucose-negative and fructose-positive colony. It contained 11 milliunits of P-glucoisomerase activity per mg of protein, thus retaining only 0.9% of P-glucoisomerase activity of the wild type. A number of mannose-negative and glucose-positive mutants was similarly obtained from *S. cerevisiae*, but none was completely devoid of P-mannoisomerase activity. Two such mutants used in this study, *PMI-J* and *PMI-332*, retained nearly 20% of the wild type P-mannoisomerase activity and contained 33 and 37 milliunits of this enzyme per mg of protein, respectively.

Enzyme Assay—Toluene-treated cells were used in most of the experiments. In some cases, cell-free extracts made by a French pressure cell were used. The methods of cell disruption, measurements of extinction, and procedures for enzyme assays were the same as in the accompanying paper (7). The only change was in the assay of P-glucomutase (EC 2.7.5.1); in addition to glucose-1-P, $NADP^+$, cysteine, 0.2 mM EDTA, and glucose-6-P dehydrogenase (EC 1.1.1.49), the reaction mixture contained 3 μM glucose-1,6-P_2. The rate of NADPH formation was linear with time at all levels of the extract used. P-Mannoisomerase was measured by determining fluorometrically the rate of formation of fructose-6-P from mannose-6-P and by measuring fructose-6-P continuously on a recorder. The incubation mixture contained 0.6 mM mannose-6-P, 0.05 mM $NADP^+$, 0.5 unit of P-glucoisomerase, 0.3 unit of glucose-6-P dehydrogenase, and the enzyme solution in a final volume of 1 ml. The enzyme extract for assay of P-mannoisomerase was made in a French pressure cell in 50 mM potassium phosphate buffer, pH 7.4, and by subsequent clarification in the centrifuge. The slight contamination of fructose-6-P in the sample of mannose-6-P was removed by prior incubation of the latter in the assay mixture lacking P-mannoisomerase. All enzyme units are expressed in international units as μmoles of substrate per min at 22–25°.

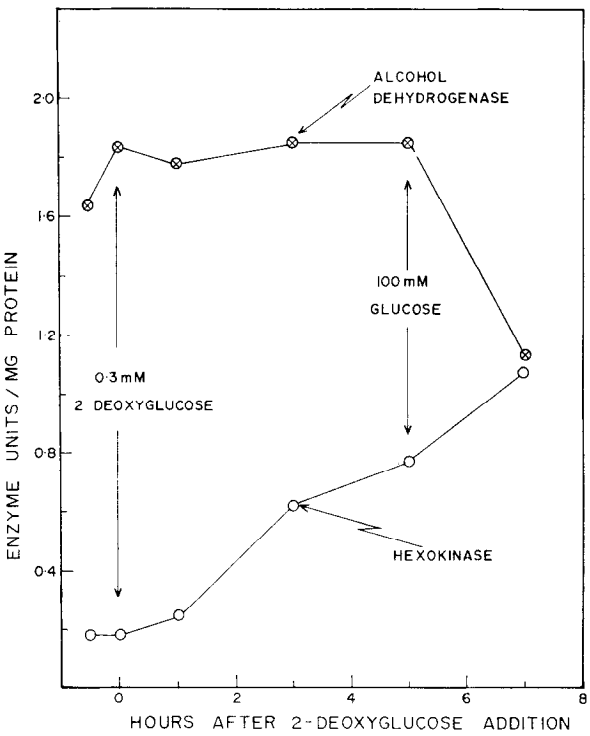

FIG. 1. Effect of 2-deoxyglucose on the rate of synthesis of hexokinase and alcohol dehydrogenase in the hybrid yeast. A culture growing on yeast extract-peptone-acetate medium was treated with 0.3 mM 2-deoxyglucose at time 0. At the instants indicated, flasks were chilled and the cells were processed for enzyme assay as described under "Methods," with the use of a French pressure cell for enzyme extraction. The cell concentration at 0 hour was 5×10^6 cells per ml; there was no significant growth during incubation in 2-deoxyglucose as assayed by cell count. Addition of 100 mM glucose at the 5th hour led to nearly 0.8 doubling during the next 2 hours. Data on each time point refer to a separate flask.

For the assay of transport activity toward carbohydrates, cells were incubated in 20 mM concentrations of the respective sugar solutions; aliquots were transferred periodically to membrane filters, the solids were washed three times with cold water, and the filter with the cells was transferred to 5% perchloric acid. Sugars were estimated in the neutralized supernatants by enzymic analysis (7).

Metabolite Assay—2-Deoxyglucose was estimated by peroxidase (EC 1.11.1.7)-coupled glucose oxidase (EC 1.1.3.4) assay (9). Phosphate esters containing this sugar analogue were estimated together as the free deoxysugar after treatment of the neutralized acid extract with 5 to 10 i.u. of purified *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) at pH 8 for 1 hour. Glucosone was estimated by measuring ADP produced by reaction with ATP and yeast hexokinase. Glycolytic intermediates were estimated fluorometrically (10). In samples containing 2-deoxyglucose and its metabolites, glucose, glucose-6-P, and fructose-6-P were assayed by NADH oxidation in a system containing α-glycero-P dehydrogenase (EC 1.1.1.8), triose-P isomerase (EC 5.3.1.1), aldolase (EC 4.1.2.7), P-fructokinase (EC 2.7.1.11), ATP, P-glucoisomerase, and hexokinase. Glucose-1,6-P_2 was estimated by the method of Passonneau *et al.* (11) with minor modifications. UDP-Glucose was estimated in the acid extract by UDP-glucose dehydrogenase (EC 1.1.1.22) (12). Mannose-6-P was assayed fluorometrically by measuring it as fructose-6-P after coupling with a preparation of P-mannoisomerase from bakers' yeast.

Chemicals—UDP-Glucose dehydrogenase, 2-deoxyglucose, fructose, galactose, and glucose-1,6-P_2 were from Sigma. Glucosone was prepared by hydrolysis of glucosazone (13); it was free of fructose or glucose but contained traces of pyruvate. 6-Deoxyglucose was prepared by mild acid hydrolysis of the β-methyl glycoside (Pierce Chemical Company, Rockford, Illinois). As determined by yeast hexokinase- and glucose-6-P dehydrogenase-coupled assay, its glucose content was below 1% of that of 6-deoxyglucose. 2-Deoxyglucose was tested for glucose impurity in the same way as was 6-deoxyglucose, except that a purified preparation of yeast glucokinase (8) was used in place of hexokinase. The content of glucose was less than 0.1%. Since these were used at millimolar concentrations or less, these were used as such. Fructose and mannose (British Drug Houses, Poole, England), which contained, respectively, 0.2 and 0.1% glucose as impurity, were treated overnight with glucose oxidase (Worthington) until the level of glucose came down to 0.002 and 0.005%, respectively, as tested for 2-deoxyglucose. These were sterilized by filtration without any attempt to inactivate the glucose oxidase. P-Mannoisomerase was purified from bakers' yeast according to Gracy and Noltmann (14) but using only the $(NH_4)_2SO_4$ fractionation step followed by DEAE-

