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Lysis of *Escherichia coli* by Glycine Is Potentiated by Pyridoxine Starvation

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Pyridoxineless mutants of *Escherichia coli* are lysed in a few hours when starved for pyridoxine in a glucose minimal medium containing glycine at 10 mM. The lysis is prevented equally well by L-alanine and by D-alanine when either is present at 0.1 mM. The lysis is potentiated by 0.5 mM L-methionine. The peculiar susceptibility of *E. coli* B to glycine-mediated lysis during starvation for pyridoxine suggests that the starvation reduces the availability of some normal antagonist of glycine, presumably alanine.

During a study of the effects of amino acids on pyridoxine-starved *Escherichia coli*, we observed that glycine and methionine appeared to cause lysis of these cells (8). Although growth inhibition and lysis of bacteria by glycine were first reported many years ago (11) and have been a subject of study by several more recent workers (10, 18, 21), the potentiation of glycine lysis by pyridoxine starvation has not been reported before to the best of my knowledge. The studies reported here were pursued because the initial observations indicated that lysis occurred in pyridoxine-starved strains in 2 or 3 h when 0.01 M glycine was used, whereas earlier reports of glycine-induced lysis of *Escherichia coli* found that this concentration of glycine was ineffective even at long times (10, 21). Maculla and Cowles used, for example, 16 h of incubation with 3 M glycine to get 77% lysis of *E. coli* (18). This report describes the observations in detail and defines the conditions for maximal lysis. It also describes both potentiation of glycine-induced lysis by certain amino acids and inhibition of glycine-induced lysis by alanine. We do not presently plan to expand these interesting observations beyond those reported here.

MATERIALS AND METHODS

Strains. The strains used represent each of the nutritionally and genetically distinct types of pyridoxineless mutants so far isolated in this lab from *E. coli* B. They have been described previously (2, 4, 6, 7, 8).

Lysis experiments. Cultures (50 ml) of the organisms were grown overnight at 37C with vigorous shaking in glucose minimal medium (3) containing 100 μg of pyridoxine per liter. In the morning 10 ml of each culture was centrifuged for 10 min at 5,000 × *g* at room temperature, washed in 0.9% NaCl, and suspended in 50 ml of fresh, identical medium. This was incubated as above until the cell density had doubled. The culture was then centrifuged and washed with 0.9% NaCl. The cells were resuspended in approximately 12 ml of fresh 0.9% NaCl. Cells were dispensed into 250-ml Erlenmeyer flasks containing 52.5 ml of glucose minimal medium with amino acids and other additions as indicated in the figures. The initial cell density was usually near 0.16 optical density units at 650 nm as measured in 18-mm culture tubes. Samples (5 ml) were withdrawn every hour for absorbancy measurements.

Miscellaneous. An optical density at 650 nm of 0.3 represents 0.15 mg of cell mass (dry weight).

RESULTS

Figure 1 shows optical densities measured when representatives of nutritionally and genetically distinct types of pyridoxineless mutants of *E. coli* B were starved for pyridoxine in the presence and in the absence of glycine. The open circles in the figures show that, in the absence of glycine, most pyridoxineless strains continue arithmetic growth at a constant rate for about 2 h after pyridoxine starvation begins and then appear to shift to a new, lower arithmetic growth rate for several hours. Wild-type *E. coli* continues normal logarithmic growth. The filled circles show, on the other hand, that shortly after 1 h of pyridoxine starvation, cultures of mutants not only slow growth but begin to lose turbidity if glycine is present. That the turbidity changes represented

