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Lethal Synthesis of Methylglyoxal by *Escherichia coli* During Unregulated Glycerol Metabolism[1]

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In *Escherichia coli* K-12, the conversion of glycerol to triose phosphate is regulated by two types of control mechanism: the rate of synthesis of glycerol kinase and the feedback inhibition of its activity by fructose-1,6-diphosphate. A strain which has lost both control mechanisms by successive mutations, resulting in the constitutive synthesis of a glycerol kinase no longer sensitive to feedback inhibition, can produce a bactericidal factor from glycerol. This toxic factor has been identified by chemical and enzymological tests as methylglyoxal. Methylglyoxal can be derived from dihydroxyacetone phosphate through the action of an enzyme which is present at high constitutive levels in the extracts of the mutant as well as that of the wild-type strain. Nine spontaneous mutants resistant to 1 mM exogenous methylglyoxal have been isolated. In all cases the resistance is associated with increased levels of a glutathione-dependent enzymatic activity for the removal of methylglyoxal. Methylglyoxal-resistant mutants derived from the glycerol-sensitive parental strain also became immune to glycerol.

The initial steps in the dissimilation of glycerol and L-α-glycerophosphate (L-α-GP) in *Escherichia coli* are outlined in Fig. 1. Glycerol enters the cell by facilitated diffusion (34) and is retained as L-α-GP after its phosphorylation by glycerol kinase, whose activity is subject to remote feedback inhibition by fructose-1,6-diphosphate (21, 44). L-α-GP enters the cell via an active transport system (20). Intracellular L-α-GP may be acted upon by two specific pyridine nucleotide-independent L-α-GP dehydrogenases: an aerobic enzyme associated with the particulate fraction, and an anaerobic enzyme found in the soluble fraction whose activity is stimulated by flavine adenine dinucleotide and flavine mononucleotide (25, 30). The expression of the genes for all the above proteins is under the control of the *glp* regulator gene (10, 28).

In previous communications (6, 44), it has been shown that cells of a mutant *E. coli* strain constitutive in the *glp* system and producing an altered glycerol kinse which is insensitive to inhibition by fructose-1,6-diphosphate are killed when exposed to glycerol during growth on succinate or casein amino acids. Zwaig and Diéguez have further shown that succinate-grown cells of this strain, suspended in an unbuffered solution of glycerol, excreted a dialyzable product which is toxic to a variety of bacterial cells, including both gram-positive and gram-negative species (43). These workers found the bactericidal product to be stable to heating at 100 C for 10 min and to have a molecular weight of less than 500 by gel-exclusion chromatography (43).

In this paper we present chemical, enzymological, and genetic evidence to show that this lethal factor is methylglyoxal (2-oxopropanal). Cooper and Anderson (7) have discovered in *E. coli* a novel enzymatic activity catalyzing the formation of methylglyoxal from dihydroxyacetone phosphate (DHAP). Data will be presented which indicate that this enzyme activity plays a role in the lethal synthesis of methylglyoxal in strains with uncontrolled dissimilation of glycerol.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains described are ultimately derived from strain 1, an *E. coli* K-12 strain with a deletion in the alkaline phosphatase structural gene (28). Strain 7, derived directly from strain 1, synthesizes the gene products of the *glp* regulon constitutively, rather than inducibly (28). Strain 43 in turn was derived from strain 7 as a mutant which produces an altered glycerol kinase insensitive to feedback inhibition by FDP (45). Methylglyoxal-resistant derivatives of these three strains were obtained by direct plating on glucose minimal media containing 1.0 mM methyl-

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glyoxal, or an appropriate concentration of the lethal factor from culture filtrates of strain 43 cells that had been challenged with glycerol.

Growth of cells and preparation of extracts. The minimal medium buffered by phosphate at *p*H 7.0 was that previously described (38). Solid media with the same mineral composition were prepared by the addition of agar (Difco) to a final concentration of 1.5%. Concentrations of carbon sources in liquid or solid growth medium were: glucose, 10 mM; glycerol, 20 mM; sodium succinate, 15 mM. Succinate minimal medium was supplemented with 0.04% casein hydrolysate. Bacterial growth at 37 C with vigorous aeration was monitored turbidometrically with a Klett colorimeter (no. 42 filter, 1 unit = 3×10^6 bacteria per ml during exponential growth).