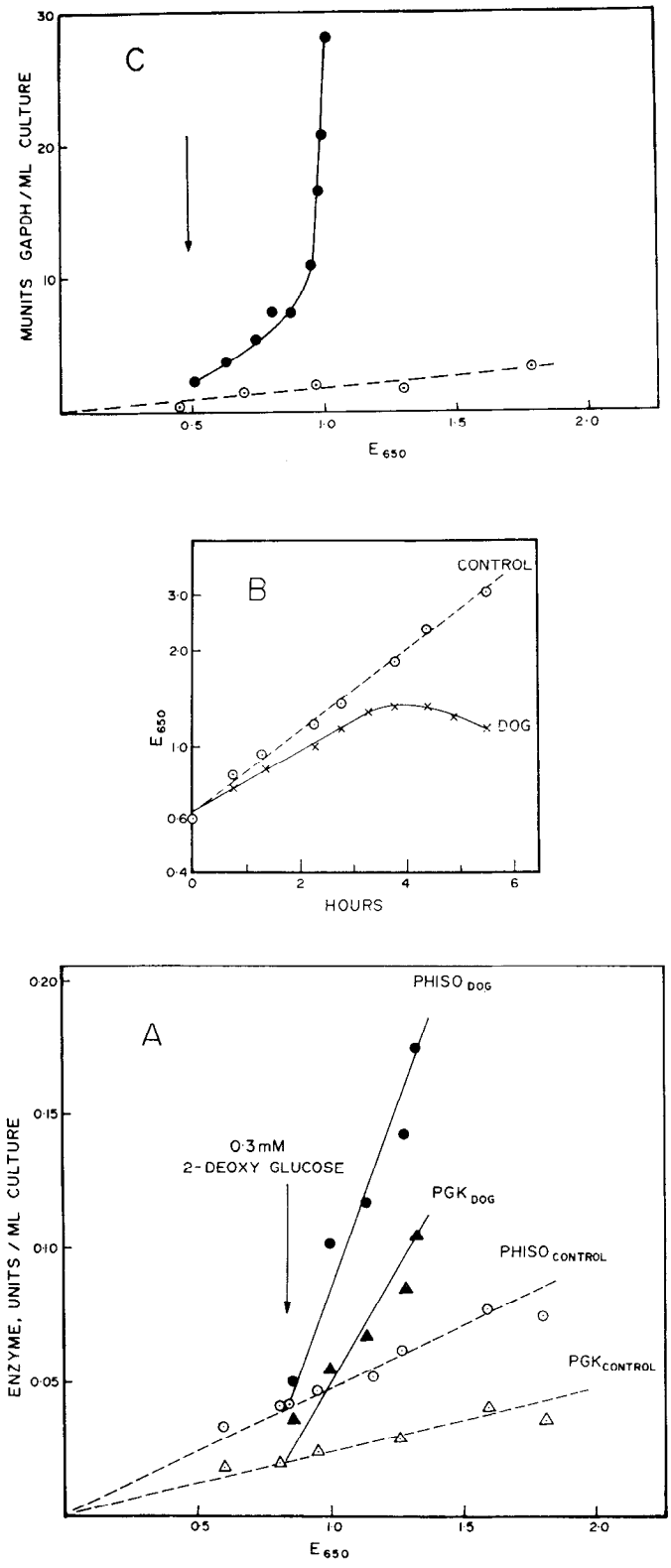

FIG. 2. Induction of P-glucoisomerase (*PHISO*), P-glycerate kinase (*PGK*), and glyceraldehyde-3-P dehydrogenase (*GAPDH*) in the hybrid yeast by the addition of 2-deoxyglucose. The subscripts *control* and *DOG* indicate the cultures without and with 2-deoxyglucose, respectively. *Munits*, milliunits. – – –, cultures without 2-deoxyglucose;——, cultures with 2-deoxyglucose. To a culture growing exponentially in yeast extract-peptone-acetate, 0.3 mM (*A* and *B*) or 0.4 mM (*C*) 2-deoxyglucose was added, as marked by the *arrows*. Nothing was added to the control cultures. At the points indicated, 10-ml aliquots were withdrawn, chilled, and centrifuged, and the pellet was treated with toluene as described elsewhere (7). Another aliquot was treated with galactose for measurements of the extinction values at 650 mμ (E_{650}). P-Glycerate kinase was assayed in samples stored for 48 hours at 0°. In *B*, growth (E_{650}) is plotted against time, counted from the moment of addition of 2-deoxyglucose. *A* and *B* refer to one experiment, and *C* refers to another.

cellulose column chromatography. The preparation was reasonably free from hexokinase and adenylate kinase (EC 2.7.4.3). Other enzymes and substrates were from Boehringer Mannheim.

RESULTS

Induction of Glycolytic Enzymes in Hybrid Yeast by 2-Deoxyglucose—The rapid and extensive metabolic transformations undergone by glucose prompted us to search for sugar analogues that could serve as inducers of glycolytic enzymes. Induction by a nonmetabolizable compound would serve to put limits on the number of compounds that might be involved in the induction. 2-Deoxyglucose satisfied some of these criteria. It is phosphorylated by yeast hexokinase (15), but its metabolism via P-glucoisomerase is negligible (16). Van Steveninck found that the major metabolite produced from this substance is 2-deoxyglucose-6-P (16); this in turn generates the following series of compounds: 2-deoxyglucose-1-P, 2-deoxyglucose-1,6-P_2, UDP-2-deoxyglucose, GDP-2-deoxyglucose, and also 2-deoxygluconic acid (17, 18). Incorporation of 2-deoxyglucose into yeast oligosaccharide has also been reported (19). Cells treated with high concentrations of this substance fail to grow (20), however, and they are characterized by a very low ATP:ADP ratio and a depleted orthophosphate pool (21). In these experiments, therefore, the concentration of 2-deoxyglucose was carefully adjusted with respect to the cell titer. If the cell to 2-deoxyglucose ratio was too high or too low, no induced synthesis of glycolytic enzymes was observed. When the ratio was too high, the cells that escaped 2-deoxyglucose outgrew the ones that took it up; when the ratio was too low, all synthetic activities stopped. Results in Fig. 1 illustrate one such experiment showing the effect of 2-deoxyglucose on the time rate of change of specific activity of two glycolytic enzymes. Before addition of 0.3 mM 2-deoxyglucose, cells were growing exponentially; the specific activity of hexokinase was steady while alcohol dehydrogenase continued to be synthesized. Treatment with 2-deoxyglucose stopped the synthesis of the dehydrogenase, but the rate of hexokinase synthesis speeded up, although cell division had virtually halted. Addition of 100 mM glucose thereafter appeared to have only marginal effects on the rate of synthesis of hexokinase, while alcohol dehydrogenase showed the characteristic dilution effect resulting from resumption of growth.

In the following series of experiments, enzyme synthesis following 2-deoxyglucose addition was followed as a function of the increase in mass of the culture. For this purpose, aliquots of the growing culture from the same flask were extracted by treatment with toluene, rather than by French pressure cell, and the results were expressed as enzyme activity per ml of the culture medium against the yeast mass, determined by the extinction at 650 mμ. The results of such experiments are indicated in Figs. 2 and 3. Fig. 2 shows the results of two separate experiments, in one of which (*A*) the increase of P-glucoisomerase and P-glycerate kinase (EC 2.7.2.3) is shown against the increase in the mass of the culture and the corresponding growth curves

