REVERSAL OF AZASERINE BY PHENYLALANINE[1]

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Kaplan, Reilly, and Stock (1959) have studied the reversal of azaserine by aromatic amino acids and have hypothesized without any direct evidence that these substances prevent the binding or penetration of the antibiotic into the cell. The present paper presents more direct evidence that this hypothesis is correct.

It is shown that azaserine acts on nongrowing cells of *Escherichia coli* strain ML35 in such a way that subsequent growth in azaserine-free medium leads to increases in permeability, lysis, and death. It has been found that phenylalanine reverses only when it is present during the azaserine treatment and has no effect when present during the subsequent growth period. These results are interpreted to mean that phenylalanine prevents the binding or penetration of azaserine into the cell, but plays no direct role in preventing the metabolic disarrangements caused by azaserine.

This work has been aided by a strain (ML35) of *E. coli* which forms β-galactosidase constitutively at high levels but lacks a permease. This strain is useful in detecting nonspecific effects on permeability.

MATERIALS AND METHODS

Organisms. *E. coli* strain ML35 was provided by Dr. Jacques Monod; *E. coli* strain 15T⁻U⁻ was obtained through the courtesy of Dr. Seymour Cohen; *E. coli* strains B and B/r were obtained from the Department of Genetics, Carnegie Institution of Washington.

Medium and growth conditions. A defined medium of the following composition was used: K_2HPO_4, 6.0 g; KH_2PO_4, 3.0 g; NH_4Cl, 1.0 g; NaCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; glycerol, 5.0 g; and distilled water, 1 liter. In the medium used for the growth of strain 15T⁻U⁻, thymine and uracil were present at final concentrations of 8 μg/ml and 40 μg/ml.

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Cells were grown overnight in defined medium at 37 C on a rotary shaker, diluted into fresh medium, and were used when logarithmic growth had been re-established. Growth was followed by measuring optical density at 420 mμ on the Lumetron colorimeter.

Phosphate buffer used for washing and as a suspension medium during azaserine treatment had the following composition: K_2HPO_4, 6.0 g; KH_2PO_4, 3.0 g; distilled water, 1 liter.

Viable counts were made by the pour plate method using nutrient agar; plates were counted after 48 hr incubation.

β-Galactosidase assay. To a 1.0-ml sample of cell suspension or supernatant was added 4.0 ml of 0.0016 M *o*-nitrophenyl-β-D-galactoside in 0.1 M Na_2HPO_4 buffer, pH 7. After incubation at 37 C for sufficient time to give an optical density at 420 mμ in the range of 0.100 to 0.400, the reaction was stopped by the addition of 5 ml of 1 M Na_2CO_3. Following centrifugation, the optical density was then converted to μg *o*-nitrophenol (ONP) with the use of a standard curve, and the β-galactosidase activity reported as μg *o*-nitrophenol released per ml sample per hr.

For total β-galactosidase, the cells were first treated with toluene. To a 1.0-ml cell sample previously adjusted to an optical density of 0.100 was added 0.1 ml of acetone-toluene 20:1. After incubation at 37 C for 15 min, *o*-nitrophenyl-β-D-galactoside was added and the assay continued as above.

Azaserine. Azaserine was a gift of Parke Davis and Company. Because this antibiotic is unstable, the dry powder and aqueous stock solutions were stored in the freezer. It was used without sterilization.

RESULTS

Effect of azaserine on permeability. Azaserine was added to exponentially growing cells of *E. coli* strain ML35 and growth, viable count, and β-galactosidase activity measured (figure 1). The latter activity measured on nontoluenized cells is an indication of nonspecific changes in