For preparation of extracts, cells growing in 250-ml cultures in 2-liter Erlenmeyer flasks were harvested at the late exponential phase, washed once by centrifugation at 3 to 4 C with 0.9% NaCl, and suspended in 5 ml of 25 mM imidazole-hydrochloride buffer (*p*H 6.6). The cells were disrupted by treatment for 5 min with a 60-w sonic disintegrator (Measuring & Scientific Equipment, Ltd., London). A dry-ice-ethanol bath at −10 C served to prevent overheating of the extracts during sonic treatment. The sonically treated crude extracts were clarified by centrifugation at 12,000 × *g* for 20 min at 3 to 4 C. Extracts were stored at −20 C.

Enzyme assays. Glycerol kinase was measured by a spectrophotometric assay at *p*H 7.5 (45). The aerobic L-α-GP dehydrogenase was assayed by coupling the oxidation of L-α-GP to the reduction of a tetrazolium dye to its formazan (30) under a condition where there is little or no contribution from the flavine-stimulated, anaerobic enzyme (25). Enzymatic production of methylglyoxal from DHAP was assayed by monitoring the increase in absorbancy at 240 nm due to the formation of *S*-lactylglutathione in the presence of excess reduced glutathione and glyoxalase I. The complete reaction mixture contained 25 μmoles of imidazole-hydrochloride buffer (*p*H 6.6), 1.6 μmoles of glutathione, 0.8 μmole of DHAP, 50 μg of glyoxalase I, and *E. coli* crude extract in a final volume of 1.0 ml. The reaction was initiated by the addition of DHAP or crude extract. Extracts were diluted in 25 mM imidazole buffer (*p*H 6.6).

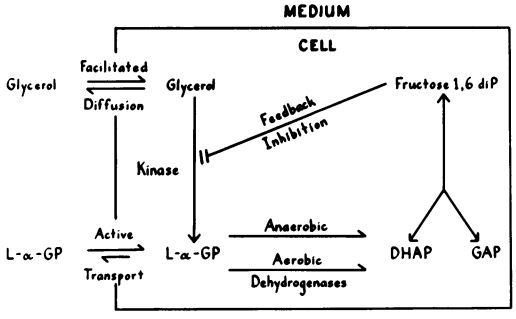

FIG. 1. *Pathways for glycerol and L-α-glycerophosphate (L-α-GP) dissimilation in Escherichia coli. DHAP, dihydroxyacetone phosphate; GAP, D-glyceraldehyde-3-P.*

Enzymatic determination of intermediates. L-α-GP was measured spectrophotometrically with the aid of rabbit muscle nicotinamide adenine dinucleotide (NAD)-linked L-α-GP dehydrogenase. Reduced NAD (NADH) production from L-α-GP-containing samples was monitored at 340 nm in 0.3 M sodium carbonate, 30 mM hydrazine, and 20 mM NAD, with 20 μg of enzyme, in a final volume of 1.0 ml at *p*H 9.5. For enzymatic determination of glycerol, the same reaction system was used but with the addition of 10 mM $MgCl_2$, 10 mM adenosine triphosphate, and 20 μg of crystalline *Candida mycoderma* glycerol kinase per ml to convert glycerol to L-α-GP. L-Lactate was estimated in the same manner as L-α-GP, with 50 μg of crystalline rabbit muscle NAD-linked L-lactate hydrogenase in place of L-α-GP dehydrogenase. DHAP concentrations were determined by measuring the extent of oxidation of NADH in a reaction system containing 0.1 μmole of NADH, 50 μmoles of $NaHCO_3$ (*p*H 8.3), and 30 μg of rabbit muscle L-α-GP dehydrogenase in a final volume of 1.0 ml. Crystalline triose phosphate isomerase (50 μg) was added to this reaction system for the determination of D-glyceraldehyde-3-phosphate. Methylglyoxal was determined spectrophotometrically by monitoring the formation of *S*-lactylglutathione catalyzed by glyoxalase I as described by Klotzsch and Bergmeyer (26).

