ACTION OF AZASERINE ON *ESCHERICHIA COLI*[1]

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Azaserine (*O*-diazoacetyl-L-serine) was first isolated from a crude broth filtrate of *Streptomyces fragilis* and found to possess marked antitumor activity (Ehrlich *et al.*, 1954; Bartz *et al.*, 1954; Stock *et al.*, 1954). The chemical synthesis of this compound was reported immediately thereafter; the synthetic *O*-diazoacetyl-L-serine was found to be identical in all physical, chemical, and biological properties with the antibiotic azaserine (Moore *et al.*, 1954). A comparison of the D and L isomers of *O*-diazoacetylserine demonstrated that the inhibitory activity resides in the isomer that contains L-serine (Nicolaides *et al.*, 1954; Stock *et al.*, 1954).

The ability of azaserine to interfere with a number of biochemical events in various biological systems suggests that the antibiotic may disrupt one or more fundamental metabolic processes (Maxwell and Nickel, 1954; Coffey *et al.*, 1954; Halvorson, 1954; Gots and Golub, 1956*b*; Dagg and Karnofsky, 1955; Barker *et al.*, 1956). One of the biosynthetic steps which appears to be most sensitive to azaserine inhibition is the synthesis of purines. In both an avian liver system (Levenberg *et al.*, 1957) and a bacterial system (Tomisek *et al.*, 1956) a marked inhibition by azaserine of the amidination of (α-*N*-formyl)-glycinamide ribotide, a purine precursor, has been demonstrated. Further studies have revealed that the inhibitory action of azaserine in these systems appears to be on the utilization of the amide-*N* of glutamine as the source of nitrogen atom three of the purine nucleus; the competitive nature of the inhibition has, thus far, been established only for the avian liver system.

The purpose of the present studies was to investigate some of the effects that are produced by azaserine upon *Escherichia coli*. Investigations were carried out on the following three levels of cellular activity: growing cells, nonproliferating cells, and cell-free extracts. Elucidation of the alterations in metabolic patterns of the bacterium would provide information about the site or sites of action of the antibiotic in this microbial system and would provide a basis for similar studies in other biological systems.

MATERIALS AND METHODS

Bacterial strains. The organisms used in these studies were wild-type *E. coli* strain W and several aromatic amino acid requiring mutants derived from it (Davis, 1951) and *E. coli* strains B wild-type and B-96, a purine requiring mutant (Gots, 1950).

Medium and bacteriological techniques. To avoid complications of the undefined effects of complex medium on preventing the inhibition by azaserine (Reilly, 1956) all experiments were performed using medium M-9 of Anderson (1946). For solid medium, agar (Difco) was added to M-9 in a final concentration of 1.5 per cent.

In preliminary studies, the ability of various compounds to block the inhibitory effect of azaserine on the growth of *E. coli* was determined by a modification of the filter paper disc procedure (Vincent and Vincent, 1944). Candidate agents were incorporated into melted M-9 agar (45 C); the mixture was seeded with the microorganism and was dispensed into petri dishes. After the contents of the dishes had solidified, filter paper discs (Schleicher and Schuell, Analytical Filter Papers, no. 740-E) that previously had been impregnated with azaserine were placed onto the surface of the medium. The plates were incubated at 37 C for 16 to 18 hr. At the end of the incubation period, the diameters of the zones of inhibition that surrounded the paper discs were measured and recorded in millimeters.

To determine the quantitative aspects of the prevention of azaserine inhibition by candidate agents, a tube dilution technique was employed.

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Solutions of azaserine were sterilized by heating in a boiling water bath for 10 min; candidate agents were sterilized by autoclaving. One drop of a 1:10 dilution of an 18-hr culture of *E. coli* strain W washed from the surface of a nutrient agar slant with 3.0 ml of saline, was added to a series of tubes containing azaserine and candidate agents in a total volume of 10 ml of M-9. After incubation at 37 C for 16 hr, the turbidity of each culture was measured in terms of optical density at 560 mμ in a Coleman Universal spectrophotometer. The optical density readings of each experiment were plotted against the amounts of azaserine in the tubes in the form of a dose-effect curve by the method of least squares. The amount of azaserine per tube that was required to cause 50 per cent inhibition of the growth of the organism was read directly from the curve.

