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Accumulation of Methylglyoxal in a Mutant of *Escherichia coli* Constitutive for Gluconate Catabolism

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A culture of a mutant of *Escherichia coli*, derepressed for gluconate catabolism, is killed by the addition of gluconate to the culture. The product responsible for this bactericidal effect was identified as methylglyoxal. Two types of mutants resistant to gluconate were isolated. One of them showed increased activity of glyoxalase I.

Gluconokinase (EC 2.7.1.12) catalyzes the first step in gluconate metabolism in *Escherichia coli* (2). Its product, gluconate-6-phosphate (G-6-P), may either be oxidized to pentose phosphate or dehydrated to 2-keto-3-deoxygluconate-6-phosphate (KDGP) (4, 9) which is in turn cleaved to pyruvate and glyceraldehyde-3-phosphate. The last two reactions are catalyzed by G-6-P dehydrase (EC 4.2.1.12) and KDGP aldolase (EC 4.2.1.14), constituents of the Entner-Doudoroff pathway (5). In addition, the operation in *E. coli* of a second gluconokinase (Hung, Orozco, and Zwaig, Bacteriol. Proc., p. 146, 1970) and two transport systems for gluconate (13) has been postulated. This sequence of reactions is summarized in Fig. 1.

Gluconate transport, gluconokinase and G-6-P dehydrase activities are induced by growth on gluconate. At least one of the transport systems, one of the gluconokinases, and G-6-P dehydrase share a common regulatory system since a constitutive mutant (strain M18) isolated in our laboratory (18) shows derepression of those activities.

Recently, mutants affecting KDGP aldolase activity have been isolated which can grow normally on glucose but are inhibited by the addition of gluconate or uronic acids to different growing media (6, 7, 14). The observed toxicity appears to be caused by the accumulation of KDGP (6, 7). In this paper we describe the lethal effect of methylglyoxal which accumulates when gluconate is added to a culture of a mutant constitutive for the gluconate catabolic system, and the selection of resistant strains, one of which shows increased activity of glyoxalase I (EC 4.4.1.5).

MATERIALS AND METHODS

Bacterial strains. Strain M1, derived from *E. coli* K12 HfrC, prototrophic, originally strain E15 of C. Levinthal, was obtained from E. Lin. Strain M18 is a spontaneous mutant isolated from strain M1 (18). Strains M61 and M62 were obtained from strain M18 as resistant to the gluconate effect.

Media and growth conditions. Cells were grown aerobically at 37 C in 25-ml cultures in 250-ml flasks with shaking. The mineral medium previously described (13) was supplemented with 1% casein hydrolyzate (CAA) or 0.4% glucose. Growth was followed turbidimetrically with a Klett colorimeter (no. 42 filter, 1 unit = 4×10^6 bacteria per ml during logarithmic growth).

Preparation of bacterial extracts. Cultures grown up to 55 Klett units were harvested by centrifugation in the cold, and the cells were washed once with 0.85% NaCl. Cells were resuspended in 2 ml of 100 mM potassium phosphate buffer, pH 7.0, for gluconokinase determination or in 2 ml of 25-mM imidazole-hydrochloride buffer, pH 6.6, for glyoxalase I assay (10). The cells were disrupted by treatment for 2 min with a sonic disintegrator (Measuring and Scientific Equipment, Ltd., London, England) and clarified by centrifugation at $12{,}000 \times g$ for 20 min at 2 C.

Enzyme assays. Gluconokinase was assayed by the procedure of Fraenkel and Horecker (8). Distinction between gluconokinases was not made. Glyoxalase I was determined according to the procedure of Freedberg, Kistler, and Lin (10).

