JOURNAL OF BACTERIOLOGY, July 1979, p. 152-160
0021-9193/79/07-0152/09$02.00/0

Vol. 139, No. 1

Physiological Effects of Seven Different Blocks in Glycolysis in *Saccharomyces cerevisiae*

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Received for publication 28 February 1979

Saccharomyces cerevisiae mutants unable to grow and ferment glucose have been isolated. Of 45 clones isolated, 25 had single enzyme defects of one of the following activities: phosphoglucose isomerase (*pgi*), phosphofructokinase (*pfk*), triosephosphate isomerase (*tpi*), phosphoglycerate kinase (*pgk*), phosphoglyceromutase (*pgm*), and pyruvate kinase (*pyk*). Phosphofructokinase activities in crude extracts of the *pfk* mutant were only 2% of the wild-type level. However, normal growth on glucose medium and normal fermentation of glucose suggested either that the mutant enzyme was considerably more active in vivo or, alternatively, that 2% residual activity was sufficient for normal glycolysis. All other mutants were moderately to strongly inhibited by glucose. Unusually high concentrations of glycolytic metabolites were observed before the reaction catalyzed by the enzyme which was absent in a given mutant strain when incubated on glucose. This confirmed at the cellular level the location of the defect as determined by enzyme assays. With *adh* (lacks all three alcohol dehydrogenase isozymes) and *pgk* mutants, accumulation of the typical levels of hexosephosphates was prevented when respiration was blocked with antimycin A. A typical feature of all glycolytic mutants described here was the rapid depletion of the intracellular adenosine 5'-triphosphate pool after transfer to glucose medium. No correlation of low or high levels of fructose-1,6-bisphosphate with the degree of catabolite repression and inactivation could be found. This observation does not support the concept that hexose metabolites are directly involved in these regulatory mechanisms in yeast.

The metabolism of fermentable sugars by the Embden-Meyerhoff pathway in *Saccharomyces cerevisiae* causes several remarkable changes in the metabolic state of yeast cells: (i) a strong increase in the glycolytic flux accompanied by incomplete oxidation and alcoholic fermentation of such sugars; (ii) repression of the synthesis of mitochondrial enzymes and cytochromes (catabolite repression); (iii) repression of the synthesis of enzymes of the glyoxylate shunt and gluconeogenesis; and (iv) even proteolytic inactivation of some repressible enzymes (8, 12, 21, 22). Although much knowledge is available concerning the regulation of glycolysis (Pasteur effect), few attempts have been made to clarify the mechanism relating the rate of glycolysis to the extent of repression of the enzymes required for growth on nonfermentable carbon sources. This repression by fermentable sugars presents one of the major problems of regulatory genetic systems in yeast. We have recently isolated a yeast mutant in which a wide spectrum of normally repressible enzymes had become resistant to catabolite repression. This effect was associated with a reduction of the glycolytic flux on glucose medium (6). To test a possible relationship between the glycolytic flux and catabolite repression, as well as inactivation of gluconeogenic enzymes, we isolated mutants with enzyme defects in glycolysis.

Several yeast glycolytic mutants have been isolated recently (3, 7, 14, 15, 23), but only mutants lacking hexokinases (16), phosphoglucose isomerase (18), or pyruvate kinase (20, 23) were characterized in detail. The common feature of all of those mutants was that growth was inhibited by glucose. Usually, this inhibition has been attributed to the accumulation of toxic levels of glycolytic intermediates, depletion of ATP, or repression of oxidative metabolism (14, 18, 20, 23). We have investigated this problem by using mutants with defects at seven different steps of glycolysis (phosphoglucose isomerase [*pgi*], phosphofructokinase [*pfk*], triosephosphate isomerase [*tpi*], phosphoglycerate kinase [*pgk*], phosphoglyceromutase [*pgm*], and pyruvate kinase [*pyk*]); a previously isolated mutant lacking all alcohol dehydrogenases (*adh*)

(3) was included in this study. A detailed investigation of metabolite accumulation allowed us to draw preliminary conclusions regarding growth inhibition in some glycolytic mutants by glucose. Moreover, the relation between intracellular levels of glycolytic intermediates and catabolite repression and inactivation was studied.

MATERIALS AND METHODS

Yeast strains. Glycolytic mutants were isolated in two strains of our collection: SMC-1B (a *his4 MAL2-8^c MAL3 SUC3*) and GR1.2-1A (α *ilv1 MAL4 SUC*). The designations *MAL* and *SUC* refer to the ability to ferment maltose or raffinose, respectively (see reference 26). Strain 4.12D (a *leu1 ade2-110 MAL4*) was used for the determination of mating type. Strains DFY 23 (a *ino1 ino4 pyk4*) and DFY 70 (α *ino leu2 pfk1*) were kindly provided by D. Fraenkel and were used as allele tester strains for mutants lacking pyruvate kinase or phosphofructokinase activity. Strain M6.1A (α *his4 adc1-11 adr2-56 adm*) lacks all three alcohol dehydrogenase isozymes and is called *adh* in the text. For gene designations *adc1*, *adr2*, and *adm*, see reference 4.