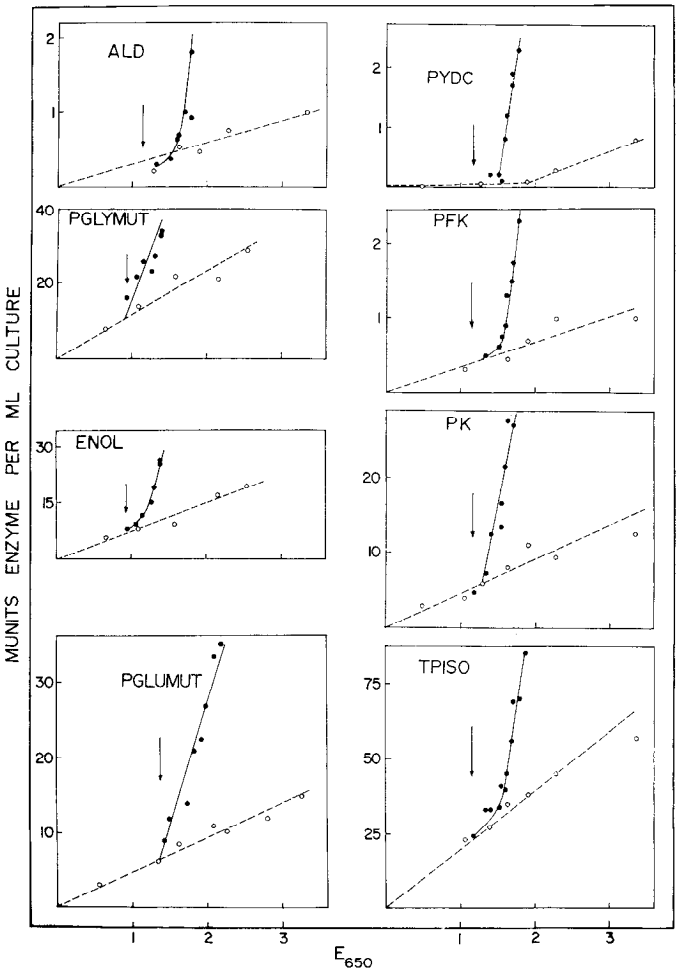

FIG. 3. Differential plots showing the appearance of glycolytic enzymes of the hybrid yeast following 2-deoxyglucose addition. Aldolase (*ALD*), pyruvate decarboxylase (*PYDC*) (EC 4.1.1.1), P-fructokinase (*PFK*), pyruvate kinase (*PK*) (EC 2.7.1.40), and triose-P isomerase (*TPISO*) were assayed on the same experimental samples; the concentration of 2-deoxyglucose used in this experiment was 0.3 mM. Aldolase, P-fructokinase, and triose-P isomerase were assayed in samples stored for 24 hours at 0°. All other enzymes were assayed in fresh samples. P-Glycerate mutase (*PGLYMUT*) (EC 2.7.5.3) and enolase (*ENOL*) (EC 4.2.1.11) were assayed in a separate experiment with 0.4 mM 2-deoxyglucose. P-Glucomutase (*PGLUMUT*) was followed in a third experiment with 0.25 mM 2-deoxyglucose. All other details are as in Fig. 2. *Munits*, milliunits.

(*B*) under these conditions. In the other experiment (Fig. 2*C*), the differential rate of glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12) synthesis in indicated. Results in Fig. 3 show the induction of all of the other glycolytic enzymes obtained from a number of separate experiments. In all of these experiments, addition of 2-deoxyglucose caused eventual lysis of the culture; the sampling was therefore continued only until the moment the extinction values did not start falling. When lysis occurred, the enzyme value also dropped. These experiments indicate that all of the glycolytic enzymes, with the exception of alcohol dehydrogenase, are induced by 2-deoxyglucose. The induced synthesis of most of these enzymes takes place with considerable delay, although P-glucomutase, P-glucoisomerase, P-glycerate kinase, and P-glycerate mutase are induced without any lag in

TABLE I

Differential rates of glycolytic enzyme synthesis in S. fragilis × S. dobzhanskii in response to 2-deoxyglucose addition

Results are expressed in milliunits of enzyme increase per unit increase in yeast mass, expressed as E_{650}. Results are calculated from the linear part of the data in Figs. 2 and 4. Control refers to the culture without 2-deoxyglucose.

Enzyme	Control	2-Deoxyglucose
	milliunits/E_{650}	
P-Glucomutase	4.7	32.2
Hexokinase[a]	5.3	60.2
P-Glucoisomerase	50.0	270.0
P-Fructokinase	0.3	7.4
Aldolase	0.3	8.2
Triose-P isomerase	19.5	155.0
Glyceraldehyde-3-P dehydrogenase	1.7	360.0
P-Glycerate kinase	30.1	170.0
P-Glycerate mutase	11.4	26.0
Enolase	7.3	16.5
Pyruvate kinase	4.6	47.4
Pyruvate decarboxylase	0.4[b]	7.6

[a] Calculated from results of a separate experiment not shown here; 0.3 mM 2-deoxyglucose was used.

[b] Maximal rate. Kinetics of synthesis was not linear.

these experiments. In another experiment, not shown here, the 2-deoxyglucose-induced synthesis of P-glucoisomerase and P-glycerate kinase in a yeast extract-peptone-pyruvate medium took place with some delay. The inhomogeneous nature of the induction pattern of glycolytic enzymes is also indicated by the varying extent of stimulation of their differential rate of synthesis. Table I presents a summary of the differential rates of glycolytic enzyme synthesis calculated from the results in Figs. 2 and 3. The highest increase in the differential rate of synthesis is in the case of glyceraldehyde-3-P dehydrogenase, amounting to more than 200-fold, whereas the differential rate of synthesis of P-glycerate mutase and enolase increased by a factor of 2.5. Generally, enzymes with a low specific activity in the uninduced state (7) are the ones which increase maximally in response to induction by 2-deoxyglucose. Under the conditions of these experiments, the rate of RNA synthesis, measured by the rate of ^{14}C-uracil incorporation into 10% trichloracetic acid-insoluble material, was inhibited 45% by 0.4 mM 2-deoxyglucose during an initial period of 30 min. 2-Deoxyglucose treatment is also known to inhibit incorporation of amino acids in proteins (22). The observed increase in the activity of enzymes therefore represents minimal values. The other feature of the induced synthesis of glycolytic enzymes by 2-deoxyglucose is that, in one experiment with the hybrid yeast, removal of this sugar analogue 2 hours after addition of 0.4 mM 2-deoxyglucose did not cause any dilution of specific activity of P-glycerate kinase by growth during a subsequent 3-hour period, as happens with metabolizable sugars (7). Growth, however, was resumed.

Induction of Glycolytic Enzymes in Hybrid Yeast by Glucosone and Related Compounds—A few experiments were performed with glucosone, following our observation that this particular substrate of yeast hexokinase inhibits growth of the hybrid yeast and causes a lowering of the ATP:ADP ratio. Although further work is necessary to decipher its metabolic fate, our preliminary observations indicated that glucosone was mimicking

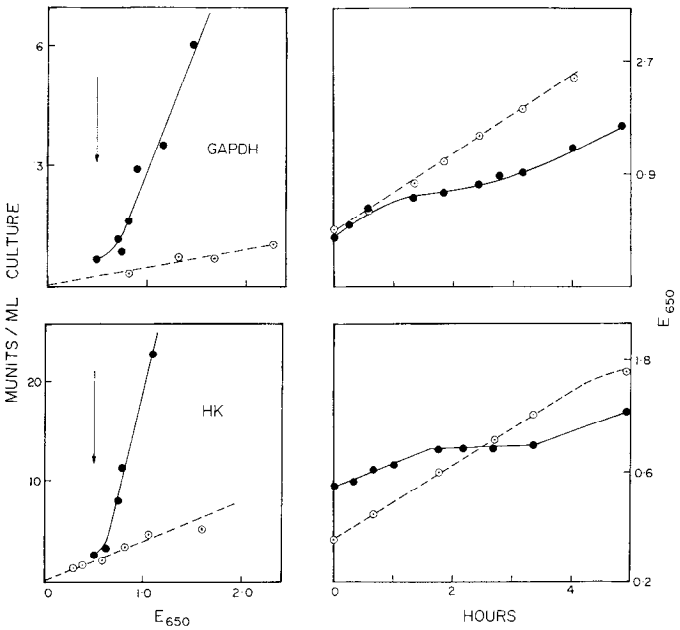

FIG. 4. Induction by glucosone of hexokinase (*HK*) and glyceraldehyde-3-P dehydrogenase (*GAPDH*) of the hybrid yeast. The two figures on the *top* refer to one experiment, and the two on the *bottom* refer to another. Figures on the *left* depict enzyme appearance and those on the *right* the growth curves under these conditions. – – –, control cultures; ——, cultures in which 1.8 mM glucosone was added at the points marked by the vertical *arrows*, corresponding to the 0 points on the right hand curves. E_{650}, extinction at 650 mμ, plotted in exponential scale. *Munits*, milliunits.

the effects of 2-deoxyglucose. Results in Fig. 4 show that glucosone induces hexokinase and glyceraldehyde-3-P dehydrogenase in the hybrid yeast. The stimulation of the differential rate in the case of the dehydrogenase is much less than with 2-deoxyglucose. The other feature of glucosone action is that growth inhibition by this substance is temporary, although enzyme synthesis is stimulated while the growth continues to be inhibited. The extent and duration of the growth inhibition, however, are somewhat different in the two experiments indicated in Fig. 4.

