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# Properties of *Escherichia coli* Mutants Deficient in Enzymes of Glycolysis

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Physiological properties of mutants of *Escherichia coli* defective in glyceraldehyde 3-phosphate dehydrogenase, glycerate 3-phosphate kinase, or enolase are described. Introduction of a lesion in any one of the reversible steps catalyzed by these enzymes impaired both the glycolytic and gluconeogenic capabilities of the cell and generated an obligatory requirement for a source of carbon above the block (gluconeogenic) and one below (oxidative). A mixture of glycerol and succinate supported the growth of these mutants. Mutants lacking glyceraldehyde 3-phosphate dehydrogenase and glycerate 3-phosphate kinase could grow also on glycerol and glyceric acid, and enolase mutants could grow on glycerate and succinate, whereas double mutants lacking the kinase and enolase required L-serine in addition to glycerol and succinate. Titration of cell yield with limiting amounts of glycerol with Casamino Acids in excess, or vice versa, showed the gluconeogenic requirement of a growing culture of *E. coli* to be one-twentieth of its total catabolic and anabolic needs. Sugars and their derivatives inhibited growth of these mutants on otherwise permissive media. The mutants accumulated glycolytic intermediates above the blocked enzyme on addition of glucose or glycerol to resting cultures. Glucose inhibited growth and induced lysis. These effects could be substantially overcome by increasing the osmotic strength of the growth medium and, in addition, including 5 mM cyclic adenosine 3',5'-monophosphate therein. This substance countered to a large extent the severe repression of β-galactosidase synthesis that glucose caused in these mutants.

The Embden-Meyerhof pathway can be regarded as a sequence of reactions producing the oxidizable, energy-yielding substrate pyruvate and several essential biosynthetic precursors such as hexose monophosphates, dihydroxyacetone phosphate, glycerate 3-phosphate, and phosphoenolpyruvate. A block in any of the enzymes between triose phosphate isomerase (TPI) and pyruvate kinase (PYK) (Fig. 1) divides this amphibolic sequence into two branches, starting from glucose and pyruvate, respectively, and functioning in opposite directions to meet the biosynthetic needs of the cell. Thus, in the absence of any other productive route, such mutants should fail to grow on glycerol, glucose, or pyruvate, and the provision of an appropriate biosynthetic precursor should elicit growth on any oxidizable substrate. Based on the logic that mutants blocked in a bidirectional sequence should require compounds at both ends for growth, we described a method that enabled the isolation of single-gene mutants of *Escherichia coli* deficient in glyceraldehyde 3-phosphate dehydrogenase (GAP), glycerate 3-phosphate kinase (PGK), or enolase (ENO) activity (15). Using a related selection procedure, Hillman and Fraenkel have also isolated similar glycolytic mutants and characterized those lacking GAP (13). In an earlier communication (16), we described the genetic characteristics of these mutations. We report here some physiological properties of these mutants, with special reference to those lacking PGK and ENO. To minimize interference from other unknown mutations harbored by the original isolates, the mutations *pgk-1* and *eno-2* were transferred by transduction, and the behavior of the transductants was compared with that of their respective isogenic parents.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains used for these studies are listed in Table 1. The mutant strains were maintained on medium X (see below). All cultures were routinely grown in medium X with aeration at 37°C for most of the experiments. The incubation of agar plates was carried out at 37°C. Cell growth in liquid medium was determined turbidimetrically at 650

nm, using a cuvette with a 1-cm path length and a Zeiss spectrophotometer, model PMQ II. An absorbance value of unity corresponded to 1.8 mg of wet *E. coli* cells, determined by measuring the $E_{650}$ of a number of suspensions whose wet weight was determined on membrane filters.

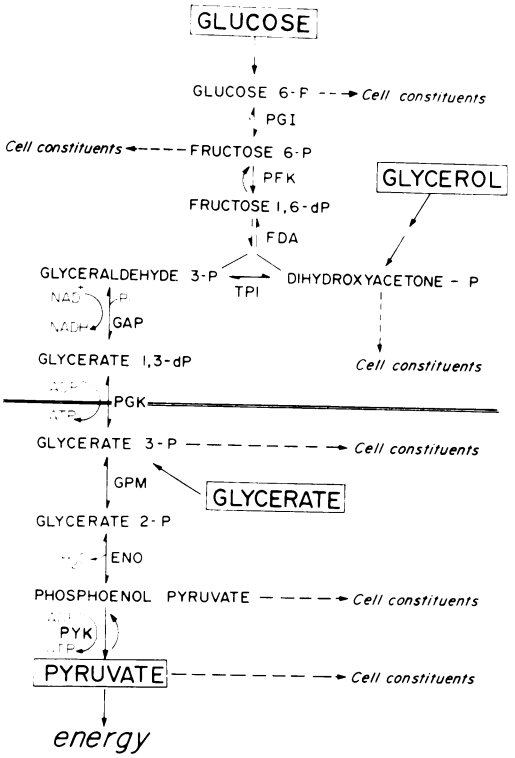


FIG. 1. *Schematic representation of the rationale for selecting mutants affected in the interconversion of glyceraldehyde 3-phosphate and PEP. For the purpose of illustration, the block has been placed at the PGK reaction. The enzyme symbols are explained in the legend to Table 2.*

**Media.** LB broth contained 1% tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl, adjusted to pH 6.7. Glucose (0.1%) was also present unless otherwise stated. The basal mineral salts medium consisted of 0.58% $Na_2HPO_4 \cdot 2H_2O$, 0.3% $KH_2PO_4$, 0.1% $NH_4Cl$, 0.05% NaCl, and 0.01% $MgSO_4$; pH was 6.7. It was supplemented with 1 μg of thiamine hydrochloride and 20 μg of the necessary L-amino acids (reagent grade) per ml. $MgSO_4$ and the carbon sources were added separately. The sugars were present at a concentration of 10 mM, glycerol, glycerol phosphate, and glycerate at 25 mM, and gluconeogenic substrates such as sodium succinate and sodium lactate at 40 mM, unless stated otherwise. All the sugars except L-arabinose, L-rhamnose, and L-fucose were of D-configuration. Glucose was obtained from Sarabhai Chemicals; the rest of the sugars were from Sigma Chemical Co. DL-α-Glycerol phosphate and the calcium salt of DL-glyceric acid were also obtained from Sigma. From a 0.1 M solution of calcium glycerate, the $Ca^{2+}$ was precipitated out with $Na_2HPO_4$. Medium X consisted of the basal medium supplemented with 1 μg of thiamine hydrochloride per ml, 20 μg of L-tryptophan per ml, 0.5% Casamino Acids, 4 mM glycerol, and 40 mM sodium succinate. For preparing solid media, 1.5% agar (Difco) was used; 1.2% Oxoid agar was used for LB broth plates.

**Isolation of PGK-ENO double mutants.** Strain T38 (*pgk-1*) was mutagenized by using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described earlier (15). After penicillin enrichment in minimal medium containing 1 mM glycerol, 25 mM DL-glycerate, and L-serine, appropriate dilutions were plated on glycerol plus succinate minimal plates containing L-serine. Colonies unable to grow on a combination of glycerol, glycerate, and L-serine were retained.

