PURINE METABOLISM IN BACTERIA

IV. L-AZASERINE AS AN INHIBITOR

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The antibiotic, L-azaserine (o-diazoacetyl-L-serine) has been shown to be a versatile disorganiser of many biological functions. Following its demonstration as an antibacterial (Coffey et al., 1954) and antineoplastic agent (Stock et al., 1954), it was also found to be a potent mutagenic agent (Hammerley and Demerec, 1955), an inducer of bacterial filamentation (Maxwell and Nickel, 1954) and phage formation in lysogenic bacteria (Gots et al., 1955), and an inhibitor of substrate induced enzyme formation in yeast (Halvorson, 1954).

The variety of biological functions so disturbed by azaserine suggests a radiomimetic type of action involving an underlying mechanism of nucleic acid dysfunction. That azaserine could inhibit the synthesis of nucleic acids was first revealed by Skipper et al. (1954), who showed that the incorporation of single carbon units into the nucleic acid purines of animal tissues was prevented by azaserine. Hartman et al. (1955) were able to pinpoint the site of inhibition in soluble fractions of enzymes from pigeon liver at the level of purine synthesis involving formylglycinamide ribotide as substrate. The intracellular accumulation of this intermediate was also reported to occur in bacteria inhibited by azaserine (Skipper, 1956). Evidence has been obtained that the inhibition is the result of an antimetabolic antagonism with the glutamine which is required for the amination of formylglycinamide ribotide to yield formylglycinamidine ribotide (Buchanan, 1956; Levenberg and Melnick, 1956). We have been studying the factors which can control and affect the biosynthesis of purines in bacterial systems. This has been facilitated by the use of purine requiring mutants which allow the accumulation of the aminoimidazole precursors of the purine nucleus. Using these systems as an index of the potential capacity for purine synthesis we have found that azaserine is a powerful inhibitor of the synthesis of the imidazole intermediates. This paper deals with a description of these inhibitory effects and the factors which influence the inhibition.

MATERIALS AND METHODS

All experiments were performed with washed nonproliferating bacterial suspensions by the methods previously described (Gots and Love, 1954). The final concentration of cells in the incubation mixtures was 0.5 mg of cells (dry weight) per ml. Glucose (0.4 per cent) served as the source of carbon and energy, and ammonium chloride (0.1 per cent) as the sole source of nitrogen. No growth occurred in the absence of the auxotrophic growth factor. Reactions were stopped by adding trichloroacetic acid.

The synthesis of 5-amino-4-imidazolecarboxamide (AICA) was followed with suspensions of the purine requiring mutant, strain B-96. This mutant was isolated in this laboratory as a derivative of Escherichia coli strain B by the penicillin selection method. Its properties have been described (Gots and Love, 1954). It accumulates AICA primarily as a riboside.

Strain W-11 is a purine requiring mutant of E. coli strain K-12 which was originally obtained from Dr. J. Lederberg. Its properties and the methods of handling it as cell suspensions have been described (Love and Gots, 1955). The diazotizable amine which it accumulates has recently been identified (Love and Levenberg, personal communication) as the riboside of 5-aminomidazole, a precursor of the corresponding ribose form of AICA. As previously described, the formation of the aminomidazole by cell suspensions of this mutant was facilitated by aeration.

The tryptophan requiring mutants, strains B-37 and B-82, were derivatives of E. coli strain B. The properties of these mutants have been

1 This investigation was supported by research grants (C-2189 and C-2790) from the National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.
described (Gots et al., 1954). The synthesis of anthranilic acid by strain B-37 and indole by strain B-82 can also be followed with cell suspensions (Gots and Rowlands, unpublished data).

An azaserine resistant mutant of strain B-96 designated as B-96/azs was isolated from survivors of azaserine action on solid synthetic minimal media containing adenine. Whereas 0.1 mg of azaserine per ml could completely inhibit the parent B-96, the resistant mutant survived the action of more than 10 μg per ml indicating a several hundred fold increase in resistance. The mutation to azaserine resistance did not affect the requirement for purines.

The aminoimidazoles in the trichloracetic acid extracts were measured as diazotizable amines by the method of Bratton and Marshall (1939). The presence of 1 mg of azaserine per ml did not interfere with this test.