Since both 2-deoxyglucose and glucosone are substrates of yeast hexokinase (15) and both seem to induce glycolytic enzymes, we have examined a few other compounds with respect to their inducing ability. In Table II are summarized the results of a number of such experiments in which we examined the induction of pyruvate kinase in the hybrid yeast. The results show that the differential rate of pyruvate kinase synthesis increases 6- to 12-fold over the control value whenever a substrate of hexokinase is incorporated in the growth medium. This inducing effect is independent of stimulation or inhibition of growth by the particular compound.

Profile of Glycolytic and Related Metabolites during Induction by 2-Deoxyglucose—Fig. 5 shows the results of an experiment in which the intracellular levels of free and phosphorylated 2-deoxyglucose were measured during incubation of the hybrid yeast under conditions described in Fig. 3. The concentrations of both the free and phosphorylated forms of this compound are characterized by a rise soon after the addition of 2-deoxyglucose and then by a gradual fall. Of the two forms, the phosphorylated compounds constitute the major fraction, its minimal level

TABLE II

Differential rate of pyruvate kinase synthesis in hybrid yeast elicited by substrates of hexokinase

Rates refer to maximal values obtained from a mean of at least four experimental points. Induction medium was yeast extract-peptone-acetate supplemented with the compounds listed here. Other details are the same as in Table I. Control refers to the culture without sugar. The concentrations of 2-deoxyglucose and glucosone were 0.3 and 1.8 mM, respectively, and 20 mM for the rest.

Experiment	Inducer	Effect on growth[a]	Control	Inducer
			milliunits/E_{650}[b]	
1	Glucose	+	12	112
	Fructose	+	12	121
	Glucosamine	–	12	116
2	Mannose	+	8	92
3	2-Deoxyglucose	–	5	47
4	Glucosone	–	5	32

[a] +, stimulation; –, inhibition of growth.
[b] See legend, Table I.

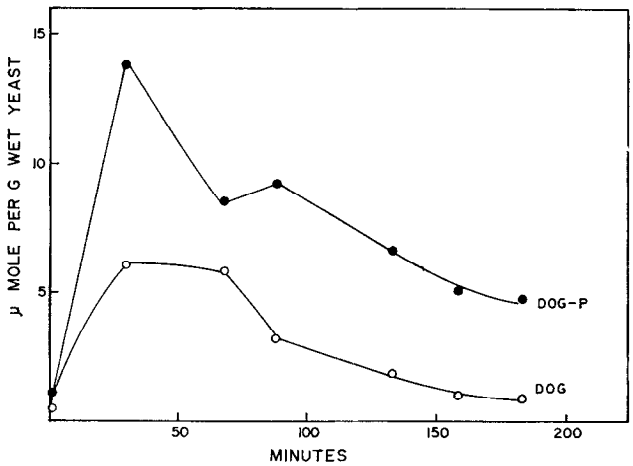

FIG. 5. Kinetics of intracellular free and phosphorylated 2-deoxyglucose during growth of *S. fragilis* × *S. dobzhanskii* in the presence of 2-deoxyglucose. A culture growing exponentially under the conditions given in Fig. 3 was treated with 0.4 mM 2-deoxyglucose (*DOG*). The initial and the final values of extinction at 650 mμ were 1.525 and 1.990, respectively. At the instants indicated, 50 ml of the culture were removed, chilled, and centrifuged, and the pellet was washed in 150 mM KCl. The pellet was extracted with 5% $HClO_4$, and the metabolites were estimated in the neutralized acid extract by procedures described under "Methods." The values of free and phosphorylated 2-deoxyglucose were corrected for glucose and glucose-6-P, respectively, both of which were in the range of 0.05 to 0.3 μmole per g of wet yeast. *DOG-P*, 2-deoxyglucose liberated by the action of alkaline phosphatase. The first sample was taken 1 min after addition of 2-deoxyglucose.

during the 3-hour period being 6 μmoles per g of wet yeast. In separate experiments, we have also measured glycolytic metabolites in cells treated with 2-deoxyglucose. For this purpose, a thicker cell suspension was used and, at various instants, aliquots were taken directly in a final concentration of 5% perchloric acid. Assay of the neutralized supernatants for glycolytic intermediates indicated that the sum of glucose-6-P, fructose-6-P, and the aldolase metabolites was less than 6% of the concentration of 2-deoxyglucose phosphates in all of the samples.

TABLE III

Effect of 6-deoxyglucose on level of glycolytic enzymes of S. cerevisiae and S. fragilis × S. dobzhanskii

Yeast cultures grown overnight in yeast extract-peptone-acetate medium were brought to exponential phase by dilution in fresh medium to which 1 mM 6-deoxyglucose (6-DOG) or 100 mM glucose was added. Exponential growth was continued for 8 hours. Cells were broken by French pressure cell.

Yeast	Additions	Enzyme[a]				
		HK	PHISO	PFK	TPISO	PK
		units/mg protein				
S. fragilis × *S. dobzhanskii*	None	0.20	0.68	0.10	2.88	0.35
	6-DOG	0.21	0.72	0.10	3.25	0.27
S. cerevisiae	None	1.65	1.30	0.14	3.70	0.72
	6-DOG	1.68	1.27	0.16	3.60	0.84
	Glucose	2.20	3.52	0.87	11.40	10.80

[a] HK, hexokinase; PHISO, P-glucoisomerase; PFK, P-fructokinase; TPISO, triose-P isomerase; PK, pyruvate kinase.

The predominant metabolites under conditions in which 2-deoxyglucose induces glycolytic enzymes in the hybrid yeast are therefore the free sugar analogue and its phosphorylated derivatives, primarily 2-deoxyglucose-6-P (16).

Biely and Bauer have obtained evidence for the formation of 2-deoxyglucose-1,6-P_2 by yeast cells treated with 2-deoxyglucose (17). It is possible that induction of glycolytic enzymes observed with 2-deoxyglucose and glucosone is due to the formation of 2-deoxyglucose-1,6-P_2 and glucosone-1,6-P_2, respectively. By analogy, the observed induction with glucose could be due to the formation of glucose-1,6-P_2. No significant increase over the control value of 0.2 μmole of glucose-1,6-P_2 per g of wet yeast was found, however, under conditions in which glucose induces glycolytic enzymes of the hybrid yeast.

Failure of 6-Deoxyglucose to Elicit Increase of Glycolytic Enzyme Activity—As shown in Fig. 5, the stimulation of differential rate of glycolytic enzyme synthesis by 2-deoxyglucose is associated with the accumulation in the yeast of considerable amounts of this substance, as well as of its phosphorylated derivatives. We have chosen to examine, therefore, the effect of a glucose analogue that cannot be phosphorylated. Results in Table III summarize such experiments with 6-deoxyglucose. Since concentrations of this sugar analogue of 10 mM or above considerably inhibited growth of the hybrid yeast, a level of 1 mM was chosen for the study. At this concentration, growth was not appreciably inhibited. The results indicate that none of the enzymes examined increases in specific activity beyond the uninduced level in either the hybrid yeast or a haploid strain of *S. cerevisiae*. Under the same conditions, glucose brings about appreciable increase in the specific activity of five glycolytic enzymes of the haploid yeast. Examination of reducing sugar (23) in the cell extracts indicated that cells grown with 6-deoxyglucose had 3 times more reducing sugar per unit weight compared with the cells grown without this substance. This suggests that glycolytic enzymes are not induced by 6-deoxyglucose, even if cells take up this glucose analogue.