**Preparation of extract and enzyme assays.** Cells were harvested at the stationary phase for preparing the crude extracts. Enzyme assays were made on 20,000 × *g* supernatant fractions from sonically

TABLE 1. *E. coli K-12 strains used*[a]

| Strain | Genotype | Source or reference |
|---|---|---|
| A | *trp leu proA his argE ilvC tyrA* | *lac*$^+$ *gal*$^+$ derivative of strain AN16 (14, 15) |
| ASm | *trp leu proA his argE ilvC tyrA rpsL* | Spontaneous streptomycin-resistant derivative of A |
| H65 | *tpi-1*[b] | Mutagenesis of A |
| K4 | *fda-1*[b] | Mutagenesis of A |
| L9 | *pgk-1*[b] | Mutagenesis of A |
| G6 | *gap-1*[b] | Mutagenesis of A |
| H73 | *gap-2*[b] | Mutagenesis of A |
| O13 | *gap-3*[b] | Mutagenesis of ASm |
| O16 | *eno-2*[b] | Mutagenesis of ASm |
| O6 | *eno-3*[b] | Mutagenesis of ASm |
| I51 | *eno-1*[b] | Mutagenesis of A |
| AT713 | *argA cysC lysA mtl xyl mal rel-1 sup-59 rpsL* | 27 |
| MA176 | *thr leu serA lysA thi rpsL* | 21 |
| T38 | *thr leu lysA pgk-1 thi rpsL* | *serA*$^+$ transductant of MA176 |
| AT3 | *argA lysA eno-2*[b] | *cysC*$^+$ transductant of AT713 |
| M15 | *thr leu lysA pgk-1 eno-4 thi rpsL* | Mutagenesis of T38 |

[a] All the strains are F$^-$. The genetic symbols used are those of Bachmann et al. (2).
[b] In addition, these strains carry all the markers of their respective parents.

disrupted extracts prepared in 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM 2-mercaptoethanol and 2 mM ethylenediaminetetraacetic acid. The glycolytic enzymes were assayed fluorimetrically (23). Low levels of enolase were assayed by the discontinuous assay procedure as previously described (15). The pyruvate kinase reaction was initiated with adenosine 5'-diphosphate instead of fructose 1,6-diphosphate (FDP). Protein content of the extract was measured by the Folin-Ciocalteu method (19), using bovine serum albumin as the standard. β-Galactosidase was measured at 37°C with toluene-treated suspensions, using a 1-ml reaction mixture in 50 mM potassium phosphate buffer, pH 7.5, containing 0.2 mg of *o*-nitrophenyl-β-D-galactoside. The reaction was arrested with 2 ml of 2 M $Na_2CO_3$. A unit of β-galactosidase is defined as the amount producing 1 μmol of substrate hydrolyzed per min at 37°C.

## RESULTS

**Growth characteristics.** The mutants were originally isolated as derivatives of the parent strain A or ASm as normal colonies on glycerol-supplemented succinate minimal medium and were unable to grow on either glycerol, glucose, or succinate as carbon source. In Table 2 are listed the specific activities of most of the glycolytic enzymes of the mutant isolates described in Table 1. The selection procedure yielded mutants lacking either GAP, PGK, ENO, or TPI. A few mutant colonies that could grow on glycerol or succinate but not on glucose were subsequently found to be incapable of growth on LB broth or on succinate minimal medium containing glucose. This phenotype was found to be due to the loss of about 90% of fructose 1,6-diphosphate aldolase activity.

In Table 3 are summarized the growth patterns of these glycolytic mutants. The GAP, PGK, and ENO mutants failed to grow on all sugars and oxidative substrates tested. When provided with an appropriate carbon source above the block, they could grow on any usable energy source below the block, but only under aerobic conditions. Their inability to grow on glucose and gluconate is also illustrated by the growth kinetics in minimal medium of strains T38 and AT3, the transductional derivatives of *pgk-1* and *eno-2*, respectively (Fig. 2). Noteworthy is the fact that the growth rates of the mutants on the permissive glycerol-supplemented succinate minimal medium were similar to those of their respective wild-type parents.

Choice of the biosynthetic supplement was limited to L-rhamnose, L-fucose, glycerol, and glycerol derivatives such as DL-α-glycerol phosphate and DL-glycerate when present at a 5 mM concentration (Tables 3 and 4). The use of glycerate deserves special mention: glycerate, which is metabolized via glycerate 3-phosphate (Fig. 1), can, in combination with glycerol, support growth of GAP and PGK mutants but not of ENO-deficient strains. On the other hand, the combination of glycerate and succinate has the contrary effect. This is in keeping with the demands of the selection procedure and can form the phenotypic basis of differen-

TABLE 2. *Specific activities of enzymes of glycolysis in wild-type and mutant strains of E. coli*[a]

| Strain | Sp act | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | PGI | PFK | FDA | TPI | GAP | PGK | GPM | ENO | PYK | ZWF |
| A | 0.690 | 0.280 | 0.065 | 1.550 | 1.200 | 2.000 | 2.000 | 0.450 | 0.700 | 0.095 |
| ASm | 0.600 | 0.250 | 0.040 | 1.500 | 1.000 | 1.700 | 1.800 | 0.375 | 0.570 | 0.050 |
| G6 (*gap-1*) | 0.670 | 0.180 | 0.045 | 1.280 | 0.010 | 2.500 | 1.400 | 0.430 | 0.390 | 0.055 |
| H73 (*gap-2*) | 0.640 | 0.235 | 0.056 | 1.460 | 0.005 | 1.720 | 1.860 | 0.530 | 0.640 | ND[b] |
| O13 (*gap-3*) | ND | ND | ND | 1.400 | 0.120 | 2.800 | 3.840 | 0.445 | 0.745 | ND |
| L9 (*pgk-1*) | 0.670 | 0.300 | 0.110 | 1.260 | 1.200 | 0.008 | 1.500 | 0.470 | 0.745 | 0.110 |
| I51 (*eno-1*) | 0.710 | 0.255 | 0.064 | 1.400 | 1.330 | 1.600 | 1.400 | 0.005 | 0.740 | 0.072 |
| O16 (*eno-2*) | 0.470 | 0.160 | 0.041 | 1.350 | 1.750 | 1.400 | 1.500 | 0.001 | 0.760 | 0.043 |
| O6 (*eno-3*) | 0.590 | 0.200 | 0.047 | 1.450 | 1.100 | 1.300 | 1.600 | 0.002 | 0.500 | 0.052 |
| H65 (*tpi-1*) | ND | ND | 0.063 | 0.015 | 1.200 | 2.100 | 2.300 | 0.680 | 0.500 | ND |
| K4 (*fda-1*) | 0.500 | 0.280 | 0.007 | 1.300 | 1.200 | 2.000 | 1.550 | 0.400 | 0.400 | 0.085 |

[a] Enzyme assays were performed with sonic extracts of stationary-phase cells, grown in medium X, as described in Materials and Methods. The specific activities are expressed in units (micromoles of product formed per minute) per milligram of protein, measured in the direction of glucose to pyruvate. PGI, GAP, and PGK were measured in the opposite direction. For aldolase, the values refer to FDP cleavage. Abbreviations: PGI, Phosphoglucose isomerase; PFK, phosphofructokinase; FDA, fructose diphosphate aldolase; TPI, triose phosphate isomerase; GAP, glyceraldehyde 3-phosphate dehydrogenase; PGK, glycerate 3-phosphate kinase; GPM, glycerate phosphate mutase; ENO, enolase; PYK, pyruvate kinase; ZWF, glucose 6-phosphate dehydrogenase.