Viability. The viable count was determined by the number of colonies which appeared after 48 hr at 37 C on heart infusion agar (Difco). Aliquots of 0.05 ml of appropriate dilutions of the sample with 0.85 per cent NaCl were spread onto the surfaces of the solidified agar with the aid of a bent glass rod.

Glutamic acid-tyrosine transaminase from E. coli strain W. The medium described by Feldman and Gunsalus (1950) was used for the production of cells which contain glutamic acid-tyrosine transaminase activity. Ten liters of the inoculated medium were incubated at 37 C for 18 hr with aeration. The cells were harvested in a Sharples centrifuge, washed twice with cold distilled water, and lyophilized. Extraction of the dried cells was carried out according to the procedure of McIlwain (1948). One part of dried cell material was ground with three parts of powdered alumina (Alcoa 301) for 3 min in a precooled mortar. Five-ml portions of 0.1 M phosphate buffer, pH 8.2, were added gradually and grinding continued until a total of 50 ml buffer had been added. The cellular debris was removed by centrifugation and discarded. To the clear supernatant was added solid $(NH_4)_2SO_4$ (6.3 g of the salt per 10 ml extract) slowly, with stirring. The precipitate which formed was collected and dissolved in water distilled from glass. This fraction was treated with solid $(NH_4)_2SO_4$, and the fraction precipitating between 33 to 66 per cent saturation with the salt was collected and dissolved in 0.067 M phosphate buffer, pH 6.9; this fraction was dialyzed for 18 hr against the above buffer. All procedures, beginning with the harvesting of the cells, were carried out at 4 C.

Transaminase activity was assayed at 37 C in a 1-ml system containing 100 μmoles *p*-hydroxyphenylpyruvic acid, 25 μmoles L-glutamic acid, 0.5 ml transaminase preparation, pyridoxal phosphate, and other additions. After incubation for 1 hr, the tubes were immersed in a boiling water bath for 5 min to stop the reaction. The extent of transaminase activity was measured by determination of the amount of tyrosine present in the reaction mixture. This was accomplished by a manometric technique in which the amount of CO_2 released by the decarboxylation of tyrosine by dried cells of *Streptococcus faecalis* strain R is measured (Bellamy and Gunsalus, 1944).

Synthesis of 5(4)-amino-4(5)-imidazolecarboxamide by E. coli strain B-96. The conditions for the formation of aminoimidazole carboxamide by nonproliferating cells of *E. coli* strain B-96 have been reported by Gots and Love (1954). The basic reaction mixture consisted of 0.02 M glucose, 0.06 M phosphate buffer, pH 7.2, 0.019 M ammonium chloride and a sample of a suspension of washed cells of *E. coli* equivalent to 5 mg (dry weight) in a total volume of 5 ml. The flasks were shaken in a water bath at 37 C for 2 hr. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 4 per cent. After centrifugation, the amount of imidazole derivatives present in the supernatant was determined by the Bratton and Marshall (1939) test for diazotizable arylamines.

Various organic compounds, including several aliphatic amino acids (0.2 μmoles/ml), aromatic acids (20 μg/ml), and heterocyclic acids (20 μg/ml) were tested for their ability to antagonize the inhibitory effect of azaserine (1.0 μg/ml) in this system.

RESULTS

Preliminary studies. It was found that the inhibitory effect of azaserine on the growth of *E. coli* could be blocked by any one of the three aromatic amino acids, i. e., L-tryptophan, L-tyrosine, and L-phenylalanine (table 1). A number of other amino acids including glycine, DL-α-alanine, β-alanine, DL-valine, DL-leucine, DL-isoleucine, DL-threonine, DL-serine, DL-methionine, DL-aspartic acid, L-glutamic acid, DL-histidine, DL-ornithine, L-citrulline, L-arginine, DL-lysine, and L-glutamine, singly did not interfere with

azaserine inhibition. This confirms previous work reported by Reilly (1954) and Maxwell and Nickel (1954).

In addition the B-vitamins, thiamine (1.0 μg/ml), pyridoxine (5.0 μg/ml), and its congeners, pteroylglutamic acid (1.5 μg/ml), *d*-biotin (1.0 μg/ml), pantothenic acid (5.0 μg/ml), and nicotinic acid (5.0 μg/ml) did not reduce the size of the zone of inhibition caused by 5.0 μg azaserine per disc.