Other determinations. Carbonyl compounds were measured spectrophotometrically after their reaction with 2,4-dinitrophenyl-hydrazine, according to the

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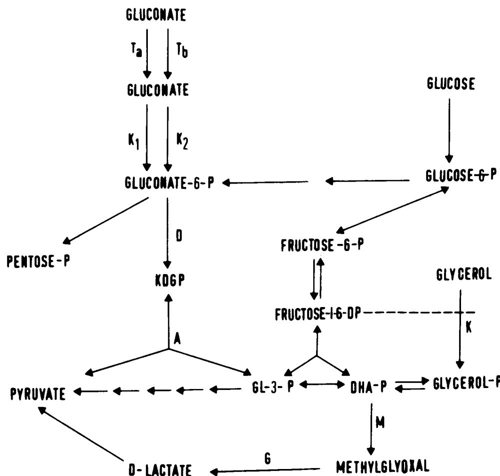

FIG. 1. *Metabolic scheme for methylglyoxal formation by E. coli strain M18. Abbreviations: Ta and Tb, transport systems for gluconate; K1 and K2, gluconokinases; D, gluconate-6-phosphate dehydrase; A, KDGP adolase; K, glycerol kinase; G, glyoxalase I; M, methylglyoxal synthase.*

procedure of Cooper and Anderson (3). Methylglyoxal was determined enzymatically with a crude extract of strain M1 by the method of Klotzch and Bergmeyer (11). Pyruvate was estimated enzymatically with crystalline lactic dehydrogenase (Sigma Chemical Co.) by the procedure of Bücher et al. (1).

Isolation of mutants. Gluconate (final concentration, 0.4%) was added to a culture of strain M18 grown on mineral medium plus CAA at 55 Klett units. The culture was left overnight, transferred to mineral medium plus CAA (1 Klett unit), and the operation was repeated once. Mutants were isolated on Antibiotic Medium 3 (Difco) plates from the overnight culture and their sensitivity to gluconate was analyzed.

RESULTS

E. coli strain M18 is constitutive for the gluconate system (18). When gluconate is added to a culture of this strain growing on mineral medium plus CAA, the growth is arrested (Fig. 2) and a rapid loss of viability is observed (Fig. 3). No lysis occurs during the next 10 h since no decrease in the optical density takes place (Fig. 2). When cells of *E. coli* strain M18 are growing on mineral medium plus glucose, however, the addition of gluconate under the conditions explained in Fig. 2, produces no toxic effect (results not shown). Since gluconokinase is partially repressed in cultures growing on glucose mineral medium (Table 1), the observed bactericidal effect of gluconate on cells growing on CAA might be related to an excessive phosphorylation which should cause the accumulation of a toxic product. Also, a similar repression effect by glucose on the transport for gluconate was observed in *E. coli* strain M18, though G-6-P dehydrase activity was not diminished (Istúriz and Zwaig, unpublished data).

The bactericidal product formed by the addition of gluconate to cells growing on CAA passes into the medium from where it can be collected and tested (Rekarte and Zwaig, unpublished data). The effect of this cell-free bactericidal product added to a culture of a normal strain for gluconate metabolism (strain M1) is similar to the observed effect of gluconate added to a culture of strain M18. This phenomenon is reminiscent of that observed with glycerol in a mutant of *E. coli* (15). A double mutant, constitutive for the glycerol phosphate system and producing a glycerol kinase insensitive to fructose-1,6-diphosphate (FDP) regulation (17), is killed when exposed to glycerol (16); the bactericidal product responsible for this effect was identified as methylglyoxal (10) which is formed from dihydroxyacetone phosphate (DHAP) through the action of methylglyoxal synthase (no EC number given) (3).

Based on these results, the bactericidal product formed in *E. coli* strain M18 was tested for its reaction with 2,4-dinitrophenylhydrazine (DNPH), a reagent for carbonyl compounds. Figure 4 shows the increase of a DNPH reactive product formed by a culture of strain M18 after the addition of gluconate. Since the DNPH reaction is not specific for methylglyoxal, a sample taken at 2 h (Fig. 4) was treated with a crude extract of strain M1 which shows glyoxa-