[b] ND, Not done.

tiating the GAP and PGK from ENO isolates. Glycerate phosphate mutase-deficient mutants would also probably behave like mutants lacking ENO. The slow growth of *gap-3* and *eno-3* on oxidative substrates deserves a comment. The leakiness of the mutations is perhaps sufficient for fulfilling the biosynthetic requirements of the cell but inadequate to serve a catabolic role. The mutant *gap-3*, for example, had 10% of the GAP activity of the wild-type parent (Table 2). Although there was no such difference between the *eno-2* and *eno-3* isolates, the latter was found to have sevenfold more enzyme than *eno-2* when a cell suspension was rapidly assayed after toluene treatment in the cold (16).

The aldolase mutant K4 and several others not listed in Table 2 were found to retain about 5 to 10% of the enzyme activity that could be the "gluconeogenic aldolase" found under these conditions of growth (26). The phenotype of these mutants closely resembles that of the temperature-sensitive aldolase mutant isolated by Böck and Neidhardt (3). The very low aldol-

TABLE 3. *Growth properties of glycolytic mutants on minimal media containing different carbon sources*[a]

| Strain | Sugar | Ox | Glp | Glp[b] + Suc | GA[b] + Suc | Glp[b] + GA | Glu[b] + Suc | LB ± Glu |
|---|---|---|---|---|---|---|---|---|
| Wild type | + | + | + | + | + | + | + | + |
| G6 (*gap-1*) | − | − | − | + | − | + | − | − |
| H73 (*gap-2*) | − | − | − | + | − | + | − | − |
| O13 (*gap-3*) | − | ± | − | + | ± | + | − | − |
| L9 (*pgk-1*) | − | − | − | + | − | + | − | − |
| I51 (*eno-1*) | − | − | − | + | + | − | − | − |
| O16 (*eno-2*) | − | − | − | + | + | − | − | − |
| O6 (*eno-3*) | − | ±[c] | − | + | + | − | − | − |
| H65 (*tpi-1*) | − | − | − | + | NT | NT | − | −[d] |
| K4 (*fda-1*) | − | + | + | + | NT | NT | − | − |
| T38 (*pgk-1*) | − | − | − | + | − | + | − | − |
| AT3 (*eno-2*) | − | − | − | + | + | − | − | − |

[a] Bacteria were streaked on minimal agar plates containing the indicated carbon sources at their respective concentrations (see Materials and Methods). Growth was recorded after 60 h of incubation. Sugars tested were glucose, fructose, mannose, mannitol, galactose, lactose, glucose 6-phosphate, gluconate, and arabinose. Abbreviations: Ox, Oxidative substrates tested such as succinate, malate, lactate, glycerate, and 0.5% Casamino Acids; Suc, sodium succinate; Glu, glucose; Glp, glycerol or DL-α-glycerol phosphate; GA, DL-glycerate; LB, represents LB broth. Symbols: +, Confluent growth; ±, poor growth; −, no growth. NT, Not tested.

[b] Present at 4 mM concentration.

[c] Not valid in the case of DL-glycerate.

[d] Several (revertant?) colonies were visible over a background of shady growth after 36 h of incubation.

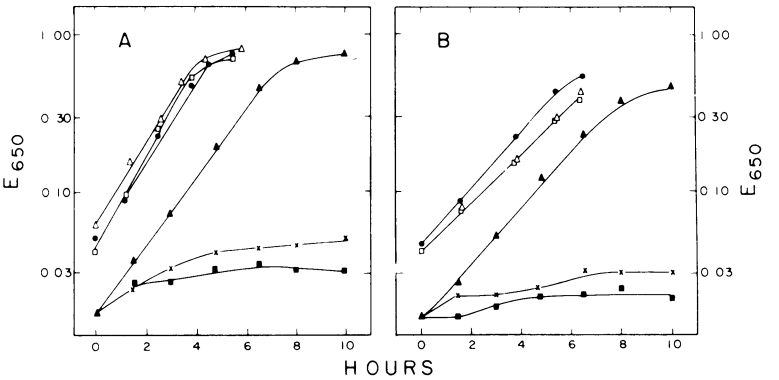


FIG. 2. *Growth kinetics of wild-type and mutant strains of E. coli in minimal media. Symbols:* (●) *Glucose, wild type;* (□) *gluconate, wild type;* (△) *glycerol + succinate, wild type;* (■) *glucose, mutant;* (×) *gluconate, mutant;* (▲) *glycerol + succinate, mutant. (A) Wild type is strain AT713, mutant is strain AT3 (eno-2); (B) wild type is strain MA176, mutant is strain T38 (pgk-1). Glycerol was used at a 4 mM concentration. The concentrations of the other carbon sources were as described in Materials and Methods. Exponentially growing cells in glycerol + succinate minimal medium were centrifuged, and appropriate dilutions in saline were used to inoculate the respective prewarmed media.*

TABLE 4. *Colony diameters on plates of succinate ± Casamino Acids media supplemented with different carbon compounds*[a]

| Supplement | Colony diam (mm) | | | |
|---|---|---|---|---|
| | T38 (*pgk-1*) | | AT3 (*eno-2*) | |
| | Supplement + glycerol | Supplement, no glycerol | Supplement + glycerol | Supplement, no glycerol |
| None | 3.0 | 0.1 | 4.0 | 0.2 |
| DL-α-Glycerol phosphate | 3.0 | 2.2 | 4.0 | 3.0 |
| DL-Glycerate | ND[b] | 0.1 | 3.0 | 0.8 |
| L-Fucose | 2.0 | 0.8 | 4.0 | 1.7 |
| L-Rhamnose | 3.0 | 2.0 | 4.0 | 2.3 |
| L-Arabinose | 1.0 | 1.0 | 0.0 | 0.0 |
| D-Galactose | 0.5 | 0.7 | 0.8 | 1.0 |
| Other sugars[c] | 0.0 | 0.0 | 0.0 | 0.0 |

[a] Agar plates contained minimal medium supplemented with 0.25% Casamino Acids and 40 mM sodium succinate. Glycerol and all the carbon supplements, when present, were at 5 mM concentration. All the supplements were treated with glucose oxidase to remove glucose. Dilutions in saline of cells growing exponentially in medium X were spread on the plates to give about 100 colony-forming units. Colonies were measured after 2 days of incubation.

[b] Not done.

[c] Other sugars used were glucose, glucose 6-phosphate, α-methyl glucoside, mannose, fructose, ribose, mannitol, and sorbitol, all of D-configuration.

ase activity of their mutant could be a consequence of the glycolytic growth conditions they used as opposed to the gluconeogenic conditions of growth used here (medium X). The observed phenotype of the TPI mutant (Table 3) is consistent with the function of this enzyme, the major discrepancy being the complete failure of these isolates to grow on glucose. The mutants reported by Anderson and Cooper (1), on the other hand, grew on glucose with a doubling time of 4 to 5 h. The difference could be due to the differential sensitivity of the strains to metabolite inhibition. Indeed, the growth of the TPI derivatives of the parental strain A was inhibited by glucose in minimal medium and less so in rich media relative to the GAP, PGK, and ENO isolates (Table 3). The inability of these glycolytic mutants to grow on LB broth (Table 3) has been found to be due to the inhibitory effect of yeast extract, likely to contain sugars and their phosphate esters (data not shown).