Preparation, concentration, and assay of the lethal factor. Late-logarithmic-phase cells of strain 43 growing in succinate minimal medium (160 to 170 Klett units) were harvested by centrifugation, suspended in an equal volume of sterile 10 mM glycerol solution made up in deionized distilled water, and incubated with shaking for 3 hr at 37 C. The incubation solution was recovered and freed from the cells by a preliminary low-speed centrifugation, followed by filtration through a membrane filter (0.45 μm pore size, HAWP type, Millipore Corp., Bedford, Mass.). The filtrate was usually concentrated 50-fold by rotary flash evaporation with reduced pressure at a bath temperature of 40 to 50 C. For factor bioassay, 0.1-ml portions of a 1:100 dilution of an overnight broth culture of strain 1 were plated on glucose minimal medium. Wells, 10 mm in diameter, were made in the seeded plates with a sterile cork borer. When 0.1 ml of 50-fold concentrated lethal filtrate was added to a well, a zone of inhibition 35 to 40 mm in diameter was observed after overnight growth at 37 C. This assay provided a convenient semiquantitative test for assessing the effect of various physical and chemical treatments on the unknown lethal factor. To prepare growth media containing the lethal factor at its original concentration, appropriate amounts of the membrane-filtered concentrated fluid were diluted into liquid or solid culture medium made up as described above.

Chromatography. In system A, Whatman no. 1 paper was developed in the ascending direction with *n*-butanol equilibrated with an equal volume of 1.5 M NH_4OH. In system B, Whatman no. 1 paper was developed in the descending direction with *n*-butanol ethanol-water (3:1:1). In systems C and D, Eastman terphthalate-backed silica gel chromatogram sheets (no. 6061) were developed in the ascending direction with chloroform and benzene, respectively. To demon-

strate glycerol and methylglyoxal, chromatograms were sprayed with or dipped in $AgNO_3$ reagent (saturated $AgNO_3$-acetone, 1:200), dried, and sprayed with or dipped in ethanolic NaOH. Carbonyl compounds were located by spraying chromatograms with 25 mM 2,4-dinitrophenylhydrazine reagent and 90 mM HCl in methanol. Carbonyl compounds chromatographed as their 2,4-dinitrophenylhydrazine derivatives were rendered visible by ethanolic NaOH.

Chemical methods. The 2,4-dinitrophenylhydrazones of carbonyl compounds present in culture filtrates were prepared by the addition of an equal volume of 10 mM 2,4-dinitrophenylhydrazine and 2 N HCl to a sample of appropriately diluted or concentrated filtrate. Protein was determined by the biuret method (17).

Chemicals. Rabbit muscle L-α-GP dehydrogenase, L-lactate dehydrogenase, and *C. mycoderma* glycerol kinase were obtained from C. F. Boehringer & Soehne, Mannheim, Germany. Glyoxalase I (from yeast), triose phosphate isomerase (rabbit muscle), and methylglyoxal were obtained from the Sigma Chemical Co., St. Louis, Mo. Methylglyoxal was purified by direct steam distillation. Uniformly labeled ^{14}C-glycerol was a product of ICN Chemical and Radioisotope Division, Irvine, Calif. Casein amino acids (salt-free, vitamin-free) were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. Glutathione was obtained from Schwarz BioResearch, Inc., Mt. Vernon, N.Y.

RESULTS

Characterization of the bactericidal product as a carbonyl compound. Although it was stable to treatment with strong acid (1 N HCl at 50 C for 15 min), the bactericidal product formed by strain 43 was readily inactivated by treatment with weak alkali, as measured by the bioassay described above. All bactericidal activity was lost when 50-fold concentrated preparations of the lethal factor were adjusted to *p*H 10 with 0.1 M Na_2CO_3. The extreme alkaline lability suggested an essential sulfhydryl or carbonyl group. The former possibility, however, was eliminated by the resistance of the biological activity to treatment with *N*-ethylmaleimide. The involvement of a carbonyl group was confirmed by the following findings. Treatment of concentrated lethal factor preparations with $NaHSO_3$ (final molarity: 0.1 M) completely inactivated the factor. A concentrated solution of the bactericidal product prepared from strain 43 gave copious orange precipitates when treated with the 2,4-dinitrophenylhydrazine reagent, whereas a control solution prepared from strain 1 contained no lethal factor and gave no precipitate. A similarly prepared concentrated filtrate from strain 43 pregrown on succinate and challenged with 0.2% glucose instead of glycerol was shown to have no bactericidal activity and gave no precipitate.