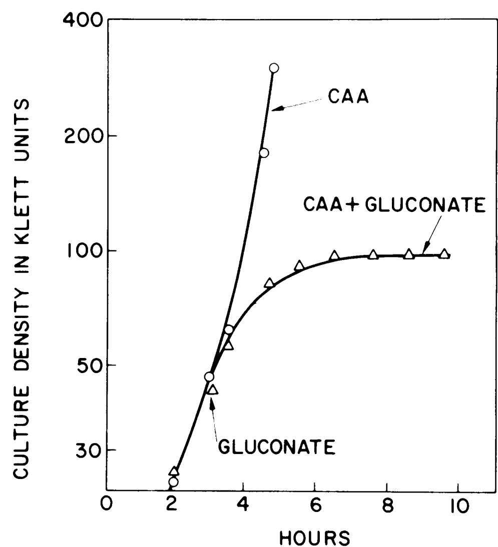

FIG. 2. *Effect of gluconate added to a culture of E. coli strain M18. Gluconate (final concentration, 0.4%) was added at the point indicated by the arrow.*

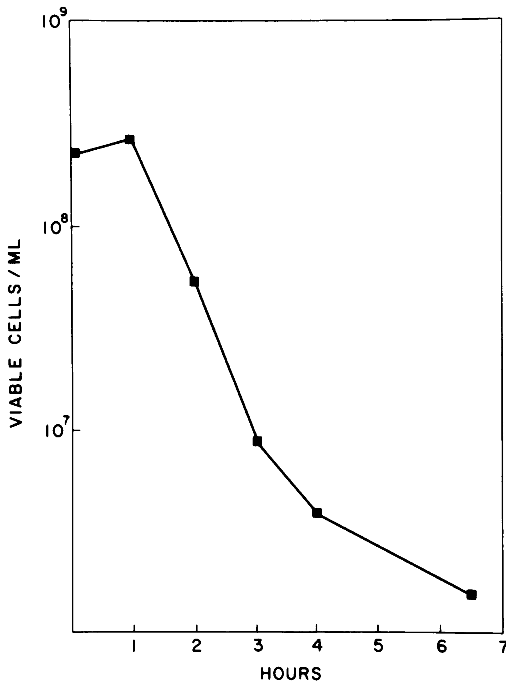

FIG. 3. *Kinetics of killing in a culture of E. coli strain M18 after the addition of gluconate. At time intervals, samples were removed from the culture of the experiment represented in Fig. 2, after the addition of gluconate (zero time) and the viability on Antibiotic Medium 3 plates determined.*

TABLE 1. *Glucose repression of gluconokinase in E. coli strain M18*[a]

Carbon source	Gluconokinase
Mineral medium plus glucose	0.690
Mineral medium plus CAA	0.948

[a] Specific activity is expressed as international units per milligram of protein.

lase I activity. This enzyme was shown to be active on methylglyoxal (10). The results of this experiment (Table 2), as well as the fact that gluconate metabolism might accumulate DHAP, the methylglyoxal precursor, strongly suggests that methylglyoxal is the bactericidal product accumulated in cultures of strain M18.

The amount of methylglyoxal found in the sample could account for only 80% of the absorption observed in Fig. 4. This result indicated that another compound besides methylglyoxal, which reacts with DNPH, was present. A possible candidate could be pyruvate since it is one of the products of KDGP aldolase reaction. Thus, a sample similar to the one analyzed for methylglyoxal (Fig. 4, at 2 h) was assayed also for pyruvate (Table 2). The color developed by an equivalent amount of pyruvate reacting with DNPH represents 14% of the absorption observed at 2 h in Fig. 4.