**Serine requirement of PGK-ENO double mutants.** In *E. coli*, L-serine was synthesized from glycerate 3-phosphate, the production of which is unimpaired in single-enzyme mutants deficient in either GAP, PGK, or ENO activity (Fig. 1). However, cells lacking the enzyme pair GAP-ENO or PGK-ENO would be unable to synthesize glycerate 3-phosphate from either end and should consequently develop L-serine auxotrophy. Mutagenized derivatives of T38 (*pgk-1*) were isolated that had simultaneously lost the ability to grow on a combination of glycerol and glycerate and needed L-serine for growth on glycerol plus succinate. Most of these had lost ENO activity. Reversion to serine independence was accompanied by the reappearance of either PGK or ENO activity. Glycerate, which should be able to supply glycerate 3-phosphate in these double mutants, could not replace L-serine. This was perhaps due to the inhibition of growth caused by glycerate, seen in glycerol- and L-serine-supplemented succinate minimal medium.

**Estimate of the gluconeogenic requirement.** What is the gluconeogenic flux of growing bacteria in contrast to their catabolic needs? We have attempted to throw some light on this question by examining the cell yield of glycolytic mutants grown on limiting amounts of carbon sources above and below the glycolytic lesion. Figure 3 demonstrates the inability of the ENO mutant to grow on either glycerol or Casamino Acids as the sole source of carbon. Similar results have been obtained with the PGK mutant. Several interesting features emerged from these growth studies. The growth rates of the mutants were nearly constant over a 400-fold range of glycerol concentration, but they did respond to varying concentrations of Casamino Acids. Apparently, this indicates a high affinity of *E. coli* for glycerol (11), shown also by the relatively sharp onset of growth plateaus as glycerol was exhausted.

There was also a linear relationship between the cell densities attained and the concentration of the limiting carbon source when the other source was present in excess (Fig. 4). The slopes representing cell mass formed per

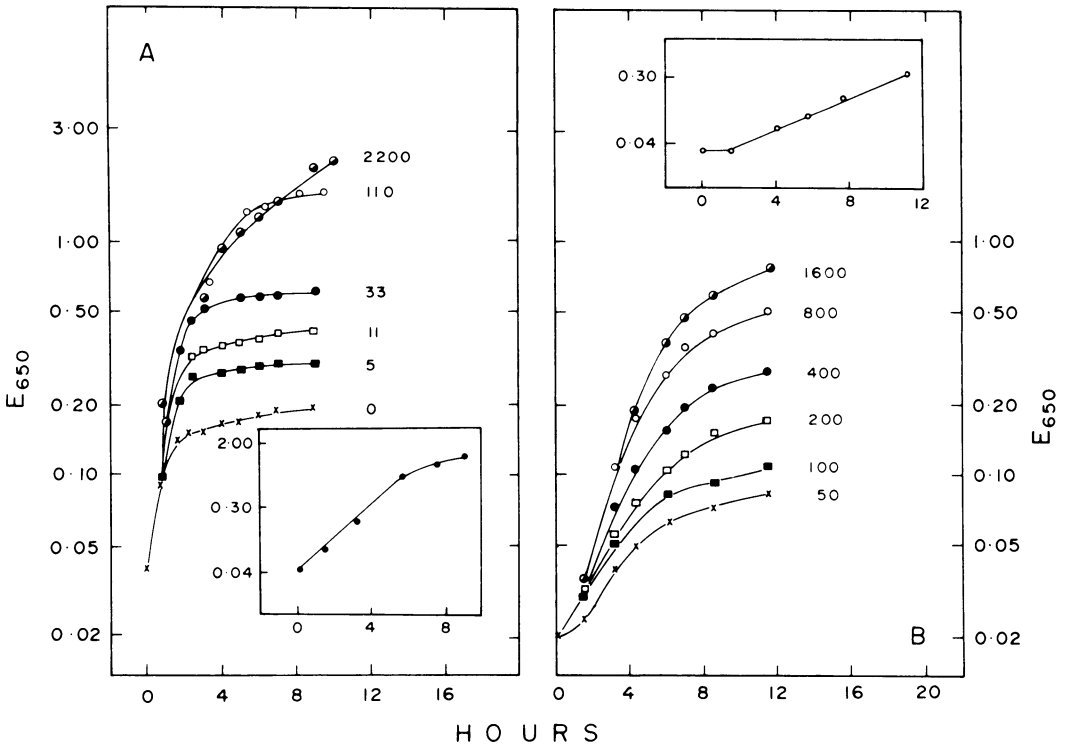


FIG. 3. *Growth of the enolase mutant AT3 (eno-2) on limiting concentrations of either glycerol or Casamino Acids in the presence of excess of the other. Numbers against the curves refer to the concentration (in micrograms per milliliter) of one of the carbon sources at a fixed concentration of the other. (A) Concentration of glycerol was varied from 0 to 2,200 μg/ml. Casamino Acids were present at 5,000 μg/ml. (B) Concentration of Casamino Acids was varied from 50 to 1,600 μg/ml. Glycerol was present at 2,200 μg/ml (25 mM). The insets refer to the growth of the wild-type parent AT713 in the absence of the variable carbon source. Cells growing exponentially in medium X were centrifuged, washed, and suspended in minimal medium containing Casamino Acids and glycerol at the indicated concentrations and supplemented with the necessary L-amino acids.*

microgram of the variable substrate differed very significantly for the two carbon sources. The gluconeogenic requirement of a growing *E. coli* cell is thus only one-twentieth of its total catabolic and other anabolic needs. These results indicate that a 1-g increase in cell wet weight requires 1 g of Casamino Acids. For 1,000 mg of wet cells, the amino acid-derived cellular components can be estimated to be approximately the following: 150 mg of protein, 20 mg of nucleic acid bases, and 30 mg of fatty acid constituents. Thus about 20% of this substrate is utilized for cell material, the remaining 80% being used oxidatively to produce energy. Lastly, the mutants grew for some time even in the absence of glycerol (Fig. 3), probably in part because of the accumulated intermediates above the block (see below).

**Choice of the biosynthetic supplement.** A different aspect of the growth of these glycolytic mutants relates to the choice of the gluconeogenic supplement. Severe inhibition of growth by a substrate whose catabolism is incomplete because of a mutationally or chemically blocked reaction is well known (4, 8, 29). In most instances, the growth inhibition has been attributed to the accumulation of phosphorylated intermediates above the metabolic block. In view of this and the anticipated accumulation of toxic metabolites, it was desirable to make a judicious choice of the sugar (gluconeogenic) precursor. Glycerol seemed to be best suited for this purpose, since its metabolism is kept slow under the feedback control of the glycolytic intermediate fructose 1,6-diphosphate (30). The ability of various other carbon sources to inhibit growth of these mutants and to serve as gluconeogenic supplements was tested. An examination of the colony diameters listed in Table 4 shows that the carbon compounds when present at 5 mM concentrations can be broadly divided into two categories: those such as α-