Characterization of the 2,4-dinitrophenylhydrazine derivative. When bactericidal factor preparations were treated with excess 2,4-dinitrophenylhydrazine reagent, only a single spot was detected on chromatograms developed in solvent systems A and D. This single spot gave a bright red color on exposure to ethanolic NaOH. R_F values of the derivative were 0.85 and 0.28, respectively, in systems A and D. When bactericidal factor preparations were treated with limiting concentrations of 2,4-dinitrophenylhydrazine, the formation of two additional derivatives was observed. Thus it seems that the unknown factor is a dicarbonyl compound; under conditions of reagent excess, a *bis*-2,4-dinitrophenylhydrazone was formed, and under conditions of limited reagent mono-derivatives were formed. *Bis*-2,4-dinitrophenylhydrazones of redistilled authentic methylglyoxal and of the unknown, prepared in parallel in the presence of excess reagent, were found to have indistinguishable chromatographic properties. Moreover, melting points of both preparations (recrystallized from water) were identical (292.5 to 293.5 C, corrected), and no depression of melting point was observed when the preparations were mixed. Both derivatives were readily dissolved in ethylacetate but could not be reextracted from ethylacetate into 1 M Na_2CO_3. The absorption spectra of the derivatives of the bactericidal product and authentic methylglyoxal were identical, both before and after treatment with alkali.

Enzymatic confirmation of the identity of the lethal factor. The release of methylglyoxal into the incubation fluid of strain 43 cultures challenged with glycerol was confirmed by specific α-ketoaldehyde assay using the glyoxalase I assay of Klotzsch and Bergmeyer (26). Enzymatically determined methylglyoxal concentrations in the filtrates (before concentration) ranged between 0.8 and 1.4 mM. The average concentration found in four separate experiments was 1.2 mM. Control filtrates from glycerol-challenged cells of strain 1 contained less than 0.01 mM methylglyoxal. Further evidence that the toxic compound was methylglyoxal was obtained from the following experiment.

An aqueous methylglyoxal solution and a preparation of the lethal factor were each divided into two portions, one maintained at neutral *p*H and the other adjusted to *p*H 11.0 with NaOH. The four solutions were kept at 3 to 4 C overnight and then assayed for α-ketoaldehyde and L-lactate content. Treatment of the methylglyoxal solution or the lethal factor preparations with alkali resulted in the loss of all α-ketoaldehyde accompanied by the formation of L-lactate, one of the predicted products of the alkaline rear-

rangement of methylglyoxal, in 20% yield (Table 1).

Furthermore, system B chromatograms of lethal factor-containing filtrates from suspensions of strain 43 cells challenged with ^{14}C-glycerol revealed the presence of only two radioactive spots: untransformed glycerol and methylglyoxal. Enzymatic determinations of intermediates were also performed on such a filtrate, the results of which are summarized in Table 2. In view of these findings, and of the demonstration below that strain 43 contains high levels of all three enzymes required for the rapid conversion of glycerol to methylglyoxal, it seems unlikely that the toxic carbonyl compound is any other than the 3-carbon α-ketoaldehyde, methylglyoxal.

Enzymatic pathway leading to methylglyoxal formation. It was shown previously that the production of the lethal factor required not only a feedback-insensitive glycerol kinase but also the synthesis of this altered enzyme at a high level (44). It has been shown also that cells possessing the above characters but lacking a functional L-α-GP dehydrogenase could no longer produce the bactericidal product (44), thus indicating that metabolism of glycerol to a product distal to L-α-GP was required. Anderson and Cooper recently demonstrated that *E. coli* mutants lacking triose phosphate isomerase could still convert glycerol to glycogen and that the extracts catalyzed the conversion of DHAP to methylglyoxal and inorganic phosphate (1, 7). We found that strains 1, 7, and 43 grown on succinate contained a similar activity at a level of 0.1 to 0.3 μmole per min per mg of soluble protein. Figure 2 presents data on some kinetic properties of the enzymatic activity for synthesis of methylglyoxal from DHAP. The relatively high K_m for DHAP observed (0.7 mM) and the complex kinetics of inhibition by inorganic phosphate are strikingly similar to those presented by Hopper and Cooper (7, 23), leaving little doubt that the same enzyme was involved in both cases (Table 3).