When a galactose-negative mutant of *S. cerevisiae, gal-3*, was examined for glycolytic enzyme induction in a yeast extract-peptone-acetate medium containing 50 mM galactose, no increased synthesis of P-glucoisomerase, P-glycerate kinase, or pyruvate kinase was observed, although galactose induces these enzymes in the wild type. The glucose-linked induction in the mutant, however, was left unimpaired. Since *gal-3* is capable of accumulating galactose intracellularly, this suggests that the inducer of these enzymes is not free galactose but some metabolite produced from it.

Effect of Glucose and Fructose on Steady State Rate of Glycolytic Enzyme Synthesis in Hexokinaseless Mutant of S. cerevisiae—The failure of 6-deoxyglucose on the one hand and the ability of hexokinase substrates on the other to induce glycolytic enzymes implicate some product of hexokinase reaction as the inducer or its metabolic precursor. The involvement of the free sugar cannot be ruled out by such experiments, however. We have therefore sought to examine this question by looking for the pattern of enzyme synthesis by sugars in a mutant lacking hexokinase. Such a mutant of *S. cerevisiae* has been obtained by selection on 2-deoxyglucose (8); this mutant, 7L1, however, retains its ability to grow on glucose by virtue of the presence of a second minor enzyme that phosphorylates glucose, although it does not grow on fructose. Nevertheless, the question whether fructose and glucose induce glycolytic enzymes in this mutant has been examined. Since the sugar-induced synthesis of glycolytic enzymes in the haploid yeast is not as marked as in the hybrid yeast, data are also presented for the wild type parent. Cells grown overnight in yeast extract-peptone-acetate medium were diluted in fresh medium containing added glucose or fructose. After about 2 hours of growth in the medium, aliquots were withdrawn periodically and assayed for all of the glycolytic enzymes. When a linear rate of enzyme synthesis was obtained, the differential rate of synthesis was estimated for all of the six culture flasks, three for the mutant and three for the wild type parent. The initial density of the cultures was so adjusted as to sustain exponential growth until the end of sampling, which was continued for 7 hours. The results are shown in Table IV.

The stimulation by glucose or fructose of the differential rate of synthesis of the glycolytic enzymes of *S. cerevisiae* is generally small. These data indicate, however, that in the wild type both of the sugars induce synthesis, whereas in the hexokinaseless mutant only glucose induces synthesis; fructose fails to bring about any perceptible increase in the rate of enzyme synthesis in the mutant. Although the basal rate of synthesis remains practically unaltered in the mutant, the extent of stimulation of this rate by glucose is reduced nearly 2-fold, except for glucokinase and P-glucoisomerase. This is consistent with the loss of nearly 90% of glucose-phosphorylating activity in the mutant clone 7L1 (8).

Since the induction of most of the enzymes with fructose is generally less than with glucose even in the wild type, we have examined the relative levels of glucose-6-P and fructose-6-P in a suspension of wild type *S. cerevisiae* treated with glucose and fructose. Such experiments indicate that glucose-treated cells have higher levels of glucose-6-P than do cells treated with fructose; the ratio of glucose-6-P to fructose-6-P is also higher.

The hexokinaseless mutant 7L1 is resistant to 2-deoxyglucose and fails to utilize this substance when a cell suspension is incubated in the presence of an oxidizable energy source, such as ethanol. The wild type parent *S. cerevisiae*, however, consumes 2-deoxyglucose under these conditions. Similarly, the addition of this sugar analogue does not cause any measurable increase in the differential rate of synthesis of either P-glucoisomerase or

TABLE IV

Effect of glucose and fructose on differential rate of glycolytic enzyme synthesis in a hexokinaseless mutant of S. cerevisiae and its wild type parent

Differential rates were estimated from plots similar to those in Fig. 3, computing from the linear portion of the plots through a minimum of four experimental points. The column marked "Control" indicates the culture without any added sugar. The experiment was completed in two installments involving two separate sets of culture. The first six enzymes listed were assayed in samples from one set, the last five from a second set. The enzymes were assayed as follows: pyruvate kinase and enolase on the day of toluenization; P-glucoisomerase, P-fructokinase, P-glycerate kinase, glyceraldehyde-3-P dehydrogenase, and P-glycerate mutase on the 2nd day; hexokinase (HK), aldolase, triose-P isomerase, and pyruvate decarboxylase on the 3rd day after toluenization. During this 48-hour period, the samples were kept over crushed ice. Results are expressed as milliunits/E_{650}.

Enzyme	Wild type			HK⁻ mutant 7L1		
	Control	Glucose	Fructose	Control	Glucose	Fructose
Hexokinase[a]	49	75	63	4	6	4
P-Glucoisomerase	45	104	85	49	115	43
P-Fructokinase	1	26	26	1	13	2
Aldolase	2	6	5	2	4	2
P-Glycerate kinase	70	340	360	90	196	100
Pyruvate kinase	36	154	154	42	100	36
Triose-P isomerase	120	200	190	140	190	140
Glyceraldehyde-3-P dehydrogenase	52	213	142	130	215	130
P-Glycerate mutase	25	112	84	34	69	36
Enolase	19	45	35	15	40	20
Pyruvate decarboxylase	8	42	40	10	17	10

[a] Includes glucokinase activity. For the mutant, the values refer only to glucokinase, as hexokinase is absent.

P-glycerate kinase of the mutant under the conditions described in Fig. 3. In contrast, the rate of P-glycerate kinase synthesis increases in the wild type parent from 138 to 640 milliunits per E_{650} in the presence of 0.3 mM 2-deoxyglucose.

Induction of Glycolytic Enzymes by Glucose in P-Glucoisomeraseless Mutant of S. cerevisiae—The observation that 2-deoxyglucose induces all of the glycolytic enzymes in the hybrid yeast points strongly to the role of some metabolite above fructose-6-P in the glycolytic sequence in this process; this does not, however, rule out the possibility that compounds at the level of fructose-6-P or some later metabolite in the glycolytic chain such as fructose-1,6-P_2 or P-enol pyruvate could also be acting as an inducer. There might be multiple inducing signals for the glycolytic enzyme system. Furthermore, the use of 2-deoxyglucose, which is identical with 2-deoxymannose, does not settle the question whether mannose-6-P, rather than glucose-6-P, is the inducer of these enzymes. We therefore chose to examine whether a P-glucoisomeraseless mutant can induce the glycolytic enzymes in response to the addition of glucose and mannose.

We have used the mutant 9520*b* for this purpose. It not only fails to grow on glucose as a sole carbon source, but it also does not grow on an otherwise permissible source of carbon, such as fructose, mannose, or acetate, if high concentrations of glucose are also present. The biochemical correlates of this phenomenon are not known. The mutant accumulates large amounts of glucose-6-P under these conditions, however. Previous studies with *E. coli* (24–26) have shown similar growth inhibition during accumulation of large amounts of phosphorylated intermediates. The mutant 9520*b*, however, could be grown at a slow rate in the presence of small concentrations of glucose, provided that another carbon source was present. As a control, we have used cells grown on acetate as an extra carbon source in the yeast extract-peptone medium. Even in acetate, the mutant grows very slowly, presumably as a result of the additional loss of nearly 85% of its respiratory activity. The inoculum culture was grown in yeast extract-peptone-acetate medium for 48 hours, centrifuged, and resuspended in a number of flasks containing the same medium. Upon resumption of exponential growth, various sugars were added to the cultures, and the differential rate of synthesis of glycolytic enzymes was determined as described for the experiment in Table IV. The results are shown in Table V. Results of a similar experiment carried out with the wild type haploid yeast are also shown so that the data are comparable.