By recycling on mineral medium plus CAA and mineral medium plus CAA plus 0.4% gluconate (see Materials and Methods), several mutants were selected from strain M18 which escaped the gluconate effect but showed similar activities for the gluconate catabolic system. Two of them (M61 and M62) were studied further. Like strain M18, both mutants produced DNPH reactive products after addition of gluconate (Fig. 4). However, in the case of strain M62, the DNPH reactive product decreased

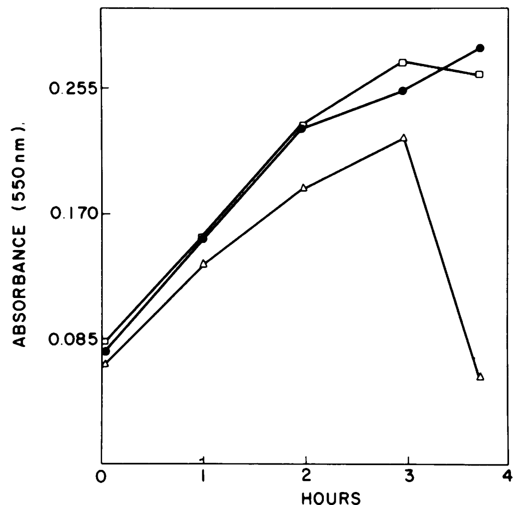

FIG. 4. *Accumulation of DNPH reactive products in cultures of E. coli strain M18 and resistant mutants after the addition of gluconate. Cells were grown on mineral medium plus CAA. At 50 Klett units, gluconate (final concentration, 0.4%) was added and samples were removed at different times, filtered on membrane filters (0.45 µm pore size; Millipore Corp.) and treated with DNPH (3). Symbols: ●, Strain M18; □, M61; △, M62.*

TABLE 2. *Methylglyoxal and pyruvate accumulation in cultures of E. coli strain M18 and in the cultures of mutant strains M61 and M62*[a]

Strain[b]	Methylglyoxal	Pyruvate
M18	1.10	1.79
M61	0.83	2.72
M62	0.72	2.39

[a] Millimolar concentration.
[b] Samples from the experiment represented in Fig. 4 were taken at 2 h.

rapidly 3 h after the addition of gluconate (Fig. 4). The analysis of the samples from the experiment represented in Fig. 4, taken at 2 h, showed somewhat reduced levels of methylglyoxal as compared with those of strain M18 (Table 2). To see whether the resistant mutants had increased capacities to utilize methylglyoxal, extracts of the mutant strains, as well as of strain M18, were tested for glyoxalase I activity. A high value of glyoxalase I activity is observed in strain M62 which could account for both the drop of methylglyoxal observed in Fig. 4 and its resistance to gluconate (Table 3). A high value for glyoxalase I activity in a mutant that escapes glycerol toxicity in the mutant referred above was previously described (10).

TABLE 3. *Glyoxalase I activity in extracts of E. coli strain M18 and in mutant strains M61 and M62*

Strain[a]	IU per mg of protein
M18	0.006
M61	0.008
M62	0.070

[a] Cells were grown on mineral medium plus CAA. IU, international units.

DISCUSSION

The addition of gluconate to a culture of a mutant derepressed of *E. coli* (strain M18) for gluconate metabolism produces the accumulation of methylglyoxal. A bactericidal product formed by glycerol in an unregulated mutant of *E. coli* has been studied (12, 15-17) which was identified also as methylglyoxal (10). Mutants from strain M18 which escape the gluconate effect have been isolated. In one case (strain M62) this resistance is apparently related to its high content of glyoxalase I activity; but, in spite of this activity, when gluconate is added to a culture of this strain, accumulation of methylglyoxal is observed in the first 3 h, followed by a sudden drop. This behaviour might be explained, for example, supposing that methylglyoxal stimulates the production of glutathione, cofactor required for glyoxalase I activity. We do not know yet the nature of the mutation involved in strain M61 which makes it insensitive to the gluconate effect. Although the lower level of methylglyoxal produced by this mutant in the presence of gluconate (Table 2) did not cause any impairment in its viability, a similar amount of methylglyoxal added to a culture of strain M1, growing in CAA, produced a rapid loss of its viability (Rekarte and Zwaig, unpublished data). Since methylglyoxal appears to exert its action through its binding to guanine residues contained in nucleic acids (12), it is not easy to envisage a change to resistance to methylglyoxal due to an alteration in the biochemical target.