Our initial supply of azaserine was obtained through the courtesy of Dr. Chester Stock of the Sloan-Kettering Institute; subsequent supplies were obtained through the courtesy of Dr. Leon Sweet of Parke, Davis & Co. All other chemicals were obtained through commercial channels.

RESULTS

Figure 1 shows the inhibitory effects of azaserine on the synthesis of AICA by cell suspensions of the purine requiring mutant, strain B-96. As little as 1 μg of azaserine per ml brought about an 80 per cent reduction in the total yield of AICA. A 50 per cent inhibition was obtained consistently within the range of 0.5 to 0.1 μg per ml. Inhibition occurred when azaserine was either present from the start or when added after synthesis had been well underway (figure 1). Since inhibition could still be demonstrated after one-half of the total amine had already been formed, the inhibition must involve something other than the reported prevention of the synthesis of enzyme (Halvorson, 1954).

In an experiment similar to the one depicted in figure 1, the cells were removed by centrifugation after an exposure of one hour to 10 μg of azaserine per ml. These were washed and resus-
pended in an equal volume of fresh reaction mixture. Synthesis of AICA by these cells resumed but at a rate and with a total yield which was 60 per cent of the control similarly treated. Thus, the inhibitory action of asaserine is partially reversible.

Since AICA formation was measured from nonspecific sources of carbon, energy, and nitrogen, it was essential to rule out a possible interference with the utilization of these nonspecific substrates. This was done by the use of tryptophan requiring mutants. Under the same conditions used for mutant B-96, the synthesis of anthranilic acid by mutant B-37 and indole by mutant B-82 was not disturbed by asaserine up to 100 μg per ml. Direct measurement of the utilization of glucose by manometric techniques also showed no inhibition by asaserine. Thus, the inhibition of AICA by B-96 is at a level beyond the utilization of the non-specific essentials for biosynthetic capacity.

It has been shown that the presence of amino acids markedly stimulates the synthesis of AICA.

The following substances allowed an increase of about 10 per cent in AICA formation inhibited by asaserine: Threonine, valine, glycine, methionine, and homo-cysteine.

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Figure 3. Effect of various amino acids and related compounds on the inhibition of AICA synthesis by asaserine. Conditions were as described in figure 1. AICA was determined after 3 hr. All additions were to concentrations of 100 μg per ml.

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Figure 4. Reversal of asaserine inhibition by phenylalanine. Conditions were as described in figure 1. DL-Phenylalanine (PhA) was added to give final concentration of 20 μg per ml.

(Gots and Love, 1954). In the presence of amino acids, as supplied by casein hydrolysate (0.1 per cent), the kinetics of inhibition by asaserine were essentially the same as obtained with ammonium salts as the sole source of nitrogen. However, in order to obtain a comparable level of inhibition, the concentration of asaserine had to be increased 100-fold in the presence of the amino acids. This can be seen in figure 2 which shows that the addition of casein hydrolysate raised the concentration of asaserine required for 50 per cent inhibition from 0.1 to 10.0 μg per ml.

An examination of the effects of single amino acids revealed a remarkable reversing action by the aromatic amino acids, phenylalanine, tyrosine and tryptophan. The inhibition by asaserine which decreased the production of AICA to 15 to 20 per cent of the uninhibited control was restored to 70 to 75 per cent of the control by the addition of any one of the aromatic amino acids. These amino acids had no effect on the
yield of AICA in the absence of azaserine. Glutamic acid and glutamine were next most active, allowing a synthesis in the presence of azaserine of 50 to 55 per cent of the uninhibited control. In this case, the uninhibited control, particularly with glutamine, was increased over that of the unsupplemented control. Except for leucine, isoleucine, and norleucine, the other amino acids had no significant reversing action on the inhibition. The effects of the amino acids are depicted in figure 3 which also includes the activity of relatives of the aromatic amino acids. Phenylpyruvic acid was the most active followed by dihydroxyphenylalanine (DOPA). \( \beta \)-Phenylserine and tryptamine were only slightly active. That the action of phenylalanine may be primarily attributed to its \( \beta \)-form is indicated by the less efficient reversing action of its \( \alpha \)-enantiomorph.