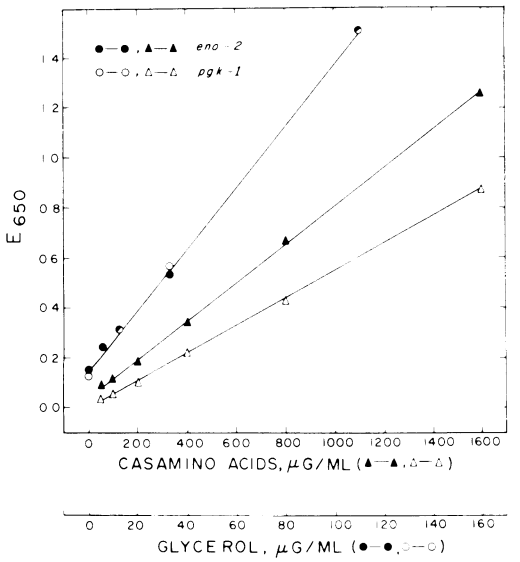


FIG. 4. *Growth yields of the mutants AT3 (eno-2) and T38 (pgk-1) as a function of varying concentrations of either glycerol or Casamino Acids when the other is present in excess. The data were obtained from the experiment for eno-2 described in Fig. 3 and a similar experiment performed with pgk-1. The values of $E_{650}$ plotted were scored after 24 h of incubation in the respective media.*

glycerol phosphate, glycerate, fucose, and rhamnose that do not inhibit growth on permissive media, and those that do. Owing to the low initial cell density on solid media, even 5 μM glucose is growth inhibitory. Therefore, to eliminate the possibility that contaminating glucose (or gluconate) in the sugar was responsible for growth inhibition, we monitored the growth kinetics in liquid medium. Due to the initial high cell density used ($E_{650}$ = 0.1), the contaminant (up to 7%) was rapidly depleted, unmasking the effect of the added sugar (see section on inhibition). Under these conditions, it was observed that α-methyl glucoside, ribose, mannitol, and sorbitol did not inhibit growth of the mutants, whereas carbon compounds such as glucose, glucose 6-phosphate, gluconate, mannose, fructose, arabinose, and galactose caused varying degrees of growth inhibition. In line with the smaller colony diameters obtained with fucose, rhamnose, and glycerate as sole carbon supplements than when glycerol was also present is the observation that in liquid media after an initial short period of rapid growth, very slow growth continued for about 8 to 10 h. After this, the growth rate of the culture increased to half of that obtained with glycerol as the supplement. Pregrowing the mutant cells with the respective carbon sources did not change the growth pattern. When glycerol was also present, normal growth proceeded. It is possible that these sugars are inhibitory in the initial stages of growth and that this effect is obscured in the presence of glycerol.

**Accumulation of glycolytic intermediates.** It was found that the GAP and PGK mutants accumulated the aldolase metabolites FDP and triose phosphates from glycerol during growth in medium X, whereas ENO mutants accumulated the two isomeric glycerate phosphates. The addition of glucose to a GAP mutant during growth caused a twofold increase in the levels of FDP and triose phosphates (Fig. 5). The major difficulty of working with a growing culture is its low cell density, which necessitates concentration of a fairly large sample by either filtration or centrifugation. The changes caused by such manipulations on the aerobic state of the culture reduce the reliability of the method for certain metabolites.

We sought to investigate the kinetics of metabolite accumulation from glycerol and glucose by using resting cells suspended in glycerol-free medium X. To exhaust the preexisting pool of metabolites, the culture was aerated for about 90 min before addition of the desired carbon source. The GAP and PGK mutants accumulated FDP and triose phosphates well above the wild-type levels from both glycerol and glucose (Fig. 5). Similarly, the ENO mutants accumulated glycerate phosphates (Fig. 6). However, the levels of the glycolytic intermediates below the block were comparable to the respective wild-type levels (Fig. 6 and 7; phosphoenolpyruvate [PEP] is not illustrated for *pgk-1*). Regarding the kinetics of accumulation, the initial rapid rate of accumulation declined after the first 10 min of incubation. A maximum difference of about twofold in the extent of accumulation from glycerol and glucose was apparent for the PGK mutant. The initial rates with glucose were consistently higher for all the mutants. The concentrations of glucose 6-phosphate measured in these experiments were of the order of 0.5 to 1.0 μmol per g (wet weight).

**Glucose utilization.** In Table 5 are compiled the data for the rates of glucose utilization under three different conditions. Within the variability expected from experiment to experiment, the gross features of these studies can be summarized as follows: the rates of glucose utilization of the mutants in buffered suspensions were severalfold lower than the corresponding wild-type rates; the rates did not substantially increase after incorporation of

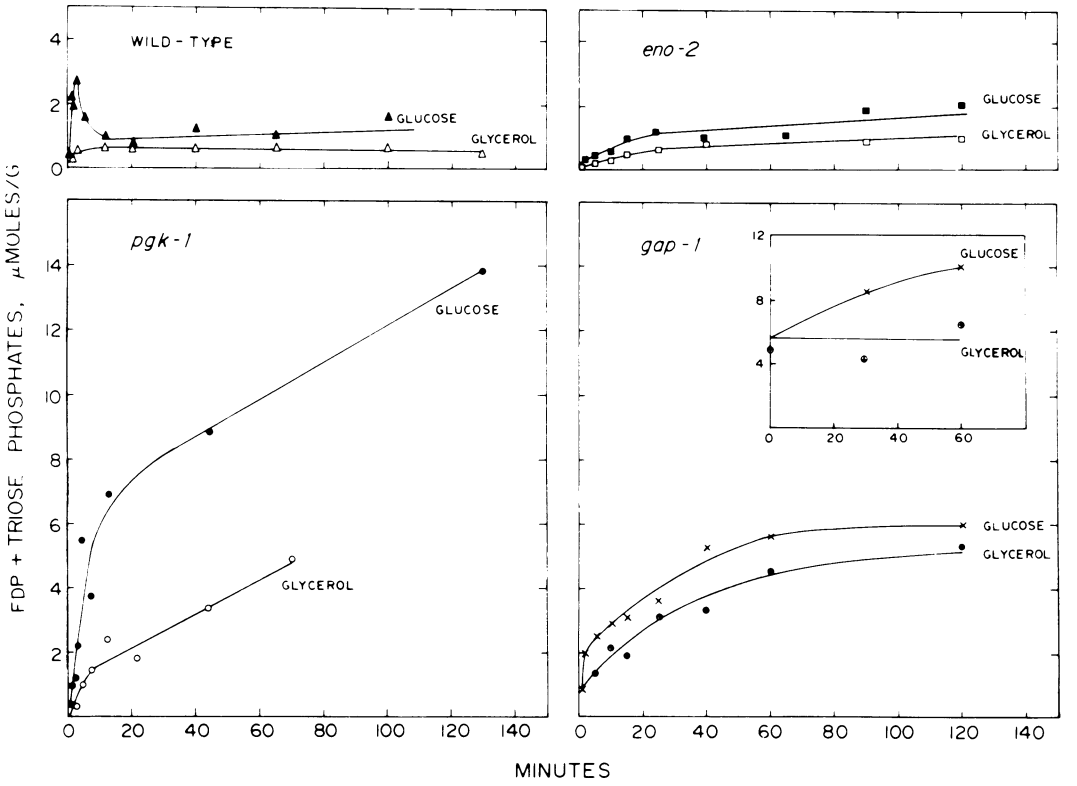


FIG. 5. *Accumulation of FDP + triose phosphates in wild-type and glycolytic mutants of E. coli on the addition of glucose and glycerol. Cells were harvested in the stationary phase, washed, and suspended in glycerol-free medium X to yield a final concentration of 30 to 70 mg of wet cells per ml. Glucose and glycerol (30 mM) were added after 90 min of aeration at 37°C. Samples (1 ml) were removed at designated intervals, added to 0.5 ml of 15% $HClO_4$, and chilled. The metabolites were assayed in the neutralized supernatants fluorimetrically (22). The exact cell concentrations were: 30 mg/ml for wild type; 35 mg/ml for gap-1; 40 mg/ml for pgk-1; and 70 mg/ml for eno-2. Weight represents the wet weight of cells. The inset refers to an exponentially growing culture. Glucose was added to the cells growing in medium X. About 40 ml of the culture was centrifuged and the cells were suspended in 50 mM potassium phosphate buffer, pH 7.4, and added to 15% $HClO_4$ (final concentration, 0.5 N).*

succinate and Casamino Acids in the incubation media. During growth in medium X, however, the rates were higher, although not as high as that of the wild type.