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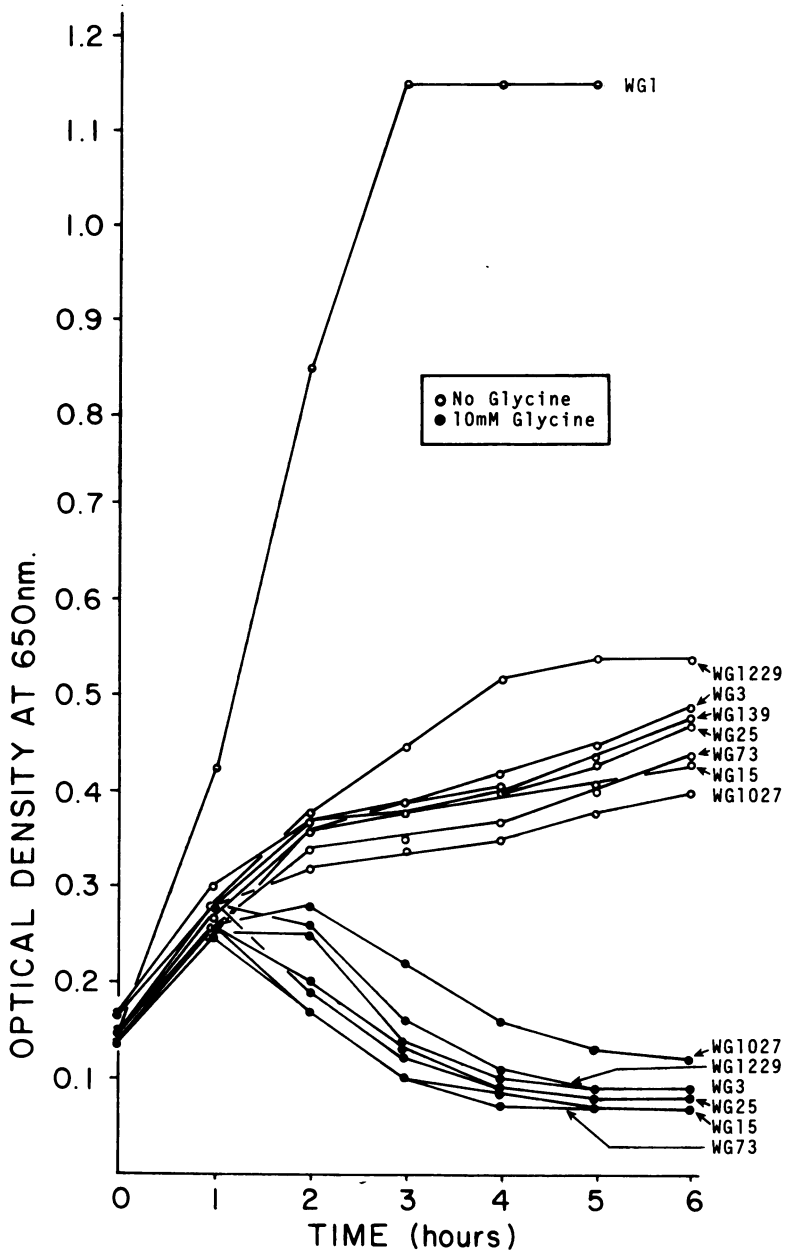

FIG. 1. *Turbidity as a function of time of starvation for pyridoxine. All strains were starved for pyridoxine beginning at 0 time. Strain WG1 is wild type Escherichia coli B. Strains WG3, WG139, WG15, WG73, WG25, WG1027, WG1229 carry pdx genes B, C, D, E, G, J, and K, respectively.*

lysis was confirmed by direct microscope examination using phase-contrast microscopy and by measurement of viable counts. The former method showed a rapid increase in swollen, odd-shaped cells similar to those seen upon penicillin treatment. The latter showed that only 5% of the viable cells present after 1 h of pyridoxine starvation in the presence of glycine remained present after 4 h of such starvation.

Figure 1 shows that wild-type *E. coli* B (strain WG1) is unaffected by glycine at this concentration. As indicated in the figure by half-filled circles, glycine-fed and glycine-starved cultures give, in this case, congruent data points. The continued small increases in cell mass during vitamin starvation have been adequately dis-

cussed by Wilson and Pardee (22).

The pyridoxineless strains in Figure 1 all behaved in a qualitatively similar way toward glycine. For that reason, a single strain, WG25, was chosen for use in the exploration of other aspects of glycine induced lysis. This strain was originally isolated after ultraviolet mutagenesis and is one of the most stable of the pyridoxineless mutants we have isolated.

Figure 2 shows that the rate of lysis of strain WG25 varies with the initial concentration of glycine. Figure 3 shows the effect of DL-alanine, DL-serine, DL-glutamine, DL-threonine, DL-methionine, L-isoleucine, and glycolaldehyde, each at 0.5 mM final concentration, upon lysis of pyridoxine-starved strain WG25 by 5 mM glycine. The data show that alanine appears to antagonize the glycine effect, while the other compounds potentiate it. The figure also shows that strain WG25 appears to be immune to glycine-induced lysis if pyridoxol is present at 10^{-3} mM. The other compounds tested were chosen because of the existence of other data that show some, mostly unexplained, relationships between pyridoxine metabolism and the test compounds (5, 6, 7, 8).

In separate experiments I found that the L-isomer of methionine appeared to be the more active isomer at potentiating glycine lysis. After 2 h of starvation, for example, cultures containing 10 mM glycine had an average optical density of 0.33 if no methionine were present but 0.30 with 0.5 mM D-methionine and 0.25 with 0.5 mM L-methionine. These optical density differences were maintained during at least 5 h of starvation, while the total optical density decreased in the usual way. Similarly, no detectable difference in effectiveness between 0.5 mM and 1.0 mM L-methionine was found.