Media and growth conditions. YEP medium (2% peptone [Difco], 1% yeast extract [Difco]), supplemented with 2 or 8% glucose, 2% glycerol-2% ethanol (YEPGE), or all of these carbon sources, was used as a basic medium. Difco agar (15 g/liter) was added to solidify media. Synthetic complete medium consisted of 2% glucose and 0.67% Difco yeast nitrogen base, supplemented with 11 amino acids, adenine, and uracil (25). Nutritional requirements were determined on synthetic drop-out medium as described by Zimmermann (25). Durham tubes with YEP-2% glucose or YEP-2% maltose were used for a rough determination of fermentation characters. Sporulation was induced on potassium acetate plates. Liquid cultures were grown aerobically by shaking at 28°C.

Mutagenesis and isolation of mutants. Stationary haploid yeast cells were mutagenized with 2% ethyl methane sulfonate in 0.1 M potassium phosphate buffer, pH 7.0, for 1 h at 28°C. The treatment of the cells was stopped by centrifugation and washing. Cells were subsequently suspended in liquid YEPGE medium to allow for mutation fixation. After 6 h, 100 to 200 cells were spread onto plates with YEPGE medium and incubated until visible colonies appeared. A total of 60,000 colonies were replica plated onto synthetic complete medium with glucose as the sole carbon source. It was assumed that glycolytic mutants would grow poorly or not at all on this medium. Altogether, 150 presumptive mutant colonies were picked up and further tested as shown below.

Preparation of crude extracts and enzyme assays. Cells grown in 5 to 20 ml of YEPGE medium were washed twice with 50 mM imidazole buffer (pH 7.0). The pellet of about 5×10^8 cells was mixed with 500 mg of glass beads (0.4 to 0.5-mm diameter) and shaken for 5 min on a Heidolph Whirlmix in a cold room as described previously (3). Imidazole buffer (2 ml) was added to the broken cells, and the mixture was centrifuged at 2,000 × *g* for 10 min. The supernatant was used as the source for all enzyme determinations described below. Glycolytic enzymes were assayed by coupling to appropriate NADP- or NADH-linked reactions, as described by Maitra and Lobo (19), but in 50 mM imidazole buffer, pH 7.0, containing 10 mM Mg^{2+}. Alcohol dehydrogenase, succinate dehydrogenase, isocitrate lyase, and fructose-1,6-biosphosphatase were determined as previously described (3, 5). Specific activities were calculated as nanomoles per milligram of protein times minutes. Protein was determined by the method of Lowry et al. (17), using bovine serum albumin as a standard.

Preparation of cellular metabolites. An appropriate amount of growing cells (equal to 20 to 50 mg, dry weight) was rapidly sampled on a membrane filter (Millipore Corp., pore size, 8 μm) and transferred into 5 ml of boiling ethanol. After 1 min, the mixture was cooled in ice and dried under vacuum within 5 min. The cell residue was suspended in 3 ml of imidazole buffer and centrifuged at 4,000 × *g* for 10 min. The clear supernatant was used for determinations of glycolytic metabolites and ATP. Determinations were carried by the method of Bergmeyer (1), using a Beckman model 25 double-beam spectrophotometer. The results obtained with wild-type cells were in close agreement with those reported by others (11).

Respiration and fermentation rates. Oxygen uptake and carbon dioxide production of whole cells were measured with a standard Warburg respirometer at 30°C in YEP medium, adjusted to pH 5.0. Growth rates were determined photometrically at 600 nm in parallel cultures.

Genetic analysis. Standard yeast genetic techniques were used for genetic analyses of mutants. The determination of nutritional requirements was not possible on synthetic medium. Therefore, the mutants had to be tested in complementation tests with normal nonmutant strains of the opposite mating type, each of which carried one of the markers segregating in the cross (e.g., *ilv1*). These markers were then identified by lack of complementation in the resulting diploids.

Chemicals. All ancillary enzymes, cofactors, and substrates were purchased from Boehringer (Mannheim, Federal Republic of Germany). Ethyl methane sulfonate was obtained from E. Merck AG (Darmstadt, Federal Republic of Germany).

RESULTS

Isolation and characterization of glycolytic mutants. Mutants with enzyme lesions in glycolysis should not grow on a medium with glucose as the sole carbon source (i.e., glucose synthetic medium), nor should they ferment glucose. Except for hexokinase, phosphofructokinase, pyruvate kinase, pyruvate decarboxylase, and alcohol dehydrogenase, all glycolytic enzyme activities are probably also indispensable for glyconeogenesis. This fact makes the search for glycolytic mutants more difficult. Therefore, YEPGE medium was considered as a permissive one for all possible mutants except those lacking aldolase and phosphoglucose isomerase activities. Lack of growth on synthetic glucose me-