In the absence of the glycolytic generation of PEP in the mutants, the lower rates of glucose utilization by the cells could result from the limitation of PEP. The failure of succinate and Casamino Acids to stimulate glucose uptake in these glycolytic mutants is not consistent with this expectation.

Results in Fig. 7 describe an experiment in which intracellular PEP levels were measured in AT3 (*eno-2*). Although the rate of glucose utilization in *eno-2* was low, the PEP levels were similar to those of the wild-type parent. Similar results were obtained with T38 (*pgk-1*).

**Growth inhibition.** The inhibition of mutant growth by abortive metabolism of substrates is well documented (4, 8, 29) and is generally ascribed to accumulation of unphysiologically high levels of metabolites. However, not much difference was observed between glucose on the one hand and the permissive carbon source glycerol on the other, as far as accumulation of the glycolytic intermediates is concerned. The substantial accumulation from glycerol of FDP + triose phosphates and glycerate phosphates makes these metabolites unlikely candidates for growth inhibition. The alternative possibility, that the 1.5- to 2-fold higher accumulation with glucose is the cause, implies a critical concentration below which no inhibition occurs. It is also possible that the inhibition is a consequence of the interference with the transport of the permissive carbon source or a severe catabolite repression of its degradative and transport enzymes by the sugar. Since these

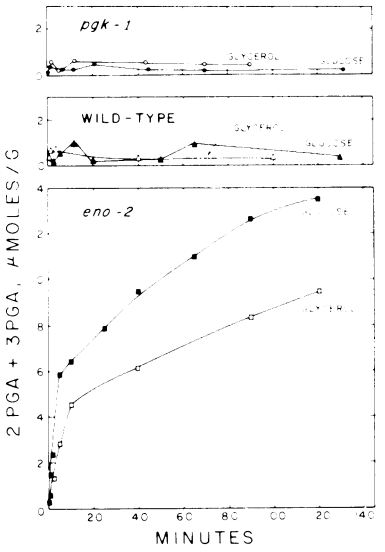


FIG. 6. *Accumulation of glycerate phosphates (2-PGA + 3-PGA) in wild-type and glycolytic mutants of E. coli on the addition of glucose and glycerol. The experiments were performed as described in the legend to Fig. 5. 2-PGA, Glycerate 2-phosphate; 3-PGA, glycerate 3-phosphate.*

glycolytic mutants carry lesions below phosphofructokinase, a stimulation of glycogen synthesis by FDP and glyceraldehyde 3-phosphate is feasible (24). In such an event, the sugar carbon would be directed toward glycogen synthesis, leading to a drainage of adenosine 5'-triphosphate (ATP) and hence impaired biosynthesis. The growth inhibition could also result from hyperproduction of the bactericidal compound methylglyoxal.

The kinetics of growth inhibition by glucose was examined in liquid medium X. The results obtained with AT3 (*eno-2*) are shown in Fig. 8. The graphs indicate that: (i) growth proceeded roughly for a generation after glucose addition; (ii) this was followed by loss of viability, part of which could be accounted for by visible lysis; (iii) glucose utilization continued even after growth inhibition commenced; and (iv) the surviving cells resumed normal growth soon after glucose was exhausted from the medium. These general features are characteristic of most of the GAP, PGK, and ENO mutants. Strain T38 (*pgk-1*) and other derivatives of MA176 were less sensitive to the bactericidal effect of glucose. Of interest is the observation with the ENO mutant (Fig. 8) that a small difference of 0.2 mM in the concentration of glucose in the two cultures caused a dramatic effect on the titer of viable cells. As seen in this experiment, the revival of an inhibited culture was largely dependent on the duration of exposure to glucose and hence indirectly on the initial ratio of glucose concentration to cell density. Not much killing was seen when the mutant cells carrying auxotrophic markers were incubated in a minimal medium with glucose as the sole carbon source. Essentially, glucose had a bactericidal effect when added to growing cultures of these glycolytic mutants, which is reminiscent of the killing action of antibiotics known to inhibit cell wall synthesis (20).

No bactericidal compound was, however, excreted into the culture medium, since the cell-free broth collected 3 h after glucose addition supported normal growth of a fresh wild-type culture. Nevertheless, we looked for the production of methylglyoxal after 3 h of exposure to 25 mM glucose and glycerol in water. The levels of methylglyoxal encountered under these conditions were between 0.01 and 0.02 mM, about fivefold lower than the inhibitory concentration (9). Methylglyoxal was measured color-

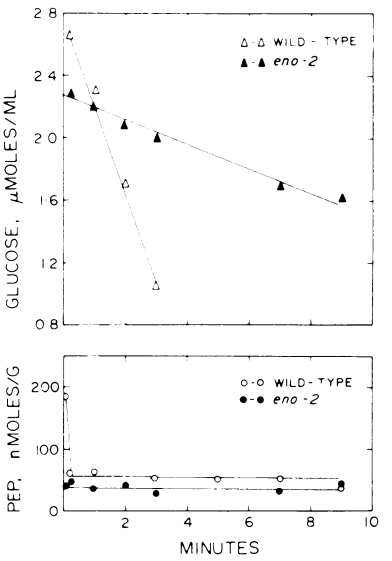


FIG. 7. *Kinetics of glucose utilization and intracellular PEP concentrations in AT3 (eno-2) and wild-type strains of E. coli. Cells were harvested in the late-exponential phase of growth, washed, and suspended in 50 mM potassium phosphate buffer, pH 7.4. After 10 min of aeration about 2.5 mM glucose was added (zero time), and 1-ml samples were quickly added into 0.5 ml of 15% $HClO_4$ and chilled. In the neutralized supernatant, glucose was estimated by the Glucostat method (Teller, Abstr. 130th Meet. Am. Chem. Soc., p. 69C, 1956), and PEP was estimated fluorimetrically (22). For comparisons all the values have been standardized for a culture of 50-mg/ml concentration. The actual cell densities used were 30 mg (wet weight)/ml for wild type and 70 mg (wet weight)/ml for eno-2.*

imetrically after reacting with 2,4-dinitrophenylhydrazine (6).

**Reversal of growth inhibition.** Growth inhibition by glucose was presumably not due to the inhibition of synthesis of any specific nutrient, since it was also seen in rich media. We observed that with strains such as T38 (*pgk-1*) and other mutant derivatives of MA176 that are not markedly affected by lysis, 5 mM cyclic 3',5'-adenosine monophosphate (cAMP) (which was without effect in the absence of glucose) largely reversed the growth inhibition caused by glucose. However, with the lysis-sensitive strain AT3 (*eno-2*), this effect of cAMP was observed only if the osmotic strength of the medium was also raised by adding 0.5 M glycerol (Fig. 9), 0.5 M sucrose, or 0.25 M NaCl (data not shown). Increasing the glucose concentration to 20 mM did not alter the pattern of growth reversal.