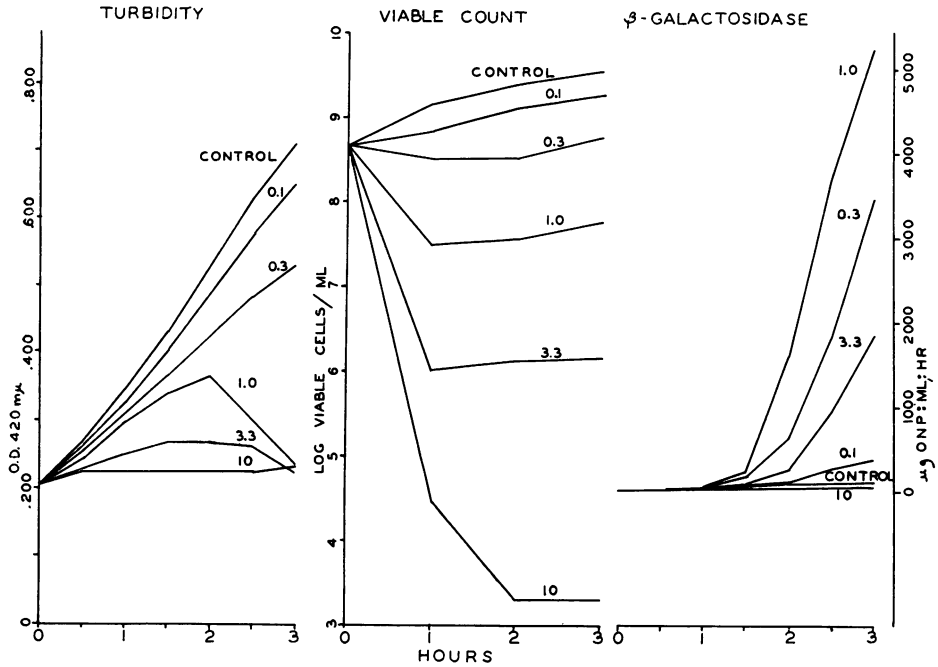

Figure 1. Effect of azaserine on growth, β-galactosidase activity of nontoluenized cells, and viable count of *Escherichia coli* strain ML35. Azaserine was added at 0 hr to give a final concentration of 0.1, 0.3, 1.0, 3.3, or 10 μg/ml.

permeability (Brock and Brock, 1959). Growth is inhibited completely by 10 μg/ml azaserine, partially by 1.0 μg/ml, and only slightly by 0.1 μg/ml. Direct microscopic counts confirmed the optical density readings, including the slight lysis at 1.0 μg/ml at later incubation times. Viable counts showed a marked lethality at the higher concentrations of azaserine, and a slight lethality even at 0.1 μg/ml. The β-galactosidase activities are quite different, however, showing the greatest increase at 1 μg/ml and less increase at either lower or higher levels. At 1 μg/ml, the increase in permeability after 3 hr is enormous, being equivalent to the full activity of toluenized cells. It should be noted that this increase is accompanied by some lysis, but that the marked increase in β-galactosidase is detectable before any drop in optical density occurs. In this system, the measurement of β-galactosidase activity of nontoluenized cells is probably a very sensitive measurement of lysis. The varying amounts of lysis at different concentrations of azaserine is interpreted to be due to the fact that some growth is necessary for this lysis to develop. At high concentrations of azaserine, growth is inhibited completely, whereas at low concentrations there is insufficient azaserine to bring about any primary action. Only at intermediate concentrations are the two factors balanced. If the carbon or nitrogen source is left out during azaserine treatment, little increase in β-galactosidase activity occurs, indicating that growth is necessary for development of leakiness.

Lysis could further be demonstrated by measuring the β-galactosidase activity of the supernatants of cells treated with various concentrations of azaserine. At 1.0 μg/ml azaserine, the increase in enzyme activity in the supernatant paralleled the increase in the whole culture, although about one-half of the enzyme activity remained sedimentable at 10,000 × *g*. Analysis of supernatants after destruction of the azaserine with acid revealed considerable amounts of 260 mμ-absorbing material. It is concluded therefore that azaserine induces lysis at appropriate concentrations and that the measurement of β-galactosidase activity is a more sensitive measurement of this lysis than optical density changes.