dium was not considered a sufficient criterion for glycolytic mutants, becuase other defects might create this phenotype (e.g., glucose uptake mutants). Therefore, 152 clones were selected and tested for glucose and maltose fermentation. Those showing normal fermentation were rejected. Isolates fermenting neither glucose nor maltose were considered to be blocked in glycolysis and not in sugar uptake since maltose enters yeast cells by an uptake system different from glucose (24). On this basis, 45 mutants were selected. We then tried to relate their growth and fermentation behavior to defects in one of the following enzyme activities: hexokinase (EC 2.7.1.1), phosphoglucose isomerase (EC 5.3.1.9), phosphofructokinase (EC 2.7.1.11), aldolase (EC 4.1.2.13), triosephosphate isomerase (EC 5.3.1.1), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), phosphoglycerate kinase (EC 2.7.2.3), phosphoglyceromutase (EC 2.7.5.3), enolase (EC 4.2.1.11), pyruvate kinase (EC 2.7.1.40), and pyruvate decarboxylase (EC 4.1.1.1). Table 1 shows enzyme activities of wild-type cells and seven phenotypic mutant classes. A strain completely lacking alcohol dehydrogenase activity (EC 1.1.1.1) was included in these experiments. Nineteen glucose nonfermenters had no specific enzymatic lesion. One mutant showed reduced activities for several glycolytic enzymes. Genetic analysis revealed, however, that this phenotype was due to a single mutational event. The latter two mutant classes were not included in the experiments described below.

The mutants were then crossed to a wild-type strain for further genetic analysis. They were all recessive. Normal 2:2 segregation of the fermentation characters was followed in at least 10 tetrads in each wild type × mutant cross. In all but one mutant, the enzymatic defect segregated along with the inability to ferment glucose. Complementation tests between mutants with the same lesion showed in all cases that they affected the same gene. Moreover, none of eight pyruvate kinase (*pyk*) mutants complemented a *pyk* mutant kindly provided by D. G. Fraenkel. This mutant is probably defective in the pyruvate kinase structural gene (D. G. Fraenkel, personal communication). No tetrads with four viable spores were obtained from the cross *pfk* × wild type. Nevertheless, it was obvious that the phosphofructokinase defect did not segregate along with the inability to ferment glucose; i.e., nonfermenting and fermenting segregants with normal phosphofructokinase levels, as well as fermenting and nonfermenting enzyme-negative segregants were observed. This was confirmed in a subsequent cross. This suggested that the original glucose-inhibited *pfk* mutant phenotype was created by two mutations, one causing a defect in phosphofructokinase and the other causing an additional and unknown block in glucose fermentation. The *pfk* mutation was considered to be leaky in vivo since it did not block glucose fermentation. It was found to be allelic to a *pfk* mutant recently isolated by Clifton et al. (7) which, quite remarkably, was also leaky in respect to glucose fermentation.

Growth inhibition by glucose. It was known from several studies with glycolytic mutants of yeast (7, 14, 18, 23) and *Escherichia coli* (10, 12) that glucose causes cessation of growth on otherwise permissive medium. This inhibition was found in most of our mutants. Figure 1 shows the effect of an addition of 2% glucose to wild-type and mutant cells growing logarithmically on YEPGE medium. Growth was reduced in *tpi* and *pgk* mutants, but completely inhibited in *pgi*, *pgm*, and *pyk* mutants (data for the latter two types not shown). *tpi* and *pgk* mutants also grew rather slowly on permissive YEPGE medium (doubling times: *tpi* mutant, 6 to 7 h; *pgk* mutant, 5 h: wild type 3 h). In contrast, the *pfk*

TABLE 1. *Specific activities of glycolytic enzymes in wild-type and mutant cells affected at various glycolytic reactions*[a]

Strain	Genotype	Sp act (nmol/min per mg of protein)							
		HXK	PGI	PFK	TPI	PGK	PGM	PYK	ADH
GR1.2-1A	Wild type	1,045	2,173	291	3,850	12,720	538	7,230	6,350
GLU128	*pgi* (4)	797	70	230	3,820	13,620	526	7,540	5,110
GLU94.4-2B	*pfk* (1)	1,154	1,852	4	2,740	12,680	428	4,940	6,350
GLU77.1-9A	*tpi* (1)	928	1,576	306	<3	11,110	323	7,890	4,522
GLU151	*pgk* (6)	940	1,380	170	3,810	300	360	5,280	4,870
GLU105	*pgm* (5)	797	1,826	223	2,490	11,730	<2	6,320	6,370
GLU36	*pyk* (8)	926	1,776	281	3,620	5,380	688	44	3,220
M6.1-1A	*adc1 adr2 adm*	769	3,550	240	3,040	10,700	500	4,710	<5

[a] Cells were grown on liquid YEPGE medium. Abbreviations: HXK, hexokinase (with glucose as substrate); PGI, phosphoglucose isomerase; PFK, phosphofructokinase; TPI, triosephosphate isomerase; PGK, phosphoglycerate kinase; PGM, phosphoglyceromutase; PYK, pyruvate kinase; ADH, alcohol dehydrogenase. The number of isolates is given within parentheses.