Although succinate-grown cells of strains 1, 7, and 43 all contained high levels of methylglyoxal synthase activity, only the constitutive strains 7 and 43 had the necessary high levels of glycerol kinase and aerobic L-α-GP dehydrogenase required to produce a flood of DHAP, the substrate for methylglyoxal synthase (Fig. 3). But under physiological conditions, inhibition of the kinase by fructose-1,6-diphosphate apparently prevented cells of strain 7 from the lethal synthesis.

Strain 7 cells grown on succinate medium and challenged with aqueous glycerol can indeed produce methylglyoxal (Table 4). Zwaig (*personal communication*) previously observed production of the toxic factor by this strain. Presumably, the osmotic shock removed the fructose-1,6-diphos-

TABLE 1. *Effect of alkali on methylglyoxal and on the lethal factor*

Prepn	α-Keto-aldehyde[a]	L-Lactate[a]
Lethal factor		
*p*H 7	51	0.5
*p*H 11	0.4	10
Methylglyoxal		
*p*H 7	34	0.6
*p*H 11	0.1	6.3

[a] Expressed in micromoles per milliliter.

TABLE 2. *Concentration of 3-carbon intermediates in filtrates containing the lethal factor*

Compound	Concn[a]
Glycerol	9.6 ± 1.2
L-α-Glycerophosphate	<0.02
Dihydroxyacetone phosphate	<0.02
D-Glyceraldehyde-3-phosphate	<0.02
Methylglyoxal	1.4 ± 0.1

[a] Expressed as micromoles per milliliter plus or minus average deviation.

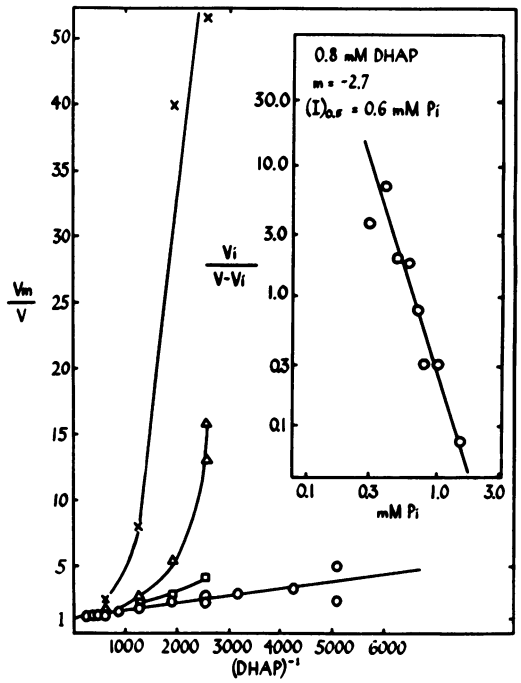

FIG. 2. *Kinetic properties of the enzymatic activity converting dihydroxyacetone phosphate to methylglyoxal found in crude extracts of strain 1. DHAP, dihydroxyacetone phosphate.*

TABLE 3. *Kinetic parameters of Escherichia coli methylglyoxal synthase*

Determination	Hopper and Cooper (23)	Present studies
K_m for DHAP	0.5 mM	0.7 mM
$(P_i)_{0.5}$[a]		0.6 mM
Response to DHAP in presence of P_i, slope of Hill plot[b]	2.9[c]	3.0[d]
Response to P_i, slope of Hill plot[e]		2.7[f]

[a] Concentration of modifier giving one-half maximal inhibition. See reference 5.

[b] Log $[V_{observed}/(V_{maximum} - V_{observed})]$ plotted against log (DHAP).

[c] (P_i), 0.3 mM.

[d] (P_i), 1.0 mM.

[e] Log of the ratio $(V_i)/(V - V_i)$ plotted against log (P_i), where V_i is the rate of reaction measured in the presence of orthophosphate, and V is the rate observed in the absence of orthophosphate. (P_i), concentration of inorganic phosphate.

[f] Dihydroxyacetone phosphate (DHAP), 0.8 mM.