Several other strains were treated to determine whether lysis occurred following azaserine treatment. These results are shown in figure 2. It can

be seen that the growth of strains B and B/r was inhibited but no lysis occurred, whereas strains 15T⁻U⁻ and ML35 did lyse. This raised the question of whether the latter two strains might be lysogenic and were being induced to a productive response by azaserine. Since Gots, Bird, and Mudd (1955) have shown that azaserine is able to induce the lysogenic *E. coli* strain K-12 (lambda), this possibility seemed likely. We had no knowledge of lysogeny in *E. coli* strains ML35 and 15T⁻U⁻, but this phenomenon can often go unsuspected unless sought for. A large number of strains of *E. coli* of varying backgrounds were tested as possible hosts for an ML35 phage, using sterile-filtered azaserine-induced lysates of ML35. In addition, a number of strains of other genera of Enterobacteriaceae were also tested as hosts. Results were uniformly negative, so that it is not possible to draw any conclusions. It is possible that the ML35 lysate contains only a defective phage, which could not be detected by conventional means. Because of the work of Gots et al. (1955) it seems simpler to assume the lysis induced by azaserine is due to an event related to lysogenicity rather than to some other cause.

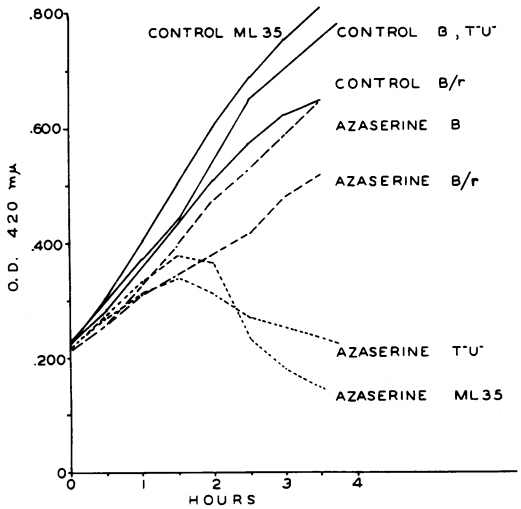

Figure 2. Effect of 1 μg/ml azaserine, added at 0 hr, on the growth of *Escherichia coli* strains ML35, 15T⁻U⁻, B, and B/r.

Reversal by phenylalanine in growing cells. Despite the uncertainty of the above observations, strain ML35 seemed to be a useful strain for studying the relationships between azaserine and aromatic amino acids, since one effect of

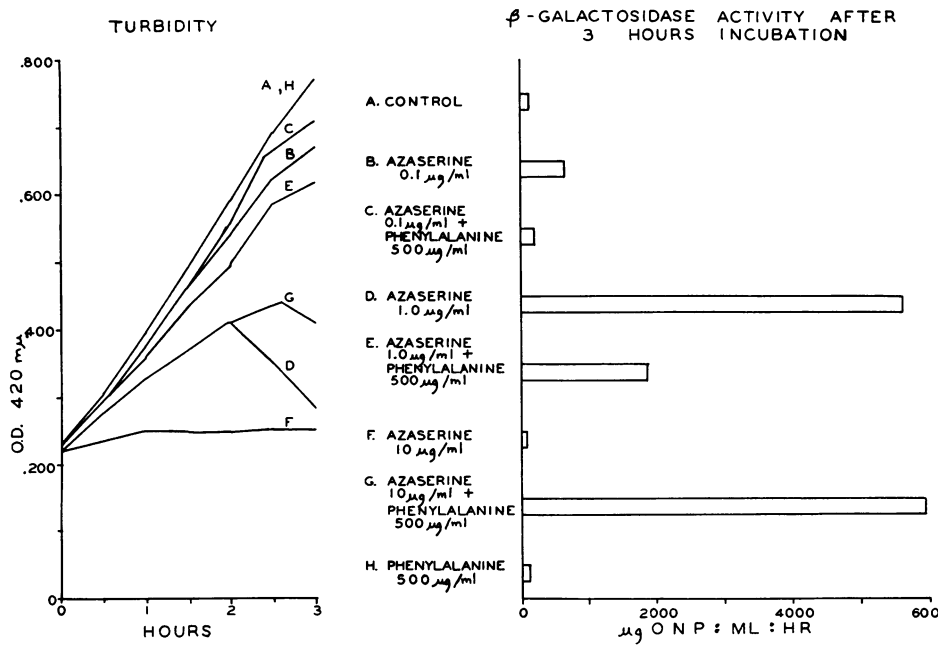

Figure 3. Effect of various azaserine concentrations with and without phenylalanine on growth and β-galactosidase activity of *Escherichia coli* strain ML35. Additions were made at 0 hr in glycerol-salts medium.