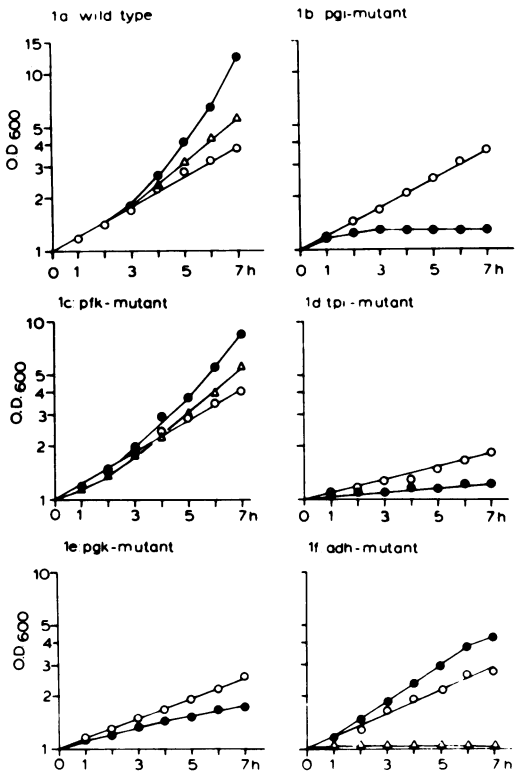

FIG. 1. *Effect of glucose on growth of wild-type cells and various glycolytic mutants. Glucose was added at 0 h to YEPGE-grown cells to give a final concentration of 2%. Growth was monitored as optical density at 600 nm (OD_{600}). Symbols: ○, YEPGE; ●, YEPGE + 2% glucose; △, YEPGE + 2% glucose + 1 μg of antimycin A per ml. Mutant designations as in Table 1.*

mutant (without the additional lesion) behaved similarly to the wild type. Growth of cells lacking alcohol dehydrogenase was initially stimulated by glucose. After prolonged incubation, however, doubling times of 6 to 10 h were usually observed (wild type, 80 to 100 min). In mutants, in which growth was stimulated by glucose (*pfk* and *adh*), we tested the effect of the respiration inhibitor antimycin A (2) on glucose growth. Glucose-growing wild-type cells were hardly affected when respiration was blocked (Fig. 1a). Of the mutants tested, cells lacking phosphofructokinase were again rather unaffected, confirming the in vivo leakiness of the *pfk* mutant. However, growth of *adh* cells on glucose stopped immediately upon addition of antimycin (Fig. 1f). This can be explained by the main physiological function of alcohol dehydrogenase in yeast, i.e., the regeneration of NAD during the fermentative breakdown of sugars. In *adh* cells, NAD regeneration is only feasible via respiration. If respiration is blocked by antimycin A, energy and carbon metabolism ceases due to a lack of NAD regeneration.

Accumulation of glycolytic intermediates and depletion of ATP. It is known from studies with yeast mutants lacking phosphoglucose isomerase (18) or pyruvate kinase (20) that considerable accumulation of glycolytic intermediates takes place upon addition of glucose to the medium. Therefore, we determined the kinetics of metabolite and ATP levels after glucose addition, hoping that such an investigation would provide some hints as to the causes of inhibition in some of the mutants. Moreover, it would allow an examination of a possible regulatory role of glycolytic metabolites in catabolite repression and inactivation.

Two percent glucose was added to cells growing on YEPGE, because this was the only permissive medium for all mutants and because the levels of most of the glycolytic metabolites are usually low in wild-type cells grown on this medium (Table 2, column 1). Addition of glucose to wild-type cells caused an increase in the content of most of the glycolytic metabolites, except 1,3-diphosphoglycerate, 2-phosphoglycerate, and phosphoenolpyruvate, which were low under all conditions. Most prominent was the 20- to 30-fold increase in fructose-1,6-bisphosphate, a typical character of hexose catabolism in yeast. A survey on metabolite levels in wild-type and mutant cells before and 2 h after the addition of glucose is given in Table 2. The observed accumulation of metabolites before a given enzymatic lesion can be considered a strong indication that enzyme tests in vitro reflect the physiological situation in vivo. In this respect, the dramatic accumulation of hexosemonophosphates in the *pfk* mutant (110 nmol [dry weight] per mg; wild type, 5.4 nmol [dry weight] per mg) and the lack of fructose-1,6-bisphosphate (Fig. 2a and b) were remarkable, because this mutant was found to be leaky in respect to glucose fermentation and growth. In a *pgi* mutant, only 70 nmol of glucose-6-phosphate (dry weight) per mg could be found after glucose addition. *tpi* mutant cells showed 6 to 10 times more dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate than did wild-type cells on glucose. The defect in triosephosphate isomerase was of special interest because this defect should allow the flow of half of the glucose carbon through glycolysis. Nevertheless, a net energy gain from glycolysis alone should not be possible, because half of the glucose carbon cannot enter the triose interconversion steps. This trait was indeed reflected in the strong accumulation of dihydroxyacetone-phosphate. As shown in Table 2, the two triosephosphates were not present in equimolar amounts. An accumulation of one of the

TABLE 2. *Effect of glucose on contents of glycolytic metabolites in wild-type cells and various glycolytic mutants*[a]