Further investigation into the nature of the effects of the aromatic amino acids was made with phenylalanine. The kinetics of the reversing action of phenylalanine are shown in figure 4. When phenylalanine was added an hour after the inhibition by azaserine had been underway, reversal of the inhibition was still obtained with an ensuing curve which paralleled the curve obtained when phenylalanine was added from the start. Efforts to show a clearcut competitive action between azaserine and phenylalanine were most disappointing. Only over a small limited range of azaserine concentrations could a semblance of competition be demonstrated. At concentrations of azaserine higher than 10 \( \mu \)g per ml, no concentration of phenylalanine (up to 1000 \( \mu \)g per ml) could restore the inhibited synthesis. Furthermore the concentrations of phenylalanine which could reverse the inhibition by amounts of azaserine below 10 \( \mu \)g per ml reached a maximum at about 50 to 100 \( \mu \)g per ml above which no additional reversal action could be demonstrated. Similar results were obtained with glutamine but this analysis was further complicated by the fact that glutamine alone increased the yield of AICA in the uninhibited control.

In an attempt to determine what metabolic
role, if any, phenylalanine might play in the synthesis of AICA, a known metabolic analogue of phenylalanine, \( \beta \)-2-thienylalanine, was examined. The surprising results are depicted in figure 5. Instead of preventing the reversal action of phenylalanine, \( \beta \)-2-thienylalanine was itself a most effective reversing agent of azaserine inhibition. Complete prevention of inhibition was obtained with 1,000 \( \mu \)g per ml.

The use of another purine requiring mutant, strain W-11, served to show that azaserine inhibits AICA formation at a level prior to the completion of the imidazole nucleus. This mutant accumulates a pentose containing aminoimidazole which is an apparent precursor of AICA (Love and Gots, 1954) and which has been identified as 5-aminoimidazole riboside. As can be seen in figure 6, the synthesis of this amine by non-proliferating cell suspensions of strain W-11 is also markedly inhibited by azaserine.

An analysis of the azaserine resistant mutant of B-96 indicated that the inhibition of AICA synthesis was related, at least in part, to the inhibition of growth. This mutant, B-96/ass, required a several hundred fold increase in azaserine concentration before its inhibition of growth was comparable to that of its susceptible progenitor. It can be seen in table 1 that the synthesis of AICA by the resistant strain is also more resistant to inhibition by azaserine. In the experiments depicted, strain B-96 required only 0.04 \( \mu \)g of azaserine per ml for a 50 per cent inhibition; the azaserine resistant strain required 1.0 \( \mu \)g per ml, a 25-fold increase. This falls short of correlating with the more than hundredfold increase in resistance with respect to growth inhibition. This lack of complete correlation would imply that the resistance phenomenon is associated only in part with a resistance to inhibition of AICA synthesis and mostly with a resistance to inhibition of other metabolic processes. It is also of interest to note that the uninhibited activity of the resistant strain consistently was 20 to 25 per cent less than that of its susceptible parent.

**DISCUSSION**

The results presented here show that the biosynthesis of the aminomimidazole precursors of the purine ring is acutely sensitive to inhibition by minute amounts of azaserine. This is in agreement with the findings of Hartman et al. (1955) where azaserine, but in much higher concentrations, prevented the conversion of formylglycinamide ribotide to inosinic acid by enzyme fractions of pigeon liver homogenates. With inhibition at the level of formylglycinamide, formation of the imidazoles would not be expected. In the pigeon liver system, glutamine acted as an efficient antagonist of azaserine inhibition which led to the implication of azaserine as a competitive inhibitor of glutamine function in amination processes (Buchanan, 1956; Levenberg and Melnick, 1956). The participation of glutamine in the amination of formylglycinamide ribotide is apparently more sensitive to azaserine inhibition than is the similar role of glutamine in amination reactions required for the synthesis of formylglycinamide ribotide.

In the bacterial systems reported here, the inhibition by azaserine was relieved much more efficiently by the aromatic amino acids and related substances than by glutamine. A frank competitive antagonism between azaserine and glutamine or the aromatic amino acids could not be demonstrated. Because of this it is difficult to assign to azaserine a competitive type of antimetabolic action with respect to glutamine and aromatic antagonists.