TABLE 1

Blocking by the aromatic amino acids of the inhibition of growth of Escherichia coli strain W by azaserine

Supplement*	Diameter of Zone of Inhibition, mm	
	10 μg Azaserine/disc†	2 μg Azaserine/disc
None	39	23
L-Phenylalanine	20	0
L-Tyrosine	24	0
L-Tryptophan	27	0

* Supplement added to M-9 agar to give 1×10^{-4} M.

† Diameter of disc, 12.7 mm.

It was observed, however, that the combination of any one of the aromatic amino acids with either L-arginine or DL-histidine exerted greater blocking of azaserine inhibition than the aromatic amino acids alone. It is noteworthy that the inhibitory effect of azaserine on the growth of a yeast *Kloeckera brevis* is best antagonized by the basic amino acids (Reilly, 1954). Moreover, Guthrie (*unpublished observations*) has observed that inhibition of *Bacillus subtilis* strain 6051 by azaserine is blocked by DL-histidine; the aromatic amino acids alone were ineffective in this regard.

Although Bennett *et al.* (1956) have reported that various purine bases block the inhibition caused by azaserine (0.08 to 0.095 μg/ml), no substantial blocking of this inhibition of growth could be demonstrated in this study. Similarly, Gots and Gollub (1956*b*) have observed that the naturally occurring purines failed to overcome the inhibition produced in wild-type *E. coli* by threshold levels of azaserine (0.05 to 0.1 μg/ml).

Studies with the aromatic amino acids and their metabolic precursors. The effect of various precursors of the aromatic amino acids (Davis, 1955) upon the inhibition of growth by azaserine are shown in table 2. With three mutants of *E. coli* that require for growth an exogenous supply of tryptophan or one of its precursors, the presence

TABLE 2

Blocking of azaserine inhibition of growth in mutants of Escherichia coli. Effect of biochemical precursors of phenylalanine, tyrosine, and tryptophan

Strain No.	Growth requirement	Supplement*	Diameter of Zone of Inhibition, mm†
83-5	L-Phenylalanine or phenylpyruvic acid	Phenylalanine	0
		Phenylpyruvic acid	0
83-8	L-Tyrosine or *p*-hydroxyphenylpyruvic acid‡	Tyrosine	0
		p-Hydroxyphenylpyruvic acid	30
M165 A-52	L-Tryptophan	Tryptophan	0
M19-2	L-Tryptophan or indole	Tryptophan	0
		Indole	30
		Indole + serine	30
M121-35	L-Tryptophan, indole, or anthranilic acid	Tryptophan	0
		Indole	30
		Indole + serine	30
		Anthranilic acid	31
W	None	None	31

* Supplement added to M-9 agar to give 1×10^{-4} M.

† Azaserine, 5.0 μg per disc; diameter of disc, 12.7 mm.

‡ Active as substitute only at pH 5.5 or when succinate is used instead of glucose as a carbon source in medium M-9.

of tryptophan in the medium completely blocked the action of the antibiotic. Neither anthranilic acid nor indole was effective.

Because the biosynthesis of tryptophan has been shown to involve the condensation of indole and serine (Umbreit *et al.*, 1946) and because, on the basis of chemical structure, azaserine might be considered to be a substituted serine, the combination of indole and serine was tested. This combination was also ineffective in overcoming inhibition by azaserine.

With *E. coli* strain 83-5 that requires either phenylalanine or phenylpyruvic acid for growth, it was found that either of the two metabolites antagonized azaserine inhibition. On the other hand, with *E. coli* strain 83-8 that requires either tyrosine or *p*-hydroxyphenylpyruvic acid for growth, only the amino acid was capable of blocking the inhibitory effect of azaserine.

Another mutant, *E. coli* strain 83-1, requires for optimal growth the presence of tryptophan, tyrosine, phenylalanine, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid. Substitution of shikimic acid for these five compounds can satisfy the total growth requirement of this mutant (Davis, 1951). The inhibitory effect of azaserine was not overcome when this mutant was grown in shikimic acid, but was blocked completely when the five end products of shikimic acid metabolism were incorporated into the medium. These results confirm and extend previously reported observations (Kaplan and Stock, 1954).

In view of the blocking of azaserine inhibition by the aromatic amino acids, more extensive studies were carried out using liquid medium. Data shown in table 3 suggest that, in the presence of any one of the individual aromatic amino acids, the type of inhibition produced by azaserine appears to be noncompetitive. This is indicated by the lack of a constant inhibitor/metabolite ratio for an over-all 8-fold increase in the concentrations of the aromatic amino acids. Individually, phenylalanine and tyrosine are more effective than tryptophan as blocking agents. In other experiments inhibition produced by levels of azaserine of 0.28 μmoles or greater could not be overcome by phenylalanine (3.9 μmoles), tyrosine (2.1 μmoles), or tryptophan (2.0 μmoles) singly or in combination.

In order to obtain further information concerning the interference with azaserine inhibition by phenylalanine, viability determinations were carried out in conjunction with turbidimetric measurements. In a typical experiment, 10-ml portions of M-9 medium were inoculated with *E. coli* strain B and incubated at 37 C with shaking. When the cultures had reached the middle of their log phase, azaserine was added to one flask to give a final concentration of 0.1 μg per ml (flask C); to another flask was added L-phenylalanine to give 16.5 μg per ml (flask B); to another flask both azaserine and phenylalanine were added (flask D); a fourth flask acted as an unsupplemented control (flask A). Viability determinations and turbidimetric measurements were made prior to and subsequent to the various additions. The results of this experiment are shown in figure 1. It may be seen that although the turbidity continues to increase in those flasks to which azaserine (flask C) and azaserine plus phenylalanine (flask D) had been added, the viable counts of such cultures are markedly different. In the presence of azaserine alone the number of viable cells dropped from an initial count of 1.9×10^8 cells per ml to a count of 5.2×10^6 cells per ml after contact with the antibiotic for 30 min, whereas the control culture increased from 1.9×10^8 cells per ml to 2.4×10^8 cells per ml. When both azaserine and phenylalanine were present, no appre-

TABLE 3

Blocking by the aromatic amino acids of the inhibition of growth of Escherichia coli by azaserine

Aromatic Amino Acid	Azaserine Required for 50% Inhibition of Growth	I/M*
μmoles	*μmoles*	
L-Phenylalanine:		
0.15	0.032	1/4.7
0.32	0.054	1/5.3
0.60	0.10	1/6.0
1.20	0.09	1/13.3
L-Tyrosine:		
0.138	0.03	1/4.6
0.276	0.042	1/6.5
0.552	0.094	1/5.8
1.10	0.078	1/14.1
L-Tryptophan:		
0.12	0.026	1/4.6
0.24	0.031	1/7.7
0.48	0.035	1/13.7
0.96	0.073	1/13.1

* I/M = (μmoles azaserine to inhibit growth 50 per cent)/(μmoles aromatic amino acid added).

ciable change in cell count was detected over a period to 90 min.

It would appear that phenylalanine protects the cells against the bactericidal section of azaserine. This protective effect, however, is not manifested as an increase in the numbers of viable cells but merely as a condition of bacteriostasis. Moreover, the cytotoxic action of the antibiotic (Maxwell and Nickel, 1954) is not prevented by phenylalanine; microscopic examination of cells from flask D at the end of incubation revealed the presence of filamentous forms to approximately the same degree as in flask C. In view of these observations, the use of only turbidimetric measurement as an index of azaserine inhibition and relief of this inhibition by aromatic amino acids may lead to false interpretations regarding the competitive or noncompetitive nature of the inhibition. The lack of correlation between turbidity and viable cell counts has been demonstrated by Cohen and Barner (1956) in the inhibition of *E. coli* by 5-bromouracil.

Cell-free glutamic acid-tyrosine transaminase from E. coli. Evidence from a number of investigations demonstrates the requirement of pyridoxal phosphate as a coenzyme for the last stages of aromatic amino acid biosynthesis (Umbreit *et al.*, 1946; Feldman and Gunsalus, 1950; Holden *et al.*, 1951). Since *p*-hydroxyphenylpyruvic acid did not overcome azaserine inhibition, whereas tyrosine did, it was conceivable that the antibiotic interferes with the amination process at the coenzyme level. Accordingly, a preparation containing a glutamic acid-tyrosine transaminase was extracted from cells of *E. coli* strain W as described under Materials and Methods. The dialyzed fraction was found to be dependent upon an exogenous supply of pyridoxal phosphate for optimal activity. A concentration of 1.0 μg per ml of pyridoxal phosphate was required for maximum transaminase activity. The effect of azaserine upon this system was studied. When azaserine was added to the system either before the addition of pyridoxal phosphate, or simultaneously with the coenzyme, the synthesis of tyrosine was not inhibited. Thus, this observation coupled with the finding that phenylpyruvic acid blocks azaserine inhibition would appear to make it unlikely that the action of the antibiotic can be attributed to an inhibition of the coenzyme function of pyridoxal phosphate for aromatic amino acid synthesis.

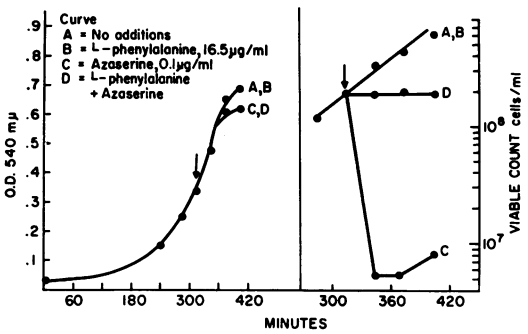

Figure 1. Effect of azaserine in the absence and presence of L-phenylalanine on culture turbidity and viability of *Escherichia coli* strain B. Arrow indicates time of additions.

Aminoimidazole carboxamide synthesis by nonproliferating cells of E. coli strain B-96. The inhibitory effect of azaserine on the synthesis of aminoimidazole carboxamide as well as the prevention of this inhibition by various aromatic acids is shown in table 4. It may be seen that phenylalanine, tyrosine, and phenylpyruvic acid were most active in overcoming this inhibition; *p*-hydroxyphenylpyruvic acid was completely inactive. In addition, the following amino acids did not overcome the inhibitory effect of azaserine in this system: glycine, β-alanine, DL-α-alanine, DL-serine, DL-threonine, DL-valine, DL-leucine, DL-isoleucine, DL-norleucine, L-arginine, L-lysine, L-glutamic acid, L-glutamine, and L-histidine. These results parallel the data obtained on the interference by the various aromatic acids with the inhibitory effect of azaserine on growing cells of *E. coli.*

In view of the blocking ability of β-2-thienylalanine in this system (Gots and Gollub, 1956*b*) the effects of a series of structural analogues of phenylalanine were investigated. In table 4 are shown the results that have been obtained with a series of halogenated phenylalanines. Although the halogenated derivatives have been shown to inhibit growth in various biological systems (Mitchell and Niemann, 1947), none of the compounds used in this study demonstrated any significant effect upon the synthesis of aminoimidazole carboxamide. All, however, with the exception of *o*-bromophenylalanine, exerted some blocking effect upon the activity of azaserine. The degree of blocking is a function not only of the size of the substituent on the aromatic ring but also of the position of the substituent

TABLE 4

Blocking activities by various aromatic and heterocyclic acids and halogenated phenylalanines on the inhibition by azaserine of the synthesis of aminoimidazole carboxamide

Supplement to Basal Mixture, 20 μg/ml*	Synthesis of Aminoimidazole Carboxamide Per Cent of Control	
	In absence of azaserine	In presence of 1.0 μg/ml azaserine
None	100	40
L-Phenylalanine	104	92
L-Tyrosine	104	92
Phenylpyruvic acid	102	80
DL-Phenyllactic acid	102	58
p-Hydroxyphenylpyruvic acid	100	40
β-3-DL-Thienylalanine	102	76
β-2-Thienylpyruvic acid	102	78
β-2-DL-Thienylalanine	100	92
β-2-DL-Furylalanine	100	68
β-2-DL-Thiazolylalanine	97	46
β-3-DL-Pyrazolylalanine	102	47
p-Fluoro-DL-phenylalanine	100	90
p-Chloro-DL-phenylalanlne	102	92
p-Bromo-DL-phenylalanine	100	85
m-Chloro-DL-phenylalanine	98	86
m-Bromo-DL-phenylalanlne	100	72
o-Fluoro-DL-phenylalanine	102	84
o-Chloro-DL-phenylalanine	98	70
o-Bromo-DL-phenylalanine	98	50