These results (Fig. 8 and 9) suggest that the inhibitory action of glucose on the growth of glycolytic mutants is expressed in two unrelated ways. One is the bactericidal action in liquid cultures due to interference in cell wall synthesis, which can be countered by an increase in the osmotic strength of the suspending medium. The other, presumably bacteriostatic in nature, can be overcome by cAMP. It is likely that glucose brings about a lowering of cAMP levels, causing greatly reduced synthesis of the transport and degradative enzymes for carbon metabolism in the mutant as illustrated below with respect to an inducible enzyme.

TABLE 5. *Rates of glucose utilization of wild-type and glycolytic mutants of E. coli*[a]

| Strain | 50 mM potassium phosphate buffer, pH 7.4 | Medium X (without glycerol) | Medium X, exponential culture |
|---|---|---|---|
| AT713 | 10.8 | ND[b] | 13.5 |
| AT3 (*eno-2*) | 1.4 | 1.8 | 5.9 |
| MA176 | ND[b] | 8.5 | 9.0 |
| T38 (*pgk-1*) | 1.4 | 0.7 | 5.0 |
| A | 12 | 7.0 | 12 |
| G6 (*gap-1*) | 1.0 | 1.6 | 11 |

[a] Rates are expressed in micromoles per minute per gram of wet cells. The values in the first two columns were obtained with heavy suspensions of resting cells in the respective media. Glucose concentrations used were 2.5 and 30 mM (see Fig. 5 and 7). For exponentially growing cells, about 1 mM glucose was used. Glucose in the medium was estimated either by the Glucostat method (J. D. Teller, Abstr. 130th Meet. Am. Chem. Soc., p. 69C, 1956) or fluorimetrically (22). For growing cultures, the rates were calculated by using the formula:

$$\frac{C_1 - C_2}{(t_2 - t_1) \times 1/2(D_1 + D_2)}$$

where $C_1$ and $C_2$ refer to the glucose concentration in the medium at two close time intervals, $t_1$ and $t_2$. $D_1$ and $D_2$ represent the cell densities in grams of wet cells per milliliter at the two time points.

[b] ND, Not done.

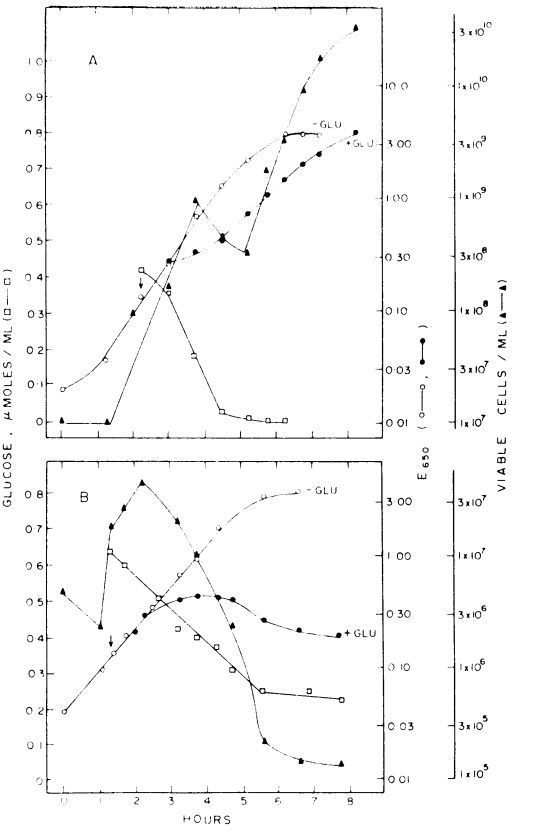


FIG. 8. *Kinetics of growth inhibition by glucose in AT3 (eno-2). To one half of an exponentially growing culture in medium X, glucose (GLU) was added at the time indicated by the arrow. Growth was monitored by following the* $E_{650}$ *and viable count of the cultures. The latter was estimated by plating appropriate dilutions in saline on minimal plates containing 4 mM glycerol, 40 mM sodium succinate, and 0.25% Casamino Acids. Glucose was estimated in chilled samples by the fluorimetric procedure, using the hexokinase and glucose 6-phosphate dehydrogenase-coupled assay (22). (A) Initial glucose concentration was 0.4 mM. (B) Initial glucose concentration was 0.64 mM.*

**Repression of β-galactosidase synthesis.** We examined the rate of induced synthesis of β-galactosidase in cultures of the glycolytic mutants and the wild-type strain in the presence of 0.5 M glycerol as the osmotic supplement and studied the effect of glucose thereon for periods extending to three generations. Exponentially growing suspensions of cells were incubated with 0.5 mM isopropyl-thio-β-D-galactoside for 5 min, after which 20 mM glucose

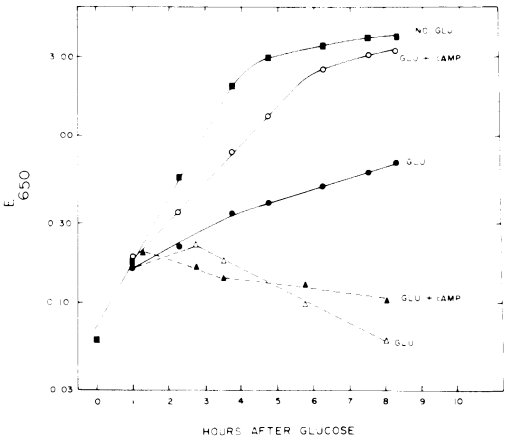


FIG. 9. *Reversal of growth inhibition. Cells of AT3 (eno-2) in medium X (dashed curves) or medium X containing 0.5 M glycerol as the osmotic supplement (solid curves) were added to prewarmed sterile flasks containing glucose or glucose + cAMP to give a final concentration of 5 mM each. Growth was then followed by measuring the absorbance at 650 nm as a function of time.*

was added. The steady-state differential rate of β-galactosidase synthesis was monitored. The enolase-negative mutant AT3 synthesized, in the absence of glucose, 1 unit of β-galactosidase per unit increase of cell mass measured as $E_{650}$, 0.055 unit/$E_{650}$ in the presence of glucose, and 0.41 unit/$E_{650}$ in the presence of glucose and 5 mM cAMP. In the wild-type parent, on the other hand, the corresponding rates of synthesis were 1 unit/$E_{650}$ in absence of glucose, 0.45 unit/$E_{650}$ in its presence, and 1 unit/$E_{650}$ when both glucose and cAMP were present together. Essentially similar results were obtained with the PGK mutant T38 and its isogenic parent. These results show clearly that one of the effects of adding glucose to growing cultures of glycolytic mutants is a severe repression of catabolite-sensitive enzymes that is substantially alleviated by cAMP.

## DISCUSSION

**General effects of glycolytic mutations.** The facultative anaerobe *E. coli* can adapt itself to grow aerobically on several carbohydrates and oxidative substrates. The Embden-Meyerhof pathway is the connecting link between the gluconeogenic and oxidative functions of the cell. The loss of either GAP, PGK, or ENO, unlike loss of the enzymes involved in the conversion of glucose to triose phosphates, breaks this link and transforms *E. coli* into an obligate aerobe whose carbon requirements for both these functions must be separately fulfilled. The introduction of such lesions divides the Embden-Meyerhof pathway into two distinct halves that can operate independently of each other without influencing the growth rates when the carbon requirements are appropriately satisfied. Under these conditions, this well-coordinated amphibolic sequence is converted into two rudimentary biosynthetic segments.