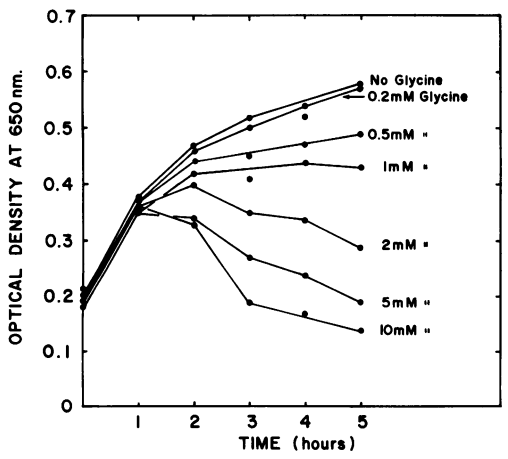

FIG. 2. *Effect of increasing glycine concentrations upon lysis of strain WG25.*

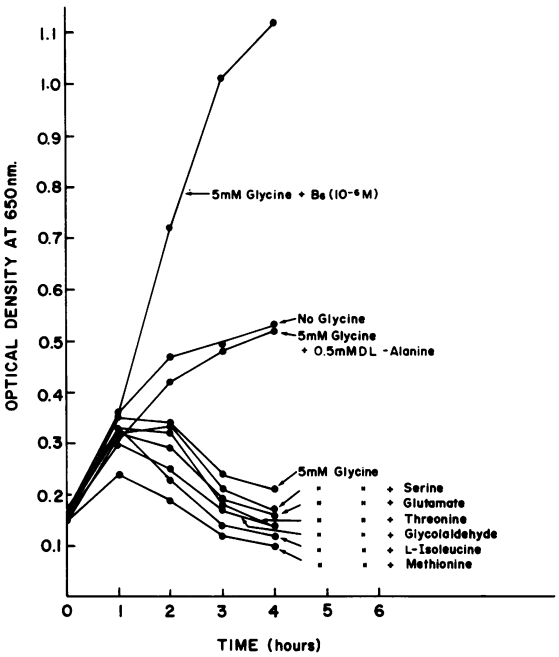

FIG. 3. *Effect of some amino acids and glycolaldehyde on glycine-induced lysis. Isoleucine was used as the L-isomer; the other amino acids were the DL-isomers. All test compounds were at 0.5 mM. B_6 refers to pyridoxal.*

L-Methionine at 0.1 mM was approximately 60% as effective as 0.5 mM L-methionine at potentiating lysis by glycine.

Presently it is not quite clear whether L-methionine is acting independently of the pyridoxine-glycine system or not. It may be, for a report has been made of methionine induced lysis in both *Salmonella typhimurium* and *Haemophilus influenzae* (9). Presently I have no explanation of this methionine effect.

Figures 4 and 5 show that both L- and D-isomers of alanine are about equally effective in antagonizing the glycine-mediated lysis, with 0.1 mM alanine being adequate for nearly total inhibition of the lysis induced by 10 mM glycine.

Additional experiments showed that D-alanine could be added to 0.5 mM final concentration any time during the first hour of pyridoxine starvation in the presence of glycine and still give the same protection against glycine lysis as seen when both amino acids were present from the start. After the first hour, the addition of DL-alanine appeared to arrest lysis at the point reached.

DISCUSSION

The data presented here show that glycine at 5 to 10 mM causes significant lysis of pyridox-

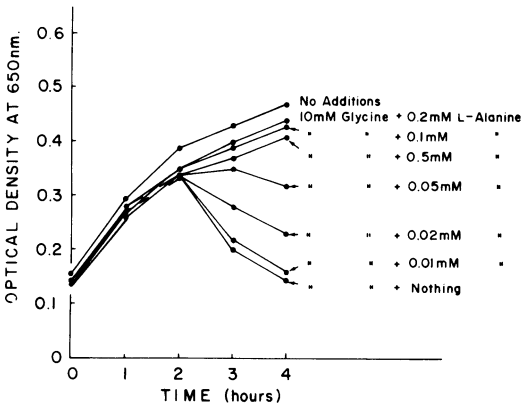

FIG. 4. *Inhibition of glycine induced lysis by* L-*alanine.*

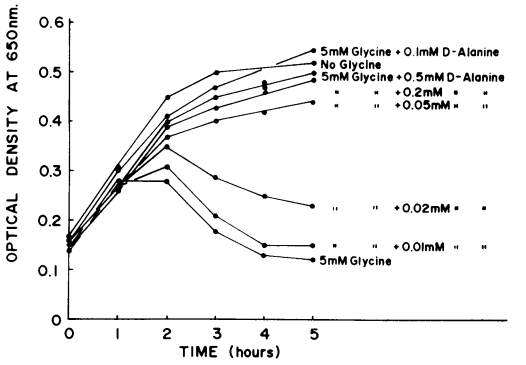

FIG. 5. *Inhibition of glycine induced lysis by* D-*alanine.*

ineless mutants of *E. coli* B when these mutants are starving for pyridoxine. In the presence of pyridoxine the mutants show no detectable response to these levels of glycine. The data showed too that L- and D-alanine at 0.5 mM are equally effective in protecting pyridoxine starved mutants from 5 mM glycine, making likely the possibility that the glycine-mediated effect results from interaction of glycine at a site that normally interacts with alanine. Finally, the data show that a few other amino acids, most notably L-methionine, appear to increase the effectiveness of glycine in its action.