* Basal mixture contains 0.02 M glucose, 0.067 M phosphate buffer, pH 7.2, 0.1 per cent NH_4Cl, and 1.0 mg/ml *Escherichia coli* strain B-96.

relative to the alanine side chain (table 5). It may be seen that the substitution of a fluoro-, chloro-, or bromo- group in the *para* position of phenylalanine results in approximately the same degree of blocking of azaserine inhibition as phenylalanine itself. When the substitution occurs at the *ortho* position, the degree of protection is a function of the size of the substituent. Thus, the order of decreasing efficiency becomes hydrogen > fluoro > chloro > bromo.

The ability of various heterocyclic acids to antagonize the inhibition by azaserine is also indicated in table 4. Substitution of a thiophene ring for the benzene ring in phenylalanine caused no decrease in blocking ability whereas substitution with either a thiazole or pyrazole ring completely abolished activity.

TABLE 5

Relative blocking efficiency by various substituted phenylalanines on the inhibition by azaserine (AZS) of the synthesis of aminoimidazole carboxamide (AICA)

Position Substituted	Group Substituted				
	—H	—F	—Cl	—Br	—OH
Para	1.0*	0.96	1.0	0.92	1.0
Meta	1.0		0.92	0.80	
Ortho	1.0	0.90	0.76	0.54	

* Relative activity = (AICA synthesized in presence of AZS and substituted phenylalanines)/(AICA synthesized in presence of AZS and phenylalanine).

0.52 = (AICA synthesized in presence of AZS)/(AICA synthesized in presence of AZS and phenylalanine).

The effects of alterations in the side chain of phenylalanine have revealed that although substitution of a carbonyl for the amino group causes only a slight decrease in blocking activity, replacement by either a methyl or a chloroacetyl radical results in a completely inactive compound. Introduction of a hydroxyl group at the β-carbon atom also results in loss of activity. Decrease in the length of the side chain by the loss of —CH_2— as in phenylglycine, or an increase by the insertion of either —CH_2—, (α-amino-γ-phenyl-*n*-butyric acid) or —O— (β-phenoxy-α-aminopropionic acid) abolishes activity.

Inversion of the amino group to give the D- isomer of phenylalanine does not destroy the blocking ability although this compound is only one half as efficient as the L- isomer.

From these results it would appear that the ability of structural analogues of phenylalanine to block the inhibitory effect of azaserine in this system is dependent upon several factors: (a) The side chain must be three carbons in length. (b) The α- position may be occupied by either an amino, a carbonyl, or a hydroxyl group. The amino group must be unsubstituted. (c) The β- carbon must be unsubstituted. (d) The benzene ring may be replaced by either a thiophene or furan ring but not by a thiazole or pyrazole ring structure. (e) Substitution by any of the halogens may be made at the *para*, *meta*, or *ortho* position on the benzene ring, but the efficiency

of *meta* or *ortho* substituted derivatives is dependent upon the size of the halogen.

DISCUSSION

It is apparent from the data that, of the compounds tested, the aromatic amino acids are the most effective in blocking the inhibition by azaserine of the growth of *E. coli*. Furthermore, the ability of these compounds to block the action of azaserine is not restricted to an actively growing system. Azaserine has been shown to be a potent inhibitor of the pathway of purine biosynthesis by nonproliferating cells of *E. coli* strain B-96. Again, the aromatic amino acids were best able to prevent this inhibition (Gots and Gollub, 1956*b*). The inhibitory effect of azaserine upon the induced synthesis of an adaptive enzyme by nongrowing yeast cells was found to be antagonized most effectively by leucine, phenylalanine, and tyrosine (Halvorson, 1954). The ability of azaserine to cause the induction of phage formation in the lysogenic *E. coli* strain K-12 is prevented by the aromatic amino acids (Bird and Gots, 1956). Consequently, it is evident that any proposal of a mechanism of action of azaserine must take into account the observations that the same amino acids that are effective as blocking agents in growing systems are equally effective in nongrowing systems.