The results indicate that there are no efficient bypass routes to glycolysis in *E. coli*, in which case growth might have resulted at least on the permissive substrate glycerol. In view of the non-phosphorylating bypass route to pyruvate (6), one wonders about the inability of this methylglyoxal pathway to support growth. The specific activity of methylglyoxal synthetase, the first enzyme of the pathway, is about twofold higher than that of fructose diphosphate aldolase (6, 9), which is the slowest step of glycolysis in this regard (Table 2). Nevertheless, synthetase activity is inhibited by inorganic phosphate and PEP; this inhibition is overcome by the substrate dihydroxyacetone phosphate. The second enzyme of the bypass, glyoxalase, is 10-fold lower in specific activity than methylglyoxal synthetase (9). It would be interesting to see whether spontaneous revertants of these glycolytic mutants, which are resistant to methylglyoxal by acquiring elevated levels of glyoxalase, grow on glycerol, or whether a subsequent event leading to alterations in the inhibitory characteristics of the synthetase can restore growth on glycerol. It is possible that growth may remain impaired due to repression of PEP synthetase (5) and the consequent PEP deficiency.

When the obligatory requirement for a gluconeogenic precursor is appropriately satisfied, these glycolytic mutants grow normally, utilizing an oxidative substrate as a source of energy. This is inferred from the almost identical growth rates obtained for the wild-type and mutant cells on glycerol-supplemented succinate minimal medium. One still wonders whether the abnormal levels of accumulated metabolites during growth influence the membrane or cell wall structure or produce other aberrations that cannot be detected by the criterion of growth rate. It is apparent that these mutants are much more sensitive to catabolite repression of β-galactosidase synthesis by glucose than is the wild type. This is contrary to expectation, and perhaps these mutants might prove to be a useful system for studying cAMP metabolism and catabolite repression.

**Accumulation of metabolites.** The results described enable us to draw some definitive

and a few speculative inferences. The glycolytic accumulation observed for the GAP, PGK, and ENO mutants is a strong indication of the operation of these glycolytic lesions in vivo. A comparison of the pattern of metabolite accumulation in the PGK and ENO mutants reveals an interesting difference in that the FDP and triose phosphate accumulation is not significant in the ENO mutant. This suggests the absence of any feedback link between the glycerate phosphates and the GAP and PGK reactions. For the PGK mutant, this accumulation is consistent with the inhibition of GAP activity by 1,3-diphosphoglycerate, the substrate of PGK. Unfortunately, the use of acidic conditions did not permit estimation of this intermediate. We suspect that its intracellular concentration will be sufficient to inhibit GAP activity. Another feature of the FDP + triose phosphate profile in the ENO-negative strain is the absence of the initial overshoot, characteristic of wild-type strains and hence of unimpaired glycolysis. Whether the decrease is a consequence of FDP activation of pyruvate kinase or ATP inhibition of phosphofructokinase, or both, remains unsettled. Another question concerns the fate of glucose, which continues to be utilized at a constant rate even when the pools of glycolytic intermediates reach a steady state (data not shown). Is there a leak past the glycolytic lesion? Or does the glucose carbon accumulate in the form of macromolecules such as glycogen and lipopolysaccharide? The synthesis of glycogen is likely to be active under the stimulatory influence of FDP (24). Somewhat surprising is the substantial accumulation of FDP and triose phosphates from glycerol in the GAP and PGK mutants. Considering the large tilt of the equilibrium in favor of FDP and the cited values of FDP and triose phosphates in other systems (12), it is reasonable to assume that about two-thirds of the total aldolase metabolites would be present as FDP. When the three aldolase metabolites taken together increase to 6 $\mu$mol of $C_6$ units per g of wet cells, the level of FDP may be as high as 4 mM. This value is well above the $K_i$ of 1 to 2 mM FDP for glycerokinase (31). The lack of accumulation of metabolites below the glycolytic blocks is also noteworthy, since these incubations were done in the presence of the gluconeogenic precursors succinate and amino acids.

It should be pointed out that an *E. coli* culture incubated in medium X has a respiration rate of about 20 nmol of $O_2$/min per mg of wet cells. Thus an aerated suspension containing 70 mg of wet cells per ml of 240 $\mu$M $O_2$ would turn anaerobic in 10 s. Nevertheless, these results have been obtained with replicate cultures and are useful for comparative purposes.

**Glucose transport and PEP.** It is believed that glucose is transported into the *E. coli* cell by the process of group translocation, with PEP serving as the phosphate donor (25). However, most of these studies have been carried out with in vitro systems. There is very little experimental evidence correlating the rates of glucose utilization by intact cells and the intracellular concentration of PEP. Kornberg and Smith (18) have speculated that an *E. coli* mutant lacking *pfkA* activity should have low levels of PEP. They observed that the low rate of $^{14}C$ incorporation from labeled glucose is stimulated in the presence of carbon sources whose catabolism is likely to be marginally affected by the *pfkA* lesion. Assuming that the accumulation of radioactivity in a metabolizing cell is a reliable measure of glucose transport (which is not always true), in the absence of any actual measurement of intracellular PEP pools, no definitive conclusions can be drawn. Attempts had been made earlier to study the effect of inhibitors of glycolysis such as iodoacetate and fluoride on $\alpha$-methyl glucoside uptake and glucose utilization (7, 17). The results suggest that the transport of these sugars is severely impaired by the poisons. However, it is preferable to work with cells in which glycolysis is blocked by mutation to avoid complications caused by multitarget inhibitors. The GAP, PGK, and ENO mutants, which cannot make PEP from glucose, lend themselves admirably to the study of glucose transport and PEP metabolism.

Our results show that the low rates of glucose utilization in these mutants are not a consequence of the limitation of PEP. One wonders whether the PEP phosphotransferase system in *E. coli* is subject to regulatory mechanisms that become amplified under conditions of impaired glycolysis. Is it possible that glucose is transported into the mutant cells by PEP-independent routes? The constant level of PEP in the cell implies that, if this metabolite is utilized as a phosphate donor in glucose transport, its rate of expenditure equals its rate of replenishment. It would be interesting to examine glucose transport in strains lacking PEP synthetase or PEP carboxykinase activity or alternatively blocked in the oxidative generation of ATP and thereby indirectly reducing the rate of PEP synthesis. Also worth examining is the effect of a *ptsI* or a *ptsH* mutation on glucose utilization and glucose-induced growth inhibition in these mutants.

**Growth inhibition.** Growth inhibition aris-

ing as a consequence of impaired catabolism of substrates is a commonly encountered phenomenon, the mechanism of which remains largely obscure. Our results suggest that the glucose-induced growth inhibition of mutants blocked in the lower segment of glycolysis is a manifestation of two independent effects: interference with cell wall synthesis and perhaps repression of catabolic and transport enzymes of the permissive substrate. In fact, glucose-resistant revertants can be obtained that are less sensitive or totally insensitive to catabolite repression by glucose (M. Irani, unpublished data). It may well be that these manifestations are more general in nature and that one or both of these effects could be responsible for the growth inhibition found in aldolase-negative strains (4, 8) and phosphofructokinase-deficient mutants (28), at least on rich media. It has been reported that added cAMP promotes growth of phosphofructokinaseless mutants on casein hydrolysate or succinate in the presence of glucose (28). Cell lysis has also been observed in the case of galactose-negative and *N*-acetylglucosamine-negative strains after addition of the non-metabolizable substrate (10, 29).

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