Two points raised specifically by these findings should be considered further, namely, how does loss of pyridoxine sensitize cells to glycine-mediated lysis and, secondly, how can L- and D-alanine be equally active in preventing lysis by glycine? These two questions will be considered separately below.

A large amount of data exists describing conditions for maximal lysis of bacterial cells, and in particular *E. coli*, by the amino acid glycine. A review of most of this early work has been made in an article by Welsch (21).

Recent reports have suggested two ways in which lysis by glycine may occur. In the first of these ways, glycine would serve as a substrate for an enzyme which normally incorporates alanine into cell wall (12, 20). In the second of these ways glycine would serve as an inhibitor of an enzyme which normally incorporates alanine into cell wall (15–17).

If glycine were to cause lysis by serving as a substrate, then one would postulate that muramyl peptides containing glycine in place of alanine could form but would be incapable of providing all of the structural properties alanine provides to make a stable cell wall. In support of the possibility of glycine serving as substrate are demonstrations by Strominger and Birge (20) and by Hishinuma et al. (12) of the accumulation of glycine-containing uridine-diphosphate-acetylmuramyl peptides during glycine lysis.

Recent work by Lugtenberg, on the other hand, has supported the second mechanism for lysis by glycine. Glycine was reported to inhibit three enzymes of murein synthesis in *E. coli* (15). These three enzymes were: (i) D-alanine:D-alanine ligase, (ii) L-alanine:D-alanine racemase, and (iii) L-alanine adding enzyme. Lugtenberg and van Schijndel-van Dam also showed that conditional mutants altered in either L-alanine adding enzyme (16) or D-alanine:D-alanine ligase (17) underwent rapid lysis when shifted to the nonpermissive temperature after first exhibiting about an hour's worth of normal growth. Lysis of mutants lacking the racemase was also reported (15). Therefore, since they showed (i) that lack of any one of these enzymes will cause cell lysis and (ii) that glycine inhibits each of these enzymes, it seemed that a satisfactory explanation of glycine lysis could be had by this mechanism simply by considering its action to be the inhibition of these three cell wall enzymes.

Against such a simple interpretation of glycine-lysis as that offered by the experiments of Lugtenberg et al., stands firstly the findings of Strominger and Birge (20) and of Hishinuma et al. (12) which were quoted above and secondly the possibility, in light of recent findings made by the Neuhaus group with gram-positive organisms (14, 19), that additional potentially glycine-sensitive enzymes than those considered by Lugtenberg may participate in cell wall synthesis. Presently, then, glycine lysis appears best explained as a result of both enzyme inhibition and also formation of glycyl peptides in place of alanyl peptides.

The question at hand then from the data presented in this work is how does loss of

pyridoxine sensitize the cells to glycine lysis? Although it is clear that many explanations might be possible, the answer may lie in a decreased ability of the cells to synthesize L-alanine as a result of the pyridoxine deficiency. Lowered L-alanine synthetic capacity would decrease the ability of the cell to furnish substrates for each of the three glycine-sensitive enzymes. This lack of normal substrates in turn would make these three enzymes less able to avoid reacting with glycine than they would be in the normal wild-type state or pyridoxine fed state. (In this argument, of course, one assumes that glycine and alanine are both acting at the same site as competitive antagonists of each other.) The lytic action of glycine then would occur as discussed above either by inhibition of one or more of the enzymes of cell wall synthesis or by its incorporation into a muramyl peptide to form a "false" or biologically inactive peptide, or both.

The second question considered above, namely how can both L-alanine and D-alanine be equally active, could be answered if one assumed that the L-alanine racemase activity is not eliminated by a few hours starvation for pyridoxine. This could occur if the L-alanine racemase of *E. coli* B is either not a pyridoxal phosphate-containing enzyme or is one of those enzymes which binds pyridoxal phosphate so tightly that a significant percentage of the cofactor remains attached during starvation for pyridoxine. Under either of these conditions the racemase would convert L-alanine to a mixture of L- and D-alanine and D-alanine likewise to a mixture of the two isomers. In this way, both forms of alanine would appear to be antagonists of glycine action but in truth only one might be. Although two reports on other *E. coli* strains contain suggestions that can be interpreted to mean that a pyridoxal phosphate requirement is not readily demonstrable for *E. coli* L-alanine-D-alanine racemase (13, 15), presently hard evidence on whether the *E. coli* B L-alanine-D-alanine racemase contains pyridoxal phosphate is totally lacking.

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