So far, in all microbial systems examined the aromatic amino acids have been able to prevent azaserine action. In addition to the inhibitions reported here, this includes inhibition of induced enzyme formation in yeast (Halvorson, 1954), induction of phage formation in lysogenic bacteria, and inhibition of growth of *E. coli* (Kaplan and Stock, 1954). In the latter case the results were interpreted along lines which implicated azaserine as an inhibitor of the biosynthesis of the aromatic

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**TABLE 1**

A comparison of the inhibition by azaserine of the synthesis of AICA by strain B-96 and its azaserine resistant derivative strain B-96/ass

<table>
<thead>
<tr>
<th>Azaserine (( \mu )g/ml)</th>
<th>B-96</th>
<th>B-96/ass</th>
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<tbody>
<tr>
<td></td>
<td>AICA</td>
<td>Inhibition</td>
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<td></td>
<td>( \text{mmoles/ml} )</td>
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<tr>
<td>0.0</td>
<td>110</td>
<td>0</td>
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<tr>
<td>0.01</td>
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<td>0.1</td>
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</tr>
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<td>10.0</td>
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<td>85</td>
</tr>
<tr>
<td>100.0</td>
<td>11</td>
<td>90</td>
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AICA was determined after 23/4 hr of incubation.
amino acids. It is difficult to extend such an interpretation to the results found here since this would imply that the synthesis of the imidazole precursors of purines depended upon the prior synthesis of the aromatic amino acids. This is unlikely and not supported by precedent. The aromatics have no influence on the yield of AICA, and at least one double mutant of strain B-96 carrying an additional deficiency in tryptophan synthesis remained unimpaired in its synthetic capacity with respect to AICA. Furthermore, the known antimetabolite of phenylalanine, β-2-thienylalanine, not only is bland with respect to its effect on AICA synthesis, but it can itself act as a most efficient countering agent of azaserine inhibitions. This would suggest that these aromatics antagonize azaserine by some nonspecific chemical interaction rather than through the restoration of an inhibited metabolic function. The lack of stoichiometric relationships between azaserine inhibition and restoration by phenylalanine would indicate that detoxification of azaserine is more than just a result of a simple chemical combination. The difference in the efficiencies of the D- and L-isomers also supports this.

It is possible that inhibition is brought about not by azaserine, but by a metabolic product of azaserine. This idea finds support in the observations of Halvorson (1954) who showed that during inhibition of his yeast systems azaserine disappeared, and serine, a possible product of azaserine catabolism, appeared. Leucine, which prevented azaserine inhibition, also prevented the catabolism of azaserine. It is conceivable that the aromatic substances may act in a similar fashion. If phenylalanine, for example, does act by preventing the catabolic "activation" of azaserine, then this "activation" would have to be a continuous process since phenylalanine can still restore synthetic activity after the cells have been exposed to the action of azaserine for at least one hour (figure 4). The results shown in this figure also serve to rule out a possible interference by phenylalanine with the penetration or absorption of azaserine.

The biosynthesis of aminimidazoles by the mutant strains studied here is a reflection, and serves only as an index, of the capacity for the de novo synthesis of purines by E. coli. The inhibition by azaserine, therefore, implies a potential for the inhibition of purine synthesis in the genetically unimpaired, synthetically sufficient wild type prototroph. However, the naturally occurring purines (adenine, guanine, etc.) have little, if any, effect in altering the course of growth inhibition of the wild type by even threshold levels of azaserine (0.05 to 0.1 μg/ml). It is apparent, therefore, that the inhibition of purine biosynthesis is not sufficient in itself to account for the inhibition of growth. Other biosynthetic processes must also be affected. That the inhibition of AICA synthesis contributes, at least in part, to the inhibition of growth is evident from the results obtained with the azaserine resistant strain.

SUMMARY

The antibiotic, L-azaserine (α-diazoacetyl-L-serine) was shown to be a powerful inhibitor of the synthesis of the aminimidazole precursors of purines by nonproliferating cells of purine requiring mutants of Escherichia coli. In the presence of amino acids a hundredfold increase in azaserine was required. The amino acids most effective in reversing the inhibition by azaserine were tryptophan, phenylalanine and tyrosine, and to a lesser degree, glutamine, glutamic acid, leucine, isoleucine, and norleucine. β-2-Thienylalanine could also prevent the inhibition by azaserine. An azaserine resistant mutant was partially resistant to the inhibition of imidazole synthesis.

REFERENCES