Metabolite	nmol/mg (dry wt) of following genotypes:															
	Wild type		*pgi*		*pfk*		*tpi*		*pgk*		*pgm*		*pyk*		*adh*[b]	
	0[c]	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2
Glucose-6-phosphate	1.4	4.3	0.9	*70.0*	2.0	*85.6*	1.1	1.3	1.6	1.3	0.8	1.5	1.0	1.5	1.1	3.4
Fructose-6-phosphate	0.5	1.1	0.4	<0.2	1.5	*24.3*	0.9	1.7	0.9	2.1	<0.2	0.3	0.2	0.4	0.5	0.6
Fructose-1,6-diphosphate	0.3	9.0	0.8	0.4	0.2	0.3	1.9	7.0	2.8	*49.0*	1.5	15.6	0.5	4.7	0.3	16.2
GAP[d]	0.2	1.1	0.2	0.2	0.2	0.4	*2.5*	*6.3*	0.4	*3.1*	0.2	0.9	0.2	0.5	0.2	2.4
DAP[e]	0.7	4.0	1.0	0.4	1.0	1.0	*16.0*	*42.7*	*2.9*	*19.2*	0.4	7.3	0.8	1.0	0.4	6.0
1,3-Diphosphoglycerate	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
3-Phosphoglycerate	0.5	3.3	0.5	0.5	<0.2	0.4	0.2	0.2	0.2	1.2	*15.7*	*59.2*	1.5	*32.8*	<0.2	3.1
2-Phosphoglycerate	<0.2	0.4	<0.2	0.2	<0.2	0.4	<0.2	0.2	<0.2	0.2	<0.2	0.2	<0.2	*1.0*	<0.2	0.2
PEP[f]	0.5	0.4	0.8	0.2	1.2	0.2	0.7	0.3	0.3	0.4	1.0	0.5	1.2	*15.6*	0.6	0.2
Pyruvate	3.3	7.0	3.0	1.5	4.1	1.9	0.8	1.1	2.3	1.8	0.8	1.0	1.0	1.1	2.0	13.4

[a] Gene designations refer to enzyme defects given in Table 1. Glucose was added to cells (initial cell densities, 2×10^7 to 4×10^7/ml) grown on YEP–2% glycerol–2% ethanol. Significantly stronger accumulation of metabolites than that in wild-type cells is indicated by italics.

[b] Complete genotype, *adc1 adr2 adm*.

[c] Time (hours) after glucose addition.

[d] GAP, Glyceraldehyde-3-phosphate.

[e] DAP, Dihydroxyacetone-phosphate.

[f] PEP, Phosphoenolpyruvate.

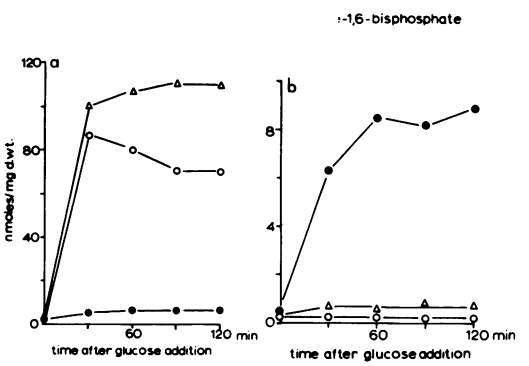

FIG. 2. *Kinetics of intracellular hexosephosphate accumulation in wild-type* (●) *and pgi* (○) *and pfk* (△) *mutant cells. Glucose was added to YEPGE-grown cells to give a final concentration of 2%. (a) Glucose-6-phosphate + fructose-6-phosphate; (b) fructose-1,6-bisphosphate.*

triosephosphates could lead to a backup of fructose-1,6-bisphosphate on glucose medium, but this was not so; only wild-type levels were observed. This might be a consequence of sufficient drainage of glyceraldehyde-3-phosphate through glycolysis. Most remarkably, the *tpi* mutant accumulated, even on YEPGE and without glucose, 10 to 20 times more triosephosphates than did wild-type cells. Although *pgk* mutants are blocked in a reaction two steps in glycolysis after the *tpi* mutant, fructose-1,6-bisphosphate accumulated on glucose medium severalfold above the wild-type or the *tpi* mutant level (Table 2 and see Fig. 4a). 1,3-Diphosphoglycerate was not found in this mutant or in wild-type cells. This could be an artifact caused by the extraction method. On the other hand, equilibrium of the preceding reaction (glyceraldehyde-3-phosphate dehydrogenase) is on the side of glyceraldehyde-3-phosphate. In mutants lacking phosphoglyceromutase (*pgm*), up to 20 times more 3-phosphoglycerate accumulated than in wild-type cells. Remarkably, this intermediate was also very high in glycerol-ethanol-grown *pgm* cells. On glucose, the *pyk* mutants produced 10 to 20 times more phosphoglycerates and phosphoenolpyruvate than did wild-type cells. Surprisingly, measurable amounts of pyruvate were found in all *pyk* mutants on glucose, but never more than 1/5 to 1/10 of the wild-type level. That may not be an indication of leakiness. More likely, the pyruvate found could be derived from amino acid catabolism when cells are grown on rich (YEP) medium. Cells lacking alcohol dehydrogenase activity did not show a significantly stronger accumulation of metabolites than did wild-type cells. Acetaldehyde was not determined quantitatively, but the strong and typical smell of this penultimate fermentation product indicated a considerable accumulation.