The differential rates of synthesis of aldolase, triose-P isomerase, glyceraldehyde-3-P dehydrogenase, P-glycerate kinase, P-glycerate mutase, enolase, and pyruvate kinase of the mutant increase very considerably by glucose and by a mixture of glucose and mannose. Fructose and mannose, singly, also bring about a significant increase in the differential rates of synthesis of these enzymes over the control; however, mannose is slightly more potent than fructose. In all of these cases, the differential rates of synthesis elicited in the mutant by mannose are somewhat lower than the glucose-induced rate in the wild type. The highest differential rate of synthesis of these enzymes in the mutant, however, is higher by a factor of 3 to 5 than the corresponding rates in the wild type yeast. The other feature of these data is that, for all of these enzymes, the differential rates of synthesis in acetate are generally lower in the mutant than in the wild type parent; for P-glycerate kinase, the decrease is as much as 13-fold.

Hexokinase and, particularly, pyruvate decarboxylase do not show significantly increased synthesis by glucose (Table V) in the P-glucoisomeraseless mutant. P-Fructokinase perhaps belongs to this category, too, as its differential rate of glucose-mediated synthesis only equals the basal rate in the wild type. On the other hand, fructose and, particularly, the combination of glucose and mannose bring about appreciable stimulation of the differential rate of synthesis of P-fructokinase. Hexokinase synthesis appears to be mediated by mannose, as shown by the significant increase in the induction rate by this sugar, singly or together with glucose. Pyruvate decarboxylase seems to be an exception in the sense that a combination of glucose and mannose gives a lower rate than does mannose or fructose alone.

Results in Table VI show the levels of sugar phosphates produced in the P-glucoisomeraseless mutant and in the wild type yeast when treated with fructose, glucose, mannose, and a mixture of glucose and mannose. The experiment was performed with nongrowing cells in a buffered suspension. These results show that in the presence of glucose the P-glucoisomeraseless mutant accumulates fairly high levels of glucose-6-P. When mannose and glucose are present together, the levels of both glucose-6-P and mannose-6-P are considerably less than when the respective sugars are present singly.

Induction of Glycolytic Enzymes by Mannose in a P-Mannoisom-

TABLE V

Effect of glucose and other sugars on differential rates of glycolytic enzyme synthesis in wild type S. cerevisiae and in a mutant lacking P-glucoisomerase

The concentrations of sugars were: glucose, 50 mM in the wild type and 5 mM in the mutant; fructose, 20 mM; mannose, 20 mM; glucose, 5 mM, and mannose 20 mM in the mixture. The control culture was without any added sugar. The entire experiment refers to a single set of culture. The enzyme assays were completed within 3 days of toluene treatment. Differential plots were made through three experimental points taken during a 6-hour period; the plots were linear for all of the enzymes except pyruvate decarboxylase. The rates for this enzyme refer to the highest rate observed in the last two samples. More than half of the added glucose remained unutilized at the end of the experiment. Other details are the same as in Table IV. PGI⁻ refers to a mutant lacking P-glucoisomerase.

Enzyme	Wild type		PGI⁻ mutant 9520*b*				
	Control	Glucose	Control	Glucose	Fructose	Mannose	Glucose + mannose
Hexokinase	30	98	32	52	48	170	200
P-Fructokinase	1.3	12.3	0.2	1.6	6.2	7.1	17.2
Aldolase	9.5	8.9	3.2	18.8	4.2	8.0	35.0
Triose-P isomerase	130	490	100	1770	230	400	1450
Glyceraldehyde-3-P dehydrogenase	80	385	15	580	120	150	1206
P-Glycerate kinase	65	230	5	780	80	100	1150
P-Glycerate mutase	18	108	10	185	40	62	350
Enolase	23	30	12	88	20	25	125
Pyruvate kinase	40	170	15	262	56	110	532
Pyruvate decarboxylase	9	33	4	3	39	29	12
Doubling time, hours	4.9	1.8	16.1	22.7	2.7	4.0	6.4

TABLE VI

Levels of ATP and sugar phosphates in a P-glucoisomeraseless mutant of S. cerevisiae and its wild type parent during incubation with sugars

Overnight cultures of the mutant 9520*b* and the wild type yeast from yeast extract-peptone-fructose were centrifuged, washed, and resuspended in 50 mM potassium phosphate buffer, pH 7.4, in 50 mM sodium acetate. Aliquots were incubated aerobically at 30° in the presence of sugars at the concentrations indicated in Table V. At the end of 30 min, the reactions were stopped in 5% perchloric acid, and metabolite assays were performed on the neutralized supernatants. The yeast concentrations were: wild type, 11.5 mg, and 9520*b*, 21.5 mg, wet weight, per ml of suspension.

Yeast	Addition to incubation mixture	Glucose-6-P	Fructose-6-P	Mannose-6-P	ATP
		μmoles/g wet yeast			
Wild type	None	0.25	<0.05	0.05	2.70
	Glucose	1.20	0.25	0.35	2.20
9520*b*	None	<0.05	<0.05	<0.05	<0.05
	Glucose	6.75	<0.05	<0.05	0.60
	Fructose	0.05	0.45	0.35	1.30
	Mannose	0.10	1.60	3.34	<0.05
	Glucose + mannose	2.80	0.50	0.84	0.40

TABLE VII

Differential rates of synthesis of hexokinase, P-glucoisomerase, and P-fructokinase in a mannose-negative mutant of S. cerevisiae and its wild type parent

The basal medium was yeast extract-peptone-acetate. Differential plots were constructed with four experimental points taken through 5.5 hours. The initial concentration of mannose was 2 mM in the mutant and 50 mM in the wild type, and that of fructose was 25 mM. Nearly 0.5 mM mannose was left unutilized in the mutant culture at the end of the experiment. The enzyme assays were completed within 24 hours of toluenization. Other details are the same as in Table V. PMI⁻ refers to the mutant deficient in P-mannoisomerase.

Enzyme	Wild type		PMI⁻ mutant *PMI-332*		
	Control	Mannose	Control	Fructose	Mannose
Hexokinase	24	62	15	35	112
P-Glucoisomerase	60	130	62	140	112
P-Fructokinase	1.7	6.0	0.7	21.8	15.9
Doubling time, hours	4.2	1.9	16.7	2.8	5.2

erase-deficient Mutant of S. cerevisiae—The above results implicate mannose or fructose (or both), rather than glucose, in the synthesis of hexokinase, P-fructokinase, and pyruvate decarboxylase. To examine this question, we have studied the induction of these and other glycolytic enzymes in a mutant strain of the haploid yeast that is deficient in the activity of P-mannoisomerase. Most of the experiments described in this section were carried out with *PMI-332*. It grew on the salt-vitamin medium (7) with glucose or fructose as the carbon source but not with mannose. It also grew very slowly in yeast extract-peptone-acetate medium. However, 2 mM mannose stimulated its growth rate in this medium. Another mutant, *PMI-J*, which grew faster in acetate and had higher basal levels of glycolytic enzymes, was also used. Mannose did not bring about any significant induction in this mutant of those glycolytic enzymes that were induced by glucose in the P-glucoisomeraseless mutant (Table V). In the other P-mannoisomerase-deficient mutants, also, the induction by mannose of the enzymes between aldolase and pyruvate kinase was very much less than

that elicited by glucose in the P-glucoisomerase mutant. Hexokinase and P-fructokinase were, however, induced by mannose. We present in Table VII the results of an experiment showing the effect of fructose and mannose on the differential rates of synthesis of a number of glycolytic enzymes in the mutant clone *PMI-332*.

It is apparent from these results that the differential rate of synthesis of hexokinase in the P-mannoisomerase mutant is increased nearly 8-fold by mannose and only 2-fold by fructose, while P-fructokinase synthesis is stimulated slightly more by fructose than by mannose. P-Glucoisomerase synthesis in the mutant is not increased by mannose or fructose any more than it is in the wild type by mannose or by glucose. The basal level in absence of sugars is also unaltered. We have not shown here data for pyruvate decarboxylase. This is because this enzyme was not found to be induced in any of the P-mannoisomerase mutants either by fructose or by mannose during 8 hours of exponential growth. Overnight cultures grown in the presence of glucose, however, had activities of pyruvate decarboxylase comparable with wild type levels.

DISCUSSION

The results reported in this paper relate to the gratuitous induction of glycolytic enzymes by the products of hexokinase reaction. Sugars fail to induce synthesis when they cannot be phosphorylated. This is borne out by the inability of 6-deoxyglucose to bring about any increase of basal specific activity in the hybrid yeast and in *S. cerevisiae*, as also by the failure of fructose or 2-deoxyglucose to stimulate the differential rate of glycolytic enzyme synthesis in the hexokinaseless mutant of the latter organism. The glucose-linked induction is also reduced as a consequence of the loss of bulk of glucose-phosphorylating activity. It is unlikely that the failure of fructose to induce is due to the loss of some undetermined transport element. This is because the normal levels of glucose in the yeast cell are very low, and incubation of the mutant with ^{14}C-fructose has shown uptake of the label at a rate 10% that of the wild type. Since fructose phosphorylation in the mutant is negligible, the bulk of the fructose should be free in the cell even if it is carried by some diffusive process. In fact, direct enzymic analysis of fructose in cells of the hexokinaseless mutant incubated in 20 mM fructose shows the presence inside the cells of 6 μmoles of fructose per g of yeast. We conclude, therefore, that the inducer of glycolytic enzymes in these cells is not the free sugar but some product of its phosphorylation.

There is one major argument against this inducer being fructose-6-P or a later metabolite in the glycolytic sequence, *viz.* that 2-deoxyglucose which cannot be converted to this compound is a potent inducer. We feel, therefore, that the inducer-like action of sugars is due really to the formation of compounds at the level of glucose-6-P.

It is likely that the inducing compound is either glucose-6-P itself or any of its metabolites whose 2-deoxy analogue can be produced from 2-deoxyglucose-6-P. The studies of Biely and Bauer (17) implicate any one or more of the following: glucose--6-P, glucose-1-P, glucose-1,6-P_2, GDP-glucose, and UDP-glucose. The exclusion of gluconate-6-P appears justified on the grounds that Bauer and Biely failed to detect the 2-deoxy analogue of this compound in an acid extract of yeast incubated with 2-deoxyglucose (18) and that gluconate has not been found to cause any increased synthesis of glycolytic enzymes in either of the *Saccharomyces* species used in these studies. Furthermore, examination of cells of both the hybrid and the haploid yeast reveals no discernible change in the level of this compound in response to glucose addition. Even when the P-glucoisomeraseless mutant accumulated over 10 μmoles of glucose-6-P per g of yeast as a result of the addition of 10 mM glucose under the conditions described in Table VI, the level of gluconate-6-P continued to be nearly the same as in cells incubated in the absence of glucose, *viz.* approximately 10 nmoles per g of wet cells. Regarding UDP-glucose, its level in the hybrid yeast during galactose metabolism (0.4 μmole per g of wet yeast) is nearly twice as much as during glucose metabolism. The kinetics of the appearance of glycolytic enzymes with glucose and galactose (7) is therefore not commensurate with UDP-glucose as the inducer. We have not measured GDP-glucose, so any comment regarding its role in induction is reserved. The kinetics of glucose-1-P changes makes it unlikely to be the inducer. This is because the changes produced by glucose in the levels of glucose-1-P in the hybrid yeast are, if at all, marginally higher than in the absence of glucose (7). On the other hand, despite the larger amounts of intracellular glucose-1-P with galactose as the growth substrate, neither the rate of appearance of the enzymes nor the delay in their synthesis is consistent with glucose-1-P being the inducer of glycolytic enzymes. As pointed out earlier, the intracellular concentration of glucose-1,6-P_2 does not change from the basal level on addition of glucose to cells of the hybrid yeast growing in yeast extract-peptone-acetate medium. It is therefore unlikely that the induction is mediated by this compound.

The inducer of glycolytic enzymes in yeasts is therefore either glucose-6-P or some unknown metabolite derived from it and presumably in equilibrium with it, or both. Incubation of yeast in the presence of 2-deoxyglucose causes accumulation of phosphate esters of which 2-deoxyglucose-6-P is the predominant constituent as shown for *S. cerevisiae* by van Steveninck (16). We therefore consider the concentrations of phosphorylated 2-deoxyglucose in Fig. 5 as reflecting primarily the levels of 2-deoxyglucose-6-P. Although the kinetics of its concentration changes does not follow that of most of the enzymes that 2-deoxyglucose induces, the intracellular concentration of 2-deoxyglucose-6-P in the steady state is considerable. Addition of glucose or galactose also causes accumulation of glucose-6-P, glucose causing a faster rise than does galactose. Glucose also causes most of these enzymes to appear much faster than does galactose. Thus, if glucose-6-P is the inducer of glycolytic enzymes, graded concentrations of this compound appear to be required for the induction of the various groups, as shown with galactose. This has been already discussed (7).

The results of experiments with the mutant lacking P-glucoisomerase (Tables V and VI) are consistent with glucose-6-P being the inducer of all of the glycolytic enzymes between aldolase and pyruvate kinase. Addition of glucose increases the rate of synthesis of these enzymes with attendant accumulation of glucose-6-P, but it causes no observable rise in the concentration of fructose-6-P or mannose-6-P. The addition of either fructose or mannose increases the levels of both mannose-6-P and fructose-6-P, although the increase in the rate of synthesis of these enzymes is much less than that with glucose. Separate experiments indicate that, in the mutants deficient in P-mannoisomerase, mannose addition causes accumulation of large amounts of

mannose-6-P, *viz.* nearly 10 μmoles per g of wet cells. But the induction level for these enzymes is much less than that with glucose. One wonders, therefore, whether the induction seen with fructose and mannose in the P-glucoisomerase mutant is due to the formation of glucose-6-P via a leak through the residual P-glucoisomerase activity. Results in Table VI show that incubation of this mutant with fructose and mannose causes a perceptible but small rise in the concentration of glucose-6-P, arising presumably through the leak. That the leak is significant is shown by the ability of galactose to support the growth of the mutant 9520*b*, albeit slowly in a yeast extract-peptone medium. Our results fail to distinguish between the possibilities of whether the observed induction by mannose and fructose is mediated by a leaky synthesis of glucose-6-P, by mannose-6-P itself, or by both. These experiments, however, suggest that the role of any other metabolite in the synthesis of these enzymes can only be minor.

Results in Table V, on the other hand, indicate that for all of the above enzymes between aldolase and pyruvate kinase, except triose-P isomerase, the differential rates of synthesis with glucose and mannose present together are considerably higher than the sum of their individual rates when either of them is present singly. This increased rate is not matched by a corresponding increase in the concentration of glucose-6-P accumulated (Table VI). In fact, the level of glucose-6-P is lower when glucose and mannose are present together than it is with glucose alone. If the levels of sugar phosphates measured during the 30-min period of incubation of the P-glucoisomerase mutant with sugars (Table VI) reflects their levels *in vivo* under the conditions of induction shown in Table V, we would interpret the data as reflecting a synergism between glucose-6-P and mannose-6-P in the induction. That is to say, a mixture of these two sugar phosphates induces more than each of them does singly. With glucose-grown wild type yeast, there would be adequate amounts of both the sugar phosphates, and constitutive induction levels would be sustained without having unphysiologically high concentrations of either glucose-6-P or mannose-6-P.