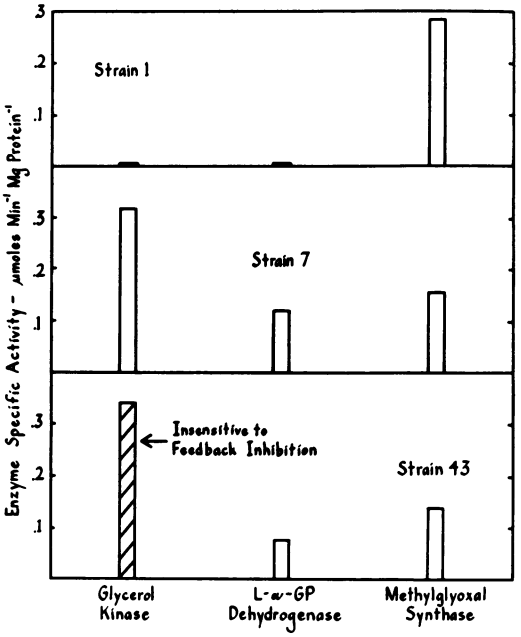

FIG. 3. *Activities of glycerol kinase, aerobic* L-α-*glycerophosphate* (L-α-GP) *dehydrogenase, and methylglyoxal synthase in Escherichia coli strains 1, 7, and 43 grown on succinate.*

phate necessary for curbing the activity of the kinase. Strain 1 cells uninduced in the *glp* system produced essentially no methylglyoxal, even in a hypotonic solution, as one would expect.

The production of methylglyoxal by strain 43 is not restricted to cells which had been pregrown in a succinate medium. Table 5 shows a variety of media which permitted the excretion of methylglyoxal upon challenge with glycerol. Succinate, lactate, and casein amino acids were among the most effective carbon sources for this purpose. The determining factor in the production of the toxic product thus appears to be a high level of the kinase rather than the nature of the carbon source per se during growth.

Mechanism of methylglyoxal resistance. When any strain of bacteria was plated on glucose minimal media containing 1 mM methylglyoxal or an equivalent concentration of the toxic product as measured by the glyoxalase I assay, spontaneous resistant mutants were found at a frequency of approximately 10^{-7}. Mutants selected from strains 1, 7, and 43 for growth in the presence of authentic methylglyoxal were found to be resistant also to the bactericidal product derived from glycerol, and vice versa. Unlike their parents, the resistant mutants actually were able to form small colonies on minimal agar containing 1 mM methylglyoxal as sole carbon source.

TABLE 4. *Methylglyoxal formation by strains 1, 7, and 43 during hypotonic exposure to glycerol*

Strain[a]	Methylglyoxal formed[b]
1	<0.02
7	0.69
43	1.45

[a] Cells grown on succinate minimal medium.

[b] Millimolar concentration found in the incubation fluid after 3 hr of hypotonic exposure to glycerol (10 mM) at 37 C.

TABLE 5. *Formation of methylglyoxal by strain 43 grown on various carbon sources*

Carbon source for growth[a]	Methylglyoxal formed[b]
Casein amino acids	1.85
Succinate	1.45
D-Lactic acid	0.88
Threonine	0.45
Mannitol	0.34
L-α-Glycerophosphate	0.26
Glycine	0.22
Lactose	0.20
Glucose	0.17
Glycerol	0.13

[a] Carbon sources present at 0.06 M carbon, with the exception of casein amino acids, present at 1.0%.

[b] Millimolar concentration found in incubation fluid after 3 hr of hypotonic exposure to glycerol (10 mM) at 37 C.

To see if the methylglyoxal resistance is associated with a new or increased capacity to utilize this compound, crude extracts of resistant and sensitive strains were examined for the presence of an enzymatic activity capable of removing methylglyoxal. Table 6 shows that such an activity could be demonstrated in vitro for all the strains, but the mutant strains were found to have from four to eight times more activity than the wild type. The activity in every case depended on glutathione, which points to glyoxalase as the responsible enzyme (27, 31). Thus it seems that resistance against methylglyoxal is acquired through an increased capacity for detoxification rather than simple tolerance for the compound.

Effect of methylglyoxal resistance on the response of strain 43 to glycerol. The question arises as to whether resistance to methylglyoxal at an external concentration of 1 mM is sufficient to protect cells of strain 43 while they generate the compound internally during exposure to glycerol. To answer this, strains 1, 7, 43 and three resistant derivatives of strain 43 were grown on succinate. At mid-exponential phase, glycerol was added to each culture. Only cells of strain 43 were killed, whereas the rest continued to grow (Fig. 4). Examination of the culture filtrates revealed significant quantities of methylglyoxal (0.20 mM) only in the case of strain 43; the other filtrates contained less than one-tenth this amount of methylglyoxal.