A typical feature of most of the mutants studied was the rapid depletion of ATP within the first 30 min of glucose incubation (Fig. 3). Even in the *pfk* mutant, the ATP content dropped from 4.6 to 0.9 nmol (dry weight) per mg, although this mutant was apparently leaky. Again, the behavior of *adh* mutant cells was different

in this respect: ATP fell only slightly within 2 h of incubation on glucose. However, after prolonged exposure to glucose medium and, hence, at reduced growth rates, ATP was hardly measurable (>0.2 nmol [dry weight] per mg). This can be explained by the fact that the energy supply of *adh* mutant cells is dependent upon the functioning of respiration. Respiratory activity is, however, strongly reduced upon prolonged growth on glucose because of catabolite repression. The ATP content in *tpi* mutants pregrown on permissive YEPGE medium was only one-third of the wild-type level. This could be a reflection of the observed high accumulation of triosephosphates in *tpi* cells on YEPGE (Table 2) and ensuing triosephosphate-wasting side reactions.

Accumulation of metabolites and operation of respiration. As shown above, the ATP pool in YEPGE-grown cells is rather small. Obviously, it cannot account for the large accumulation of phosphorylated glycolytic intermediates in the various mutant types. Except for the leaky *pfk* mutant, all mutants were apparently unable to generate a net ATP gain through glycolysis; therefore, glucose and fructose-6-phosphate could be phosphorylated at the expense of either energy reserves (e.g., polyphosphates [22]) or oxidative phosphorylation. Consequently, mitochondrial electron transport was inhibited by antimycin A before or after the addition of glucose to derepressed cells, and the kinetics of accumulation of some representative metabolites were followed in wild-type and *pfk*, *pgk*, and *adh* mutant cells. As shown in Fig. 4a, inhibition of respiration completely prevented the accumulation of fructose-1,6-bisphosphate in *pgk* cells. Moreover, antimycin A induced a rapid loss of more than 95% of this metabolite. Inhibition of respiration in the *pfk* mutant caused a reduced accumulation of hexosemonophosphates (Fig. 4b), but the levels were still well above those found in wild-type cells. Sugar-phosphate accumulation was not significantly affected in wild-type cells upon addition of antimycin A. *adh* mutant cells behaved similarly to cells (data not shown). These results showed that intact respiration is necessary both to generate and to maintain the high levels of hexosephosphates (and possibly of other glycolytic intermediates) observed in mutant cells. Consistent with these results, it could be shown that respiration rates of mutant cells were hardly affected within 3 h after addition of glucose (Table 3).

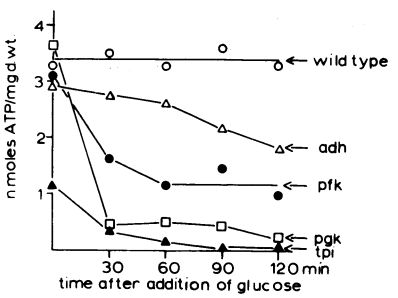

FIG. 3. *Kinetics of ATP content in wild-type cells and various glycolytic mutants after addition of glucose to YEPGE-grown cells. Glucose was added to give a final concentration of 2%. adh, pfk, pgk, and tpi refer to enzyme defects given in Table 1.*

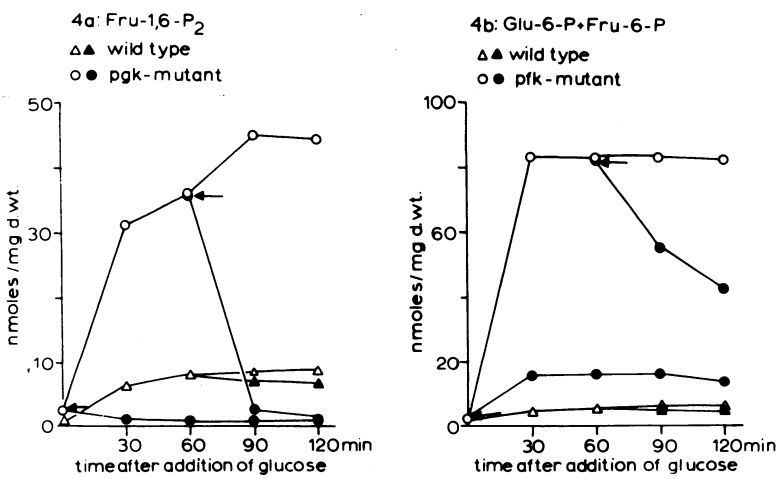

FIG. 4. *Effect of antimycin A-inhibited respiration on hexosephosphate accumulation in wild-type cells and pgk and pfk mutants. Open symbols indicate cells grown without antimycin A. Solid symbols indicate cells grown in the presence of 1 μg of antimycin per ml. Arrows indicate when the antibiotic was added. In all cases, glucose was added to YEPGE-grown cells to give a final concentration of 2%.*

TABLE 3. *Oxygen uptake of wild-type cells and various glycolytic mutants*[a]

Cells	Oxygen uptake (μmols of O_2 per h × 10^7 cells)	
	0 h	3 h
Wild type	0.80	0.56
pgi	1.00	0.76
pfk	1.10	0.86
pgk	0.70	0.71

[a] Glucose was added to YEPGE-grown (derepressed) cells to give a final glucose concentration of 2%. 0 h = 1-h interval before addition of glucose. 3 h = 1-h interval after a 3-h incubation on glucose.