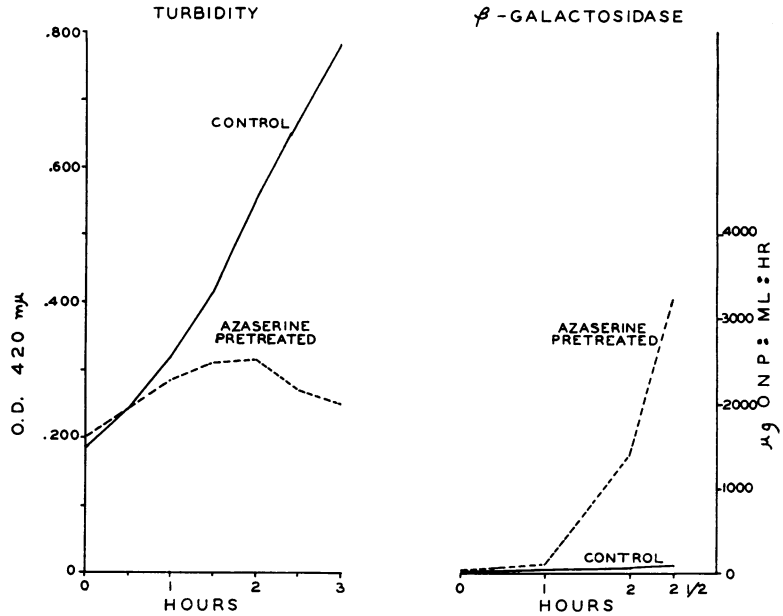

Figure 4. Effect of 1 μg/ml azaserine treatment for 1 hr at 37 C in phosphate buffer on subsequent growth and β-galactosidase activity of *Escherichia coli* strain ML35 in glycerol-salts medium.

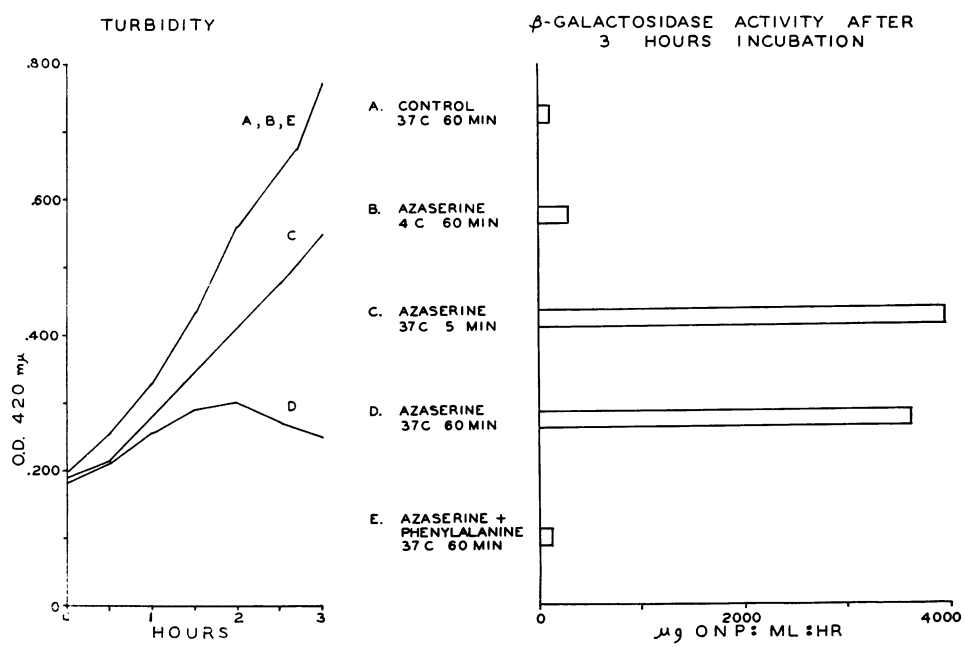

Figure 5. Effect of conditions of azaserine treatment in phosphate buffer on subsequent growth and β-galactosidase activity of *Escherichia coli* strain ML35 in glycerol-salts medium. Azaserine, 1 μg/ml; phenylalanine, 500 μg/ml.