TABLE 6. *Glutathione-dependent transformation of methylglyoxal by extracts of methylglyoxal-sensitive and methylglyoxal-resistant strains of Escherichia coli*

Strain	Parent	Sensitive/ resistant	Relative activity[a]
1	K-12	Sensitive	1.0
291	1	Resistant	4.4
292	1	Resistant	6.9
293	7	Resistant	4.8
294	7	Resistant	3.8
295	7	Resistant	7.7
296	7	Resistant	5.3
297	43	Resistant	5.9
298	43	Resistant	5.7
299	43	Resistant	4.0

[a] The activity of strain 1 (0.011 μmole transformed per min per mg of soluble protein) is set at 1.0. The activity observed in the absence of glutathione was less than 0.002 μmole per min per mg of protein. The reaction mixtures contained 0.25 μmole of methylglyoxal, 3.3 μmoles of reduced glutathione, 50 μmoles of potassium phosphate buffer (*p*H 6.6), and *E. coli* crude extract containing 50 to 300 μg of protein in a final volume of 0.5 ml. After 15 min of incubation at 30 C, the reaction mixtures were treated with 2,4-dinitrophenylhydrazine reagent for 15 min as described. Finally, 2.0 ml of 2.5 N NaOH was added, and the amount of methylglyoxal remaining was quantitated on the basis of the absorption of the *bis*-2,4-dinitrophenylhydrazone in alkali at 550 nm.

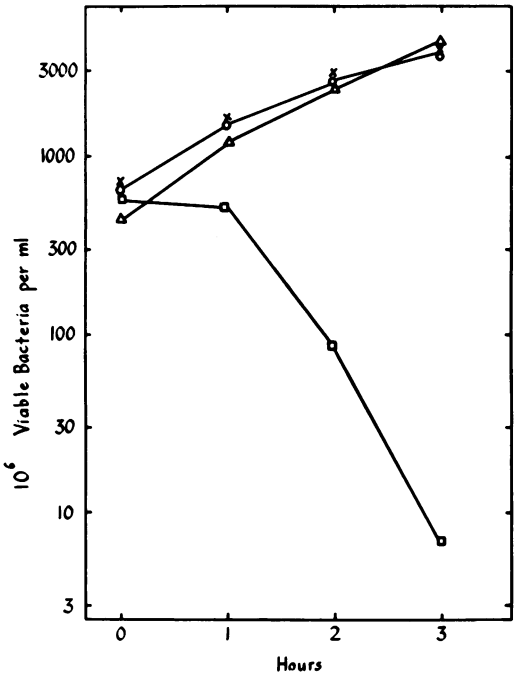

FIG. 4. *Immunity of methylglyoxal-resistant derivatives of strain 43 to glycerol. Succinate cultures of strains 1, 7, 43, 297, 298, and 299 were grown at 37 C. At mid-exponential phase, glycerol was added to a concentration of 20 mM. The number of viable bacteria per milliliter in each culture was determined by plating on rich nutrient agar immediately before and at 1, 2, and 3 hr after the addition of glycerol to each culture. Symbols: strain 1* (○), *strain 7* (△), *strain 43* (□), *strain 297* (×). *The behavior of strains 298 and 299, independently isolated methylglyoxal-resistant derivatives of strain 43, was identical to that of strain 297.*

DISCUSSION

For many years, methylglyoxal was considered to be a possible intermediate in normal glycolysis (8, 19, 33, 35). However, the experiments of Lohmann (31), in which NAD rather than glutathione restored the ability of dialyzed muscle extracts to convert glycogen to lactic acid, and the observed broad specificity of the glyoxalase system for a large number of α-ketoaldehydes (11) led to the denial of methylglyoxal as an intermediate of the major glycolytic pathway (9, 27). Cooper and his co-workers (7, 23) recently demonstrated that methylglyoxal synthase is present in *E. coli* and that this enzyme can provide a *dephosphorylating* pathway for DHAP

and thus is responsible for gluconeogenesis from glycerol in a mutant lacking triose phosphate isomerase. Hence, both their studies and those presented here show that under certain circumstances methylglyoxal is an important intermediate in a side pathway of glycolysis (Fig. 5). As stated by Hopper and Cooper (23), the striking inhibition of methylglyoxal synthase by inorganic phosphate suggests that the physiological task of this enzyme is to replenish intracellular inorganic phosphate when its concentration is low at the expense of phosphorylated glycolytic intermediates. The presence of such an enzymatic activity in higher organisms might explain the conversion of FDP to methylglyoxal and inorganic phosphate observed by a number of workers some 40 years ago (4, 24, 39, 40).