Catabolite repression and inactivation in glycolytic mutants. An as yet unsolved problem of carbohydrate metabolism in yeast is the triggering of repression of enzymes involved in oxidative metabolism and the induction of proteolytic decay of gluconeogenetic enzymes. A simple hypothesis would state that glycolytic intermediates play an important role in the induction of catabolite repression and inactivation, acting as low molecular effectors. This hypothesis is supported by the 4- to 30-fold increase in several intermediates such as fructose-1,6-bisphosphate and, to a lesser extent, glucose-6-phosphate, dihydroxyacetone-phosphate, and 3-phosphoglycerate, when cells are shifted from a YEPGE to a glucose-YEP medium. This view is not supported by the properties of the leaky *pfk* mutant described above. In this mutant, levels of all glycolytic intermediates below the phosphofructokinase step were equally low in cells grown on nonrepressing YEPGE and repressing YEP-glucose medium. Nevertheless, succinate dehydrogenase, isocitrate lyase, and fructose-1,6-bisphosphatase were fully repressed (Table 4).

Finally, we tested whether specific enzymatic defects had any influence on the glucose-induced inactivation of fructose-1,6-bisphosphatase, which bypasses the phophofructokinase reaction when gluconeogenesis is necessary. Strongly reduced inactivation was observed in cells lacking phosphoglucose isomerase (Fig. 5). On glucose, very low levels of fructose-1,6-bisphosphate were found in the *pfk* mutant, but very high levels were found in the *pgk* mutant. Despite this difference, fructose-1,6-bisphosphatase was inactivated in both mutants to the same extent, i.e., 50%; whereas in wild-type cells, inactivation was 81%.

DISCUSSION

The loss of a given enzymatic activity in a mutant, as determined in crude extracts, and the concomitant accumulation of certain glycolytic metabolites in mutant cells supplied with glucose provide sufficient evidence for the physiological relevance of these enzymatic defects. The lack of inhibition by and even the efficient utilization of glucose by our *pfk* mutant was surprising since our isolate retained only 3% of the wild-type phosphofructokinase activity, as determined in the crude extract. Nevertheless, the strong accumulation of hexosemonophosphates reflected reduced enzymatic activity in vivo. The behavior of the leaky *pfk* mutant seems to be relevant for a current explanation of growth inhibition by glucose found in other glycolytic mutants, especially those lacking phosphoglucose isomerase (7, 18; this paper). Maitra (18) assumed that growth inhibition in a *pgi* mutant resulted from an accumulation of toxic concentrations of glucose-6-phosphate. The high levels of hexosemonophosphates (up to 110 nmol [dry

TABLE 4. *Catabolite repression of succinate dehydrogenase (SDH), isocitrate lyase (ICL), and fructose-1,6-bisphosphatase (FbPase) in wild-type and pfk mutant cells*[a]

Cells	Sp act (nmol/min per mg of protein)					
	SDH		ICL		FbPase	
	r	dr	r	dr	r	dr
Wild type	4.8	78	1.5	177	1	64
pfk mutant	3.7	69	1	211	2	70

[a] r, Cells grown on YEP-8% glucose (repressed cells); dr, cells grown on YEP-3% ethanol (derepressed cells).

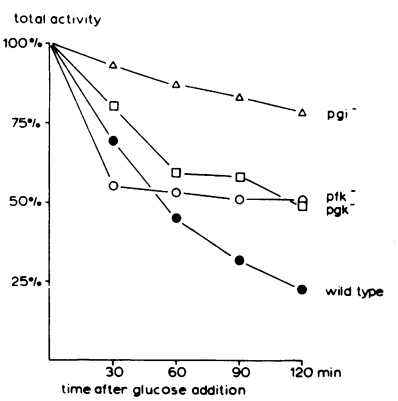

FIG. 5. *Glucose-induced inactivation of fructose-1,6-bisphosphatase on wild-type cells and mutants with defects in phosphoglucose isomerase (pgi), phosphofructokinase (pfk), and phosphoglycerate kinase (pgk), respectively. Cells were pregrown on YEP-ethanol. Values were calculated as total activity (specific activity times relative dry weight; dry weight at 0 min was taken as 1.0). Initial specific activities (nanomoles per milligram of protein times minutes): wild type, 140; pgi mutant, 132; pfk mutant, 73; and pgk mutant, 142.*

weight] per mg) found in our glucose-grown *pfk* mutant does not support this assumption. The only relevant difference between *pgi* and *pfk* mutant cells seems to be the ability of *pfk* cells to catabolize glucose via the Embden-Meyerhoff pathway. Moreover, ATP depletion upon addition of glucose was not complete, as it was found in *pgi* and other glycolytic mutants. Finally, the growth of *pfk* mutant cells on glucose in the presence of the respiration inhibitor antimycin A proved definitely that *pfk* cells were able to generate sufficient ATP y glycolysis alone. These results suggest that complete ATP depletion in *pgi* mutant cells is the primary effect leading to growth cessation on glucose.