azaserine, permeability change, could be measured so simply and precisely. Results of studies using phenylalanine as a reversing agent are shown in figure 3. It can be seen that at a fixed level of phenylalanine of 500 μg/ml, 1 μg/ml of azaserine is reduced in activity to about 0.1 μg/ml, while 10 μg/ml of azaserine is reduced to 1 μg/ml of activity. The ability of phenylalanine to reduce the effectiveness of azaserine is reflected not only in a reduction of growth inhibition but also in a reduction in permeability increase. Viable count studies showed that phenylalanine also reversed the lethal effects of azaserine. These results confirm those of Kaplan et al. (1959). Further studies on phenylalanine reversal are presented below.

Action of azaserine on nongrowing cells and eversal by phenylalanine. Because of the resemblance of certain of the effects of azaserine to an unbalanced growth phenomenon, studies were carried out in which cells were treated with azaserine under conditions which did not permit growth and then placed in complete medium. It was readily shown that even when the cells were treated in phosphate buffer, they exhibited the characteristic permeability increase when subsequently allowed to grow in complete medium. The action of azaserine on *E. coli* in phosphate buffer had also been shown earlier by Gots et al. (1955). In a typical experiment, the results of which are shown in figure 4, log phase cells were washed twice with cold phosphate buffer, resuspended in buffer, and treated with 1 μg/ml azaserine for 1 hr at 37 C on a shaker. A 10-fold drop in viable count occurred routinely under these conditions. After treatment, the cells were

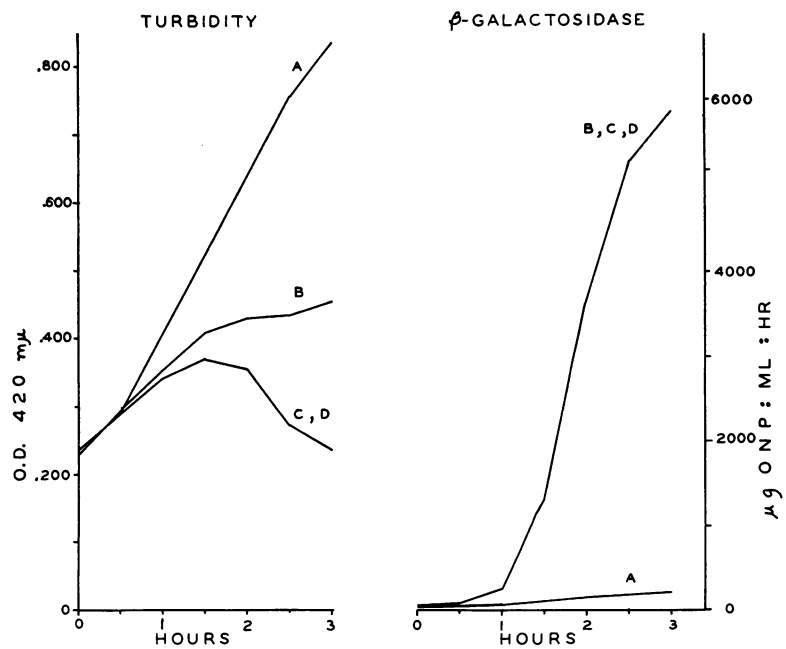

Figure 6. Effect of growth in phenylalanine before or after azaserine treatment for 1 hr at 37 C in phosphate buffer on growth and β-galactosidase activity of *Escherichia coli* strain ML35. Azaserine, 1 μg/ml; phenylalanine, 500 μg/ml.