Experiments designed to test the hypothesis that azaserine interferes with the utilization of the aromatic amino acids have yielded data the interpretation of which is questionable. This is a result of the observation that in liquid medium the growth of *E. coli* is abnormal whenever azaserine is present. Microscopic examination revealed the presence of greatly elongated filaments. Similar observations have been noted by Maxwell and Nickel (1954). Furthermore, these morphological changes were not influenced by the presence of the aromatic amino acids. Therefore, the turbidity of such treated cultures, as determined by optical density readings, may not reflect a true index of inhibition of growth as well as blockage of this inhibition. From structural considerations, however, it would be difficult to assign to azaserine the role of a competitive inhibitor of the three aromatic amino acids; a structural relationship usually has been found to be associated with competitive antagonism (Woods, 1940; Shive, 1950).

Alternatively, attempts to demonstrate an inhibition by azaserine with aromatic amino acid biosynthesis either at the coenzyme level or at the common biochemical precursor level have been unsuccessful. Furthermore, since the antibiotic is as effective against synthetically incompetent cells as it is against synthetically competent ones, then its site of action does not lie in the pathway of aromatic amino acid biosynthesis. This would be in accord with the view expressed by Gale (1952). Further evidence which would tend to rule out a direct interference by azaserine with aromatic amino acid biosynthesis stems from the finding that a number of phenylalanine derivatives are as efficient as phenylalanine in blocking azaserine inhibition of aminoimidazole carboxamide synthesis. Such would not be the case if the synthesis of the aromatic amino acids were a prerequisite for purine biosynthesis. Moreover, Gots (1955) has reported that if a secondary mutation is induced in *E. coli* that results in a growth requirement for tryptophan, the synthesis of aminoimidazole carboxamide by these cells is not affected.

It is suggested, therefore, that the ability of the aromatic amino acids to antagonize azaserine action in *E. coli* may be due to a nonspecific effect and is not, of necessity, related to the addition of metabolic end products of an inhibited reaction.

The results of this investigation may be interpreted by proposing that the penetration of the antibiotic through the cell membrane or the binding of azaserine by a cell wall component is prevented in some manner by the aromatic amino acids. This proposal would also explain the ability of the various heterocyclic acids to overcome the action of azaserine. The equivalence of the benzene, thiophene, and furan rings has been noted for a number of systems. Thus, Jacquez and Mottram (1953) found that β-2-thienylalanine, β-2-furylalanine, and *p*-fluorophenylalanine could serve as amino donors in transamination reactions with phenylpyruvic acid using heart tissue growing in tissue culture. Griffen *et al.* (1953) found that β-2-thienylalanine and D-phenylalanine would substitute for L-phenylalanine as substrates in the production of a melano-pigment by cells of *Bacterium salmonicida*.

Evidence that azaserine is under the control of an amino acid active transport system has been demonstrated by Jacquez (1957). Azaserine was found to be more actively concentrated than the "normal" amino acids by Ehrlich ascites cells *in vitro*. Furthermore, the concentrative uptake of

azaserine was antagonized by tryptophan, glutamine, and glycine.

Recently, Gots and Gollub (1956*a*) have succeeded in obtaining a cell-free extract from *E. coli* which is capable of synthesizing the ribotide of aminoimidazole carboxamide. By analogy with the investigations performed with a similar system from pigeon liver (Levenberg *et al.*, 1957), it might be anticipated that azaserine would be a potent inhibitor in this bacterial system. It may be equally anticipated that, if our proposal is correct, the aromatic amino acids would be ineffective as blocking agents since the integrity of the cell has been destroyed.

SUMMARY

Azaserine has been found to inhibit markedly the growth of *Escherichia coli*. At submaximal levels, the antibiotic apparently inhibits the cell division mechanism; this results in the formation of filamentous forms of the microorganism. The aromatic amino acids were able to block partially the growth inhibitory action of the antibiotic but not its effect upon cell division.

Azaserine was found to inhibit also the synthesis of aminoimidazole carboxamide by nonproliferating cell suspensions of *E. coli* strain B-96. This inhibition was blocked not only by tyrosine and phenylalanine but by a number of aromatic and heterocyclic acids. The structural requirements necessary for a compound to be active in overcoming this inhibitory effect of azaserine have been determined.

A proposal is given by which the results of this investigation may be interpreted.

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