Lam and Marmur (14) recently isolated yeast mutants affected at various steps of the triosephosphate interconversion (*pgk*, *pgm*, and *pyk*). We isolated an additional mutant type lacking triosephosphate isomerase (*tpi*). All of these mutants were found to be effectively inhibited by glucose. It was suggested (14) that addition of glucose to *pgk*, *pgm*, and *pyk* mutants causes depression of oxidative phosphorylation or depression of glycerol and ethanol uptake or both, which otherwise could be used as energy sources by these mutants. This seems unlikely for two reasons. First, reduction of respiratory activity in yeast cells can only be observed in growing cells; i.e., the decreasing rate of synthesis of respiratory enzymes causes reduced respiratory activity (6). Addition of glucose to mutant cells, however, inhibited growth within 1 or 2 h, i.e., before an effective dilution of preexisting respiratory enzymes could have taken place. Second, respiration rates were hardly affected upon glucose addition in *pgi*, *pfk*, and *pgk* cells. Obviously, there is neither an effective inhibition of oxidative phosphorylation in the mutants nor a considerable effect on glycerol and ethanol uptake which would result in decreasing respiration rates as well. The effect of antimycin A (Fig. 4a) shows that oxidative phosphorylation is necessary for the high accumulation of fructose-1,6-bisphosphate in the *pgk* mutant.

A somewhat surprising result was the considerable amount of triosephosphates in *tpi* mutant cells (18.5 nmol [dry weight] per mg; wild type, 0.9 nmol [dry weight] per mg) and of 3-phosphoglycerate in a *pgm* mutant when grown on permissive YEPGE medium. Similar results were obtained by Irani and Maitra (13) in *pgk* and glyceraldehyde-3-phosphate dehydrogenase mutants of *E. coli*. Once again, our data suggest that the cause of growth inhibition is not primarily due to the accumulation of toxic levels of triosephosphates on glucose. On the other hand, Clifton et al. (7) were able to select mutants affected in the first steps of glycolysis (hexokinase and phosphofructokinase) as "revertants" on glucose-pyruvate of a pyruvate kinase-deficient mutant. Partial epistasis of those secondary lesions over the pyruvate kinase defect would suggest that accumulation of phosphoglycerates and phosphoenolpyruvate is responsible for growth inhibition by glucose, at least in *pyk* mutants.

An unique feature of all glycolytic mutants, except those lacking alcohol dehydrogenase, is the rapid depletion of ATP on glucose. This does not seem to be due to an inhibition of oxidative phosphorylation. Possibly, the consumption of ATP by glucose-phosphorylating enzymes and by phosphofructokinase is so high that nothing remains for other essential ATP-requiring cellular processes. The expenditure of ATP by phosphorylating reactions can be compensated for in wild-type cells by the triosephosphate interconversions, leading to the steady-state level of ATP. The equilibrium is disturbed in most of the glycolytic mutants, because of the lack of a glycolytic net production of ATP. The leaky *pfk* mutant, which accumulated high levels of phosphorylated compounds and showed a rather low level of ATP on glucose, cannot be taken as counterevidence for this interpretation, because it was indirectly shown that glycolytic ATP production is feasible in this mutant. These considerations suggest that ATP depletion is the primary effect leading to growth cessation of glycolysis mutants on glucose.

No direct conclusions can be drawn concerning the influence of various levels of certain glycolytic intermediates on catabolite repression and inactivation. Because of the strong increase in fructose-1,6-bisphosphate after a shift of wild-type cells from a nonfermentable carbon source to glucose, the latter metabolite could be regarded as a likely candidate for a metabolic effector regulating carbon catabolite repression. The complete repression of oxidative and gluconeogenic enzymes at low levels of fructose-1,6-bisphosphate in glucose-grown *pfk* cells does not support this suggestion. No clear relationship was found between low or high levels of fructose-1,6-bisphosphate and inactivation of fructose-1,6-bisphosphatase in *pgi*, *pfk*, and *pgk* cells. The reduced inactivation rate observed in *pgi* mutants (and in most other glycolytic mutants) can be considered as a secondary effect of a severe disturbance of cellular processes, probably through the depletion of ATP on glucose. Inactivation was, however, normal in the *pfk* mutant, at least within 60 min after glucose addition, despite a very low level of fructose-1,6-bisphosphate. This leaves only the hexoses themselves as being involved in the induction of catabolite repression and inactivation. Working

with a yeast mutant with reduced hexokinase activity, Entian (9) recently suggested that a conformational or aggregational change of a hexose-phosphorylating enzyme by its substrate is involved in the initial reactions in catabolite inactivation.

ACKNOWLEDGMENTS

We thank F. K. Zimmermann and M. Grossmann for stimulating discussions and many suggestions and Ingrid Scheel and Susanne Janowsky for their skillful technical assistance.

This work was supported by Deutsche Forschungsgemeinschaft.

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