Legend:

Pretreatment	Treatment	Posttreatment
A. Glycerol-salts	Phosphate	Glycerol-salts
B. Glycerol-salts + phenylalanine	Azaserine	Glycerol-salts
C. Glycerol-salts	Azaserine	Glycerol-salts + phenylalanine
D. Glycerol-salts	Azaserine	Glycerol-salts

again washed twice, resuspended in complete medium, and growth and β-galactosidase activity were measured. Following the azaserine treatment, lysis and the characteristic marked increases in β-galactosidase activity occurred. These results show that an irreversible reaction of azaserine occurs during treatment in phosphate buffer, and that cells so treated undergo all of the characteristic responses that are exhibited by cells treated under growing conditions.

The irreversible reaction of azaserine with cells was studied further. The results presented in figure 5 show the turbidity and enzyme changes of cells pretreated with azaserine in phosphate buffer under varying conditions, washed, and suspended in complete medium. It can be seen that the action of azaserine is analogous to a binding or active transport phenomenon. It is quite rapid, being almost complete in 5 min. It does not occur in the cold. It is completely inhibited by phenylalanine. These results seem to show conclusively that phenylalanine reverses azaserine by preventing its uptake.

This point was explored further by using cells either grown in phenylalanine before treatment or grown in phenylalanine after treatment. These results are shown in figure 6. It can be seen that cells grown first in phenylalanine are partially protected from subsequent azaserine treatment. On the other hand, cells treated with azaserine in the absence of phenylalanine are not protected when they are subsequently grown in the presence of phenylalanine. This seems to show that azaserine is not acting by affecting aromatic amino acid metabolism, but that aromatic amino acids prevent the binding or uptake of azaserine.

DISCUSSION

It can be concluded from the present work that phenylalanine reverses azaserine action by preventing its binding or uptake into the cell. This verifies the hypothesis of Kaplan et al. (1959). These workers also showed that a number of other aromatic amino acids, including some amino acid analogues, also reversed azaserine action. Since these compounds block by preventing uptake, it might be inferred that the uptake of azaserine and aromatic amino acids involves the same transport system. Although azaserine seems to differ considerably in structure from the aromatic amino acids, this difference may be only superficial. Although its exact electronic configuration has not yet been determined (Karrer, 1950), the diazoacetyl ester grouping is highly unsaturated, with probably a dense electron cloud. Because of this it might easily resemble the electron rich aromatic rings and thus might be concentrated by the aromatic amino acid concentrating system. Azaserine might be a useful agent for studying aromatic amino acid transport. At any rate, it seems clear that azaserine is not acting inside the cell through a pathway related to aromatic amino acids, since if this were so, cells treated first with azaserine should be protected by subsequent growth in phenylalanine-containing medium.

SUMMARY

Azaserine reacts irreversibly with cells in phosphate buffer and brings about characteristic changes when these cells are subsequently incubated in growth medium. The antagonism of L-azaserine by phenylalanine has been shown to be due to a blocking of this binding or uptake of azaserine by the cells. When cells treated first with azaserine are used, phenylalanine has no effect on its action. Phenylalanine will reverse if it is present during azaserine treatment and has only a partial effect if the cells are grown in phenylalanine before treatment.

Some effects are described of azaserine on *Escherichia coli* strain ML35, a mutant which forms β-galactosidase constitutively and lacks a permease for β-galactosides. Some of the effects noted are probably secondary effects due to induction of phage production in this presumably lysogenic strain.

REFERENCES

BROCK, T. D., AND M. L. BROCK 1959 Effect of novobiocin on permeability of *Escherichia coli*. Arch. Biochem. Biophys., **85**, 176–185.

GOTS, J. S., T. J. BIRD, AND S. MUDD 1955 L-Azaserine as an inducing agent for the development of phage in the lysogenic *Escherichia coli*, K-12. Biochim. et Biophys. Acta, **17**, 449–450.

KAPLAN, L., H. C. REILLY, AND C. C. STOCK. 1959 Action of azaserine on *Escherichia coli*. J. Bacteriol., **78**, 511–519.

KARRER, P. 1950 *Organic chemistry*. Original translation by A. J Mee. 4th English ed., pp. 293–294. Elsevier Pub. Co., Inc., New York.