In bacteria, methylglyoxal can also be formed from glycine and threonine via aminoacetone (16, 18, 22, 41, 42). Subsequent catabolism of methylglyoxal in these cases has been shown to take place by means of the glyoxalase system, or in some cases by way of an NAD-linked dehydrogenase (22, 42).

It is beyond reasonable doubt that the toxic product excreted by *E. coli* strains hyperactive in glycerol dissimilation is methylglyoxal. The toxicity of millimolar levels of this compound to *E. coli* was noted and studied several years ago by Együd and Szent-Györgyi (12, 14), although no resistant mutants were found (13). Because of the growth inhibition properties, methylglyoxal (or another α-ketoaldehyde) was thought to be the universal inhibitor "retine," and the ubiquitous glyoxalase system was thought to be the promoting element "promine" in their theory on naturally occurring regulators of cell growth and division (36, 37). Some preliminary chemotherapy studies showed that methylglyoxal could indeed inhibit the growth of ascites tumors (2, 3, 15).

Whatever the advantages of producing methylglyoxal from DHAP or from other metabolites, the toxicity of the α-ketoaldehyde requires that the enzymatic pathways leading to its formation be well controlled, or that the cell be well equipped to dispose of the compound. The maintenance of a high constitutive level of methylglyoxal synthase, as found in *E. coli* cells under a variety of growth conditions by Cooper and his co-workers (7, 23) and by us, is probably made possible by the combination of a high K_m of the enzyme for DHAP, the dramatic third-power inhibition of its activity by inorganic phosphate, and the presence of the glyoxalase system.

The target of the lethal effect of methylglyoxal is likely to be a component of protein synthesis, as shown by the in vitro work of Szent-Györgyi and collaborators (14, 32) and in the in vivo experiments of Zwaig and Diéguez (43). However, there is also evidence for methylglyoxal inhibition of succinate oxidase and a number of other enzymes (13, 29). Sulfhydryl enzymes may be particularly sensitive to methylglyoxal (29, 36). It has also been proposed that the charge-transfer properties of methylglyoxal might cause this compound to have drastic effects on the secondary structure of cell membrane and cytoplasmic proteins at high concentrations (36).

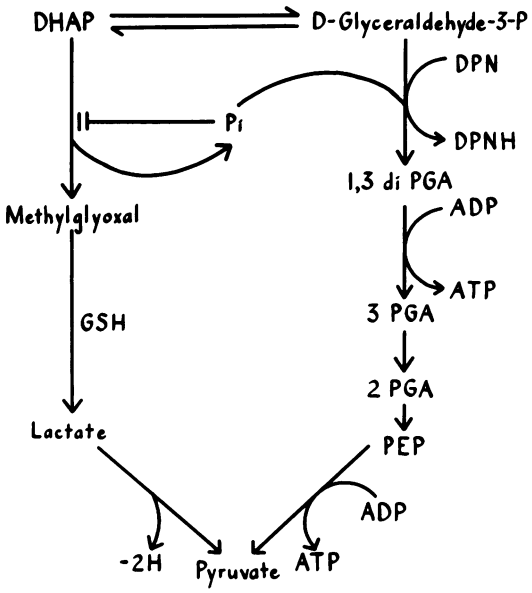

FIG. 5. *Methylglyoxal by-path in glycolysis in Escherichia coli. DHAP, dihydroxyacetone phosphate; GSH, glutathione; DPN, nicotinamide adenine dinucleotide; DPNH, reduced nicotinamide adenine dinucleotide; PGA, phosphoglyceric acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; PEP, phosphoenolpyruvate.*

Methylglyoxal may in fact kill cells by more than one mechanism. What is certain is that the wild-type cells must possess an array of control mechanisms to prevent the suicidal synthesis of this compound. In the case of glycerol dissimilation, it took the removal of two safeguards, feedback inhibition and specific repression (44), to reveal the latent danger of improperly channeled metabolic flow.

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