The other enzymes not so far discussed are hexokinase, P-fructokinase, and pyruvate decarboxylase. As seen from results with the P-glucoisomeraseless mutant, glucose does not induce these enzymes. Experiments with the mutants partially lacking P-mannoisomerase throw some light in that direction. Hexokinase synthesis is stimulated considerably by mannose both in the P-glucoisomeraseless and the P-mannoisomerase-deficient mutants, which implicates induction by mannose-6-P. Fructose slightly stimulates its synthesis in the P-mannoisomerase-deficient mutant, presumably by generating mannose-6-P. The synthesis of P-fructokinase is stimulated by fructose and mannose in both of the mutants, while the presence of glucose and mannose together elicits maximal induction in the P-glucoisomeraseless mutant. The induction of pyruvate decarboxylase, however, was not seen in exponentially growing cultures of the P-mannoisomerase-deficient mutant in the presence of either fructose or mannose, despite the observation that these sugars induce this enzyme in the P-glucoisomeraseless mutant. The experiments with cycloheximide (7) indicate that, for P-fructokinase and pyruvate decarboxylase, induction is not the sole determinant of their steady state level. These enzymes are subject to decay inside the cells, and addition of glucose appears to stabilize them against inactivation. Without a knowledge of the mechanism of this process, any conclusion about their inducers can only be tentative. It is interesting to note here that all of the three enzymes, hexokinase, P-fructokinase, and pyruvate decarboxylase, which do not appear to be induced by glucose-6-P, decrease in specific activity by dilution on removal of galactose from a previously induced culture with a one-generation delay, unlike the other enzymes (7). These three enzymes also happen to be the ones which, unlike the others, are truly glycolytic, *i.e.* they perhaps have no role in gluconeogenesis. For hexokinase and P-fructokinase, this is already indicated from the known physiology of glucose 6-phosphatase and fructose diphosphatase (27), whereas for pyruvate decarboxylase, no instance of its functional reversibility is recorded.

Regarding P-glucoisomerase, results in Table VII indicate that mannose-6-P could be its inducer, as addition of mannose doubles the rate of its synthesis in the mutant deficient in P-mannoisomerase. This does not, however, rule out the hypothesis that glucose-6-P is not the inducer. We have not indicated in Table V the changes in the residual activity of this enzyme. Such results did indicate that the residual P-glucoisomerase activity increased nearly 3-fold with glucose and about 7-fold with glucose and mannose. This suggests that P-glucoisomerase perhaps belongs to that category of enzymes that are induced by glucose-6-P. Experiments with 2-deoxyglucose are consistent with this.

One possible interpretation of the observation that 2-deoxyglucose induces glycolytic enzymes would be that the induction is favored by a low ATP:ADP ratio, as this sugar analogue is known to decrease ATP and increase ADP levels (21). Experiments with the P-glucoisomeraseless mutant indicate that this is not so, however. This mutant has a very low ATP:ADP ratio, about 2% of that of the wild type yeast grown in absence of sugars. And yet the differential rate of glycolytic enzyme induction in absence of sugars is very much reduced compared with the wild type.

As pointed out earlier (7), the level of glycolytic enzymes in yeasts grown in the absence of added sugars fluctuates within only narrow limits. The observation that a rise in the intracellular concentration of glucose-6-P and mannose-6-P following growth on sugars increases their activity poses the question whether the basal rate of enzyme synthesis in the absence of sugars is also controlled by the level of these metabolites. It can be seen from the results in Table V that a P-glucoisomeraseless mutant growing on acetate has significantly reduced rates of synthesis of those glycolytic enzymes that appear to be induced on glucose addition. This is particularly evident in the case of P-glycerate kinase. The levels of mannose-6-P and glucose-6-P are also reduced (Table VI) compared with those of the acetate-grown cultures of the wild type. We therefore propose that glucose-6-P is required for the basal synthesis as well. The regulation of β-galactosidase synthesis suggests (28) the following modes of constitutive enzyme synthesis: (*a*) an i^- or O^c state (or both) of the regulator and operator genes; (*b*) a loose interaction between the repressor and the operator; and (*c*) sustained availability of the inducer from internal metabolic pools with a wild type constitution of regulator and operator genes. Possibility *a* appears unlikely for systems that demand a quantitative modulation in response to inducing stimulus. The enzyme level in such situations would be essentially uncontrolled. Of the other two situations, our results seem to favor Possibility *c*. If the uninduced levels were previously set by the dissociation

constant of the repressor-operator complex as indicated in *b*, modulation would be permitted only in the direction of increased synthesis; the level of synthesis in absence of externally added glucose would not have decreased. If therefore the observed induction of glycolytic enzymes in response to the addition of sugars involves increased mRNA synthesis, it is very probable that the basal synthesis in the absence of sugars is triggered by internally generated sugar phosphates required for the initiation of transcription.

P-Glucomutase, however, falls outside the category of enzymes that are induced by glucose-6-P and mannose-6-P. In the experiment reflected in Fig. 3, it is induced by 2-deoxyglucose without any lag. It is also induced by galactose without any delay, although glucose does not seem to induce this enzyme (7). It could, however, be induced by glucose-1-P, since 2-deoxyglucose and galactose cause increased accumulation of 2-deoxyglucose-1-P (17) and glucose-1-P (7), respectively.

The central position of glucose-6-P at the crossroad of metabolism ensures the supply of the inducer under diverse growth conditions. During growth on acetate, for example, the levels of glucose-6-P and mannose-6-P are less than when sugars are present (Table VI); the need for glycolytic enzymes also is reduced. Sugars increase the steady state levels of both of the sugar phosphates, and this in turn would induce more glycolytic enzymes. The induction of these enzymes by a phosphorylated intermediate rather than by the free sugar maintains all of the associated advantages of a product-inducible system, as has been discussed by Hayashi and Lin (3), while the requirement of relatively high levels of glucose-6-P for induction of the complete system ensures against abortive induction in response to transient supply of carbohydrates, as would be the case if free sugar were the inducer. The synthesis of glucokinase and a number of other glycolytic enzymes in rat liver in response to the administration of insulin has been ascribed to the inducing action of this hormone *per se* (29, 30). Williamson observed a large increase in the concentration of glucose-6-P within 30 min of perfusion of rat heart with insulin (31). If such a rise occurs in the liver following insulin administration, it remains to be seen whether this is causally related to the induction of glycolytic enzymes.

The term "inducer" has been used here in the broadest connotation without any implication as to its site of action. While the inhibitory effect of cycloheximide on the synthesis of these enzymes in *S. cerevisiae* (7) indicates mediation of protein synthesis in this process, we have been unable to define further the locus of action of these sugar phosphates. The inducing effect could be at transcriptional level, as has been shown for the *lac* operon (28), or at translational level. In the latter case, the sugar phosphates might in some manner facilitate completion of the active enzyme molecules on the polyribosome. We have so far failed to detect any increase of glycolytic enzyme activity by incubating the polyribosomal fraction from uninduced cultures with a mixture of sugar phosphates under conditions permitting amino acid incorporation into proteins (32). The possibility that glucose-6-P removes an internal repressor has not been ruled out, however. There are several lines of evidence against the possibility that pyruvate acts as an end product repressor of glycolytic enzymes, as suggested for *Clostridium* (33). Growth of the hybrid yeast in the yeast extract-peptone-acetate medium produces a few millimoles of pyruvate in the broth which, when supplemented with glucose, yields even higher levels of pyruvate. Further addition of pyruvate to a culture growing in yeast extract-peptone-glucose medium is without effect on the rate of enzyme synthesis. When cells are grown on a yeast extract-peptone medium containing 50 mM pyruvate, the differential rate of synthesis of these enzymes is no less than when acetate replaces pyruvate. The stimulation of the differential rate by 2-deoxyglucose is also of the same order in these two media. If any other repressor is involved, however, *e.g.* 3′,5′-cyclic AMP, which glucose helps to remove (34, 35), the data presented here fail to throw any light in that direction. The prediction then would be that 2-deoxyglucose also removes that repressor.

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