The effect of gluconate on the derepressed mutant for the gluconate system studied here and the similar phenomenon observed previously with glycerol, permits one to infer an interpretation related to the evolution of regulatory mechanisms. The acquisition of regulatory systems is generally seen as a way to conserve energy. Alternatively, it could also be explained as a way to prevent accumulation of metabolites with lethal or inhibitory activity.

LITERATURE CITED

1. Bücher, T., R. Czok, W. Lamprecht, and E. Latzko. 1965. Pyruvate, p. 253-259. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis. Academic Press, Inc., New York.
2. Cohen, S. S. 1951. Gluconokinase and the oxidative path of glucose-6-phosphate utilization. J. Biol. Chem. **189**:617-628.
3. Cooper, R. A., and A. Anderson. 1970. The formation and catabolism of methylglyoxal during glycolysis in *Escherichia coli*. Fed. Eur. Biochem. Soc. Lett. **11**:273-276.
4. Eisenberg, R. C., and W. J. Dobrogosz. 1967. Gluconate metabolism in *Escherichia coli*. J. Bacteriol. **93**:941-949.
5. Entner, N., and N. Doudoroff. 1952. Glucose and gluconic acid oxidation of *Pseudomonas saccharophilia*. J. Biol. Chem. **196**:853-862.
6. Faik, P., H. L. Kornberg, and E. McEvoy-Bowe. 1971. Isolation and properties of *Escherichia coli* mutants defective in 2-keto-3-deoxy-6-phosphogluconate aldolase activity. Fed. Eur. Biochem. Soc. Lett. **19**:225-228.
7. Fradkin, J. E., and D. G. Fraenkel. 1971. 2-keto-3-deoxygluconate 6-phosphate aldolase mutants of *Escherichia coli*. J. Bacteriol. **108**:1277-1283.
8. Fraenkel, D. G., and B. L. Horecker. 1964. Pathways of D-glucose metabolism in *Salmonella typhimurium*. J. Biol. Chem. **239**:2765-2771.
9. Fraenkel, D. G., and S. R. Levisohn. 1967. Glucose and gluconate metabolism in an *Escherichia coli* mutant lacking phosphoglucose isomerase. J. Bacteriol. **93**:1571-1578.
10. Freedberg, W. B., W. S. Kistler, and E. C. C. Lin. 1971. The lethal synthesis of methylglyoxal by *Escherichia coli* during unregulated glycerol metabolism. J. Bacteriol. **108**:137-144.
11. Klotzsch, H., and H. U. Bergmeyer. 1965. Methylglyoxal, p. 283-284. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis. Academic Press, Inc., New York.
12. Krymkiewicz, N., E. Diéguez, U. D. Rekarte, and N. Zwaig. 1971. Properties and mode of action of a bactericidal compound (= methylglyoxal) produced by a mutant of *Escherichia coli*. J. Bacteriol. **108**:1338-1347.
13. Nagel de Zwaig, R., N. Zwaig, T. Istúriz, and R. S. Sánchez. 1973. Mutations affecting gluconate metabolism in *Escherichia coli*. J. Bacteriol. **114**:463-468.
14. Pouysségur, J. M., and F. R. Stoeber. 1971. Étude du rameau dégradatif commun des hexuronates chez *Escherichia coli* K12. Eur. J. Biochem. **21**:363-373.
15. Zwaig, N., and E. Diéguez. 1970. A bactericidal product

obtained from a mutant of *Escherichia coli*. Biochem. Biophys. Res. Commun. **40**:1415-1422.
16. Zwaig, N., W. S. Kistler, and E. C. C. Lin. 1970. Glycerol kinase, the pacemaker for the dissimilation of glycerol in *Escherichia coli*. J. Bacteriol. **102**:753-759.
17. Zwaig, N., and E. C. C. Lin. 1966. Feedback inhibition of glycerol kinase, a catabolic enzyme in *Escherichia coli*. Science **153**:755-757.
18. Zwaig, N., R. Nagel de Zwaig, T. Istúriz, and M. Wecksler. 1973. Regulatory mutants affecting the gluconate system in *Escherichia coli*. J. Bacteriol. **114**:469-484.