Mechanism of Adenine Inhibition in Adenine-Sensitive Mutants of *Salmonella typhimurium*

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ABSTRACT

DALAL, FRAM R. (University of Pennsylvania, Philadelphia), RONALD E. GOTS, AND JOSEPH S. GOTS. Mechanism of adenine inhibition in adenine-sensitive mutants of *Salmonella typhimurium*. J. Bacteriol. 91: 507-513. 1966.—The inhibition of growth of *Salmonella typhimurium* by adenine was studied with three adenine-sensitive mutants. These mutants were acutely sensitive to inhibition by adenine, were prototrophic in their growth requirements, and represented mutational events in three different genetic loci. In all cases, inhibition by adenine was relieved noncompetitively by thiamine (or its pyrimidine moiety), pantothenate (or its pantoyl moiety), and methionine alone or, more efficiently, in the presence of lysine. Kinetics of reversal indicated that adenine inhibited the synthesis of the reversing agents, probably at the level of a common factor required for their syntheses, such as the folic acid coenzymes. Support for this inference has been found by the facts that one of the mutants was identified as a partial auxotroph for *p*-aminobenzoic acid, and sulfadiazine could sensitize the wild type to acute inhibition by adenine.

Mutant strains of *Salmonella typhimurium* have been found whose growth is acutely inhibited by minute amounts of adenine. Those studied here are prototrophic, but others have been found among mutants which are auxotrophic for purines (20). In all cases, the inhibition creates a growth requirement for thiamine. The prevention of adenine bacteriostasis by thiamine was observed in *Aerobacter aerogenes* by Brooke and Magasanik (1) and in *Salmonella* by Kalle and Gots (11). Moyed (14) later attributed the growth inhibition in *Aerobacter* to a specific inhibition of the synthesis of the pyrimidine moiety of thiamine. Results presented in this paper indicate that this cannot be the sole explanation for the type of inhibition found with the *Salmonella* mutants, since other compounds have been found, including pantothenic acid and methionine, which are as effective as thiamine in relieving growth inhibition by adenine. A possible common denominator which could link these various reversing agents under unit control may be the folic acid coenzymes which mediate single-carbon transfers. All of these reversing agents are products of such transfer.

It has been known for several years that folic acid deficiencies, created either by mutation

(e.g., in a *p*-aminobenzoic acid auxotroph) or by the action of antimetabolites (such as sulfonamides), can sensitize *Escherichia coli* to inhibition by adenine (8, 12). A similar sensitization to adenine inhibition has been demonstrated in *S. typhimurium*, and this inhibition is relieved by the very same end products, thus providing further support for the inference that adenine is involved as an inhibitor in some stage of folic acid metabolism.

MATERIALS AND METHODS

Bacterial strains. Seven mutant strains of *S. typhimurium*, capable of growth in a minimal salts-glucose medium but acutely inhibited by adenine, were obtained from the collection of M. Demerec. These were isolated during routine screening for auxotrophic mutants. Only three of these strains (*ads-2*, *ads-4*, and *ads-5*) remained sufficiently stable for further study. The others showed a high rate of mutation to adenine resistance. Mutants *ads-4* and *ads-5* were derived from the wild type, strain LT-2, by treatment with 2-aminopurine, and mutant *ads-2*, by treatment with diethylsulfate.

Growth studies. The organisms were incubated at 37 C, with shaking, in Klett tubes containing 5 ml of salts-glucose medium E of Vogel and Bonner (19), with appropriate supplements. Growth was measured

turbidimetrically in a Klett-Summerson photoelectric colorimeter with a green filter (no. 54). At the end of each incubation period, all tubes showing growth were routinely streaked on minimal agar containing adenine (10 $\mu\text{g}/\text{ml}$), to check for the presence of adenine-resistant mutants that may have arisen during the incubation period. Very occasionally, mutation to adenine resistance was observed, particularly when incubation was extended to 36 hr. When this happened, the results were discarded and the experiment was repeated. The results presented are those in which no such mutations were found and which were consistently reproducible. The period of incubation and size of inoculum are indicated in the legends to tables and figures. All chemicals used to supplement the media were obtained from commercial sources.

Transduction studies. These experiments were carried out with the transducing phage, PLT-22 (HI). Production and assay of donor phage lysates followed standard procedures (20). Transductions were routinely performed by infecting recipient bacteria in nutrient broth with the donor phage at a multiplicity of 10 phages per bacterium. After an adsorption period of 10 min, 0.1-ml samples of the mixture were plated on minimal glucose-agar containing adenine (20 $\mu\text{g}/\text{ml}$). Transductants were scored after 48 hr of incubation.

Incorporation of adenine-8- C^{14} . Radioactive incorporation of adenine-8- C^{14} by logarithmically proliferating cells of LT-2 and its adenine-sensitive mutants was studied as described previously (10). The growth medium contained 40 $\mu\text{g}/\text{ml}$ of adenine. The specific activity of adenine-8- C^{14} in the medium was 0.024 $\mu\text{C}/\mu\text{mole}$. Thiamine, when included, was added at a concentration of 5 $\mu\text{g}/\text{ml}$.

RESULTS

Inhibition by purines. Of the purines tested, adenine was the most efficient in inhibiting growth of the *ads* mutants (Table 1). Hypoxanthine was a better inhibitor than either guanosine or xanthine for strains *ads-2* and *ads-5*, but strain *ads-4* was not inhibited by any of these purines under the conditions of the experiment. Adenosine was also a good inhibitor but required twice the molar concentration of adenine to give a comparable degree of inhibition. The size of inoculum was very critical in this and subsequent experiments; if great care was not maintained in its regulation, large variations were observed. For instance, the adenine sulfate concentration for 50% inhibition of the wild-type strain with an initial inoculum of 10^4 cells per milliliter was 20 $\mu\text{g}/\text{ml}$, and no inhibition was observed up to 500 $\mu\text{g}/\text{ml}$ with an initial inoculum of 10^6 cells per milliliter.

Transduction analysis. Table 2 gives the results of reciprocal transduction experiments designed for the recovery of adenine-resistant recombinants. In all cases, the *ads* mutants were transduced readily to the wild-type level of adenine

TABLE 1. Inhibition of *Salmonella typhimurium* LT-2 and *ads* mutants by purines*

Purine	Amount required for 50% inhibition ($\mu\text{g}/\text{ml}$)			
	LT-2	<i>ads-2</i>	<i>ads-4</i>	<i>ads-5</i>
Adenine.....	200	0.4	3.5	0.4
Guanosine.....	> 500	40	> 500	10
Hypoxanthine...	> 500	6	> 500	5
Xanthine.....	> 500	75	> 500	15
Diaminopurine...	200	100	200	150

* The inoculum was 10^6 cells per milliliter, and the period of incubation was 18 hr. (Guanosine was used instead of guanine because of solubility problems at higher concentrations.)

TABLE 2. Reciprocal transductions with *ads* mutants*

Recipient	Donor					
	WT	<i>ads-2</i>	<i>ads-4</i>	<i>ads-5</i>	<i>purC-305</i>	<i>purD-312</i>
<i>ads-2</i>	41	0	40	59	36	42
<i>ads-4</i>	424	234	0	315	318	106
<i>ads-5</i>	550	289	78	0	170	85
<i>purC-305</i>	380	340	231	227	0	134
<i>purD-312</i>	320	370	127	330	390	0

* Number of adenine-resistant colonies appearing on minimal medium containing adenine (20 $\mu\text{g}/\text{ml}$) after transduction procedure as described in text. The numbers are averages for three plates, and have been corrected for non-transduced controls. WT = wild type.

resistance. Results are also given for transductions with phage grown on the wild type as donor and with other representative mutants as recipients. These serve to show that there are no unusual properties of the phage preparations with respect to transducing potentials, or of the recipient cells with respect to acceptor capacities. Transduction of *ads-2* was less efficient with all donors. This could be due to either the terminal location of the gene on the transducing fragment, thus decreasing the probability of its incorporation, or to a high degree of dominance of the sensitive allele, which would result in a lag in the phenotypic expression of the recombinants. The results also show that the three *ads* mutants are apparently nonallelic, and thus represent mutational events in three different nonlinked functional groups. Comparison was also made with a series of purine auxotrophs having a pleiotropic additional requirement for thiamine (20). The results indicate that these are not allelic forms of

the *ads* mutants (see *purD* and *purC* mutants in Table 2).

Reversal of adenine inhibition. In preliminary auxanographic tests, carried out on agar medium, vitamin-free casein hydrolysate was found to reverse adenine inhibition in all of the *ads* mutants. When the constituent amino acids of casein hydrolysate were tested individually, methionine alone was found to be partially effective, but complete reversal was obtained with a mixture of methionine and lysine. Besides casein hydrolysate, several vitamins were also tested; of these, thiamine, calcium pantothenate, and, in the case of *ads-5*, *p*-aminobenzoic acid were found to permit growth in the presence of adenine. These results were confirmed in liquid medium (Table 3). The pyrimidine moiety of thiamine (2-methyl-4-amino-5-hydroxymethylpyrimidine) could replace thiamine to varying extents in the three mutants, whereas the thiazole moiety (4-methyl-5-beta-hydroxyethylthiazole) had no effect in any case. Similarly, the pantoil moiety of pantothenic acid, and not the β -alanine moiety, was found to be active (Table 3). *p*-Aminobenzoic acid reversed adenine inhibition in strain *ads-5* and, in fact, enhanced growth even in the absence of adenine. This relative requirement for *p*-aminobenzoic acid indicates that strain *ads-5* is a "leaky" *p*-aminobenzoic acid auxotroph. This was confirmed by the fact that, unlike the other two

TABLE 4. *Reversal of adenine inhibition in ads mutants of Salmonella typhimurium by certain amino acids**

Adenine ug/ml	Other additions (20 μ g/ml)	Growth of mutants		
		<i>ads-2</i>	<i>ads-4</i>	<i>ads-5</i>
0	None	100	100	100
20	None	0	0	0
20	Glycine	(20)	0	0
20	L-Methionine	(80)	(80)	100
20	L-Lysine	0	0	0
20	L-Methionine + L-lysine	100	100	130
20	DL-Homocysteine	0	0	0
20	DL-Homocysteine + L-lysine	(80)	(70)	(90)
20	Cystathionine	0	0	0
20	Cystathionine + L-lysine	(40)	(70)	(60)
20	DL-Homoserine	0	0	0
20	DL-Homoserine + L-lysine	0	0	0
20	α, ϵ -Diaminopimelic acid	0	(10)	(20)
20	α, ϵ -Diaminopimelic acid + L-methio- nine	(100)	(90)	100

* Conditions are the same as given in Table 3. The cystathionine used was a mixture of L and L-allo forms.

TABLE 3. *Reversal of adenine inhibition in ads mutants of Salmonella typhimurium by certain vitamins and their moieties**

Adenine μ g/ml	Other addition (2 μ g/ml)	Growth of mutants		
		<i>ads-2</i>	<i>ads-4</i>	<i>ads-5</i>
0	None	100	100	100
0	<i>p</i> -Aminobenzoic acid	100	100	130
20	None	0	0	0
20	<i>p</i> -Aminobenzoic acid	0	0	130
20	Thiamine	90	100	110
20	B ₁ -pyrimidine	100	(70)	(40)
20	B ₁ -thiazole	0	0	0
20	Calcium panto- thenate	100	100	100
20	Pantoil lactone	(70)	(50)	(70)
20	β -Alanine	0	0	0

* The inoculum was 5×10^6 cells per milliliter, and the period of incubation was 20 hr; parentheses indicate that growth was negligible at 20 hr, but reached the level indicated after 36 hr. Results are expressed in terms of percentages of the growth obtained in minimal medium.

mutants, *ads-5* accumulated substantial amounts of a nonacetyltable, diazotizable arylamine which behaved like the riboside of 4-amino 5-imidazole carboxamide on chromatograms and also showed increased accumulation in the presence of glycine and glutamic acid (7).

Known precursors of methionine could not reverse adenine inhibition by themselves (Table 4), but, in the presence of lysine, both homocysteine and cystathionine were effective, though to a lesser extent than methionine. Homoserine had no effect, even in the presence of lysine. α, ϵ -Diaminopimelic acid, a precursor of lysine, had only a very slight effect, either by itself or with methionine. Thymidine, another product of one-carbon transfer, had no effect on adenine inhibition, either by itself or in the presence of sub-optimal concentrations of thiamine.

A more detailed analysis was made by studying the effect of various concentrations of adenine on the growth response of mutant *ads-2* to thiamine, calcium pantothenate, and methionine in the absence or presence of lysine. The dose response to thiamine (Fig. 1) was linear at all levels of adenine concentrations, though increasing concentrations decreased the slope of the

FIG. 1. Dose response to thiamine of *Salmonella typhimurium* strain *ads-2* in the presence of adenine. The figures on the curves represent micrograms per milliliter of adenine. The inoculum was 10^5 cells per milliliter, and the period of incubation was 18 hr.

curves. A maximal effect was reached at 25 $\mu\text{g/ml}$, where the thiamine requirement for half-maximal growth was 2 $\text{m}\mu\text{g/ml}$. At a sufficiently high level of thiamine (5 $\text{m}\mu\text{g/ml}$), however, growth reversal of inhibition was complete, irrespective of the level of adenine (up to 250 $\mu\text{g/ml}$). This is the pattern that would be expected for a noncompetitive antagonist, indicating that adenine inhibits the synthesis of thiamine or, more likely, its pyrimidine moiety. On the other hand, with calcium pantothenate (Fig. 2), not only the slope, but also the maximal growth obtained, decreased with increasing concentrations of adenine. Again, the absence of a horizontal shift in the dose-response curve indicates that adenine also inhibits the synthesis of pantothenate (or its pantoic moiety), and the decrease in plateau levels with increasing adenine concentrations means that an additional limiting factor, probably thiamine synthesis, is imposed. That the effects of thiamine and calcium pantothenate are synergistic can be seen from Table 5.

Dose-response curves for methionine (in the presence of 20 $\mu\text{g/ml}$ of lysine) were similar to those for thiamine; the maximal effect of adenine was reached at 25 $\mu\text{g/ml}$, and concentrations above this did not alter the curve. At this level, the requirement of methionine for half-maximal growth was 7 $\mu\text{g/ml}$. In the absence of lysine, it

FIG. 2. Dose response to calcium pantothenate of *Salmonella typhimurium* strain *ads-2* in the presence of adenine. Conditions as in Fig. 1.

TABLE 5. Combined effect of thiamine and calcium pantothenate in reversing adenine inhibition in *Salmonella typhimurium* strain *ads-2**

Adenine $\mu\text{g/ml}$	Other additions (amt, $\text{m}\mu\text{g/ml}$)	Growth (Klett units)
0	None	300
50	None	0
50	Thiamine (1)	75
50	Ca pantothenate (250)	31
50	Ca pantothenate (1,000)	31
50	Ca pantothenate (5,000)	46
50	Ca pantothenate (250) + thiamine (1)	132
50	Ca pantothenate (1,000) + thiamine (1)	217
50	Ca pantothenate (5,000) + thiamine (1)	217

* The inoculum was 10^5 cells per milliliter, and the period of incubation was 18 hr.

took 48 hr instead of 20 hr to obtain essentially the same growth response.

The noncompetitive nature of the antagonisms also serves to rule out the possibility that the antagonists might be operating at the permease level to prevent adenine from entering the cell. The kinetics and concentrations involved would not fit such an interpretation. At any rate, an experiment was run to check this point. The uptake of adenine-8- C^{14} by logarithmically growing suspensions of *ads-2*, *ads-4*, and *ads-5* was found to be unaffected by thiamine at 1,000 times the

TABLE 6. Sensitization of *Salmonella typhimurium* LT-2 to adenine inhibition by sodium sulfadiazine and the reversal of this effect*

Adenine $\mu\text{g/ml}$	Na sulfadiazine $\mu\text{g/ml}$	Growth (Klett units) in the presence of					
		No other addition	Thiamine (2 $\mu\text{g/ml}$)	L-Methionine (20 $\mu\text{g/ml}$)	L-Methionine + L-lysine (20 $\mu\text{g/ml}$, each)	Ca pantothenate (2 $\mu\text{g/ml}$)	<i>p</i> -Aminobenzoic acid (2 $\mu\text{g/ml}$)
0	10	0 (0)	12 (125)	21 (270)	21 (258)	0 (54)	274 (325)
200	0	0 (155)	286 (320)	224 (300)	290 (354)	298 (354)	7 (175)
10	3	0 (0)	22 (232)	68 (274)	254 (320)	25 (248)	230 (282)
10	0	238 (285)					
0	3	157 (290)					
0	0	295 (350)					

* The inoculum was 10^8 cells per milliliter, and the incubation was for 18 and 24 hr; the latter figures are given in parentheses.

concentration required to effectively prevent inhibition, and proceeded at essentially the same rate (between 2,300 and 2,800 counts per minute per unit increase in optical density at 540 $m\mu$) as the uptake by the wild-type strain LT-2, either in the absence of thiamine or in its presence.

Sensitization of strain LT-2 to adenine inhibition by sulfadiazine. Sodium sulfadiazine inhibits growth of the wild type and the *ads-2* and *ads-4* mutants to about the same extent (3.0 to 4.5 $\mu\text{g/ml}$ of sulfadiazine for 50% inhibition). Mutant *ads-5* is far more sensitive to inhibition by sulfadiazine (50% inhibition at 0.07 $\mu\text{g/ml}$), as would be expected of a leaky *p*-aminobenzoic acid auxotroph. If the locus of the inhibitory effect of adenine is indeed at some stage in folic acid metabolism, then sodium sulfadiazine, by interfering with *p*-aminobenzoate utilization and hence the synthesis of the folate compounds, should greatly increase the sensitivity of wild-type *S. typhimurium* to inhibition by adenine. This was indeed found to be the case (Table 6). Moreover, the same compounds that relieve adenine inhibition of the *ads* mutants were also effective against inhibition in the wild type, whether produced by a high concentration of adenine (200 $\mu\text{g/ml}$) by itself or by a low concentration of adenine (10 $\mu\text{g/ml}$) in the presence of suboptimal levels of sodium sulfadiazine (3 $\mu\text{g/ml}$). *p*-Aminobenzoic acid had no effect on inhibition by adenine alone, but it effectively prevented sulfadiazine from sensitizing. The inhibition produced by sulfadiazine by itself at higher concentrations was completely reversed only by *p*-aminobenzoic acid; the other compounds acted more slowly and to a lesser extent.

DISCUSSION

The results show that *S. typhimurium* can be acutely sensitized to inhibition by adenine by at

least three different genetic alterations. The facts that one of these mutations apparently involves an impairment in the synthesis of *p*-aminobenzoic acid and that sulfonamides can create a similar phenotypic sensitization of the wild type serve to focus the site of inhibition at the level of folic acid metabolism. Further evidence that the inhibitory effect of adenine is due to its action at some stage in the formation or the interconversion and utilization of folic acid coenzymes is indicated by the fact that all major compounds capable of reversing adenine inhibition are either products of single-carbon transfer or, as in the case of *p*-aminobenzoic acid, are involved in the synthesis of folic acid. Studies under current investigation in this laboratory indicate a similar pattern with adenine-sensitive mutants which are auxotrophic for purines.

Formate- C^{14} has been shown to be incorporated into the pyrimidine moiety of thiamine (4, 6, 15), presumably into its 2-methyl or 5-hydroxymethyl group, through the mediation of folic acid coenzymes. It is the pyrimidine rather than the thiazole moiety of thiamine that counteracts the inhibitions studied here. Similarly, *p*-aminobenzoate is required for the conversion of γ -ketoisovalerate to ketopantoate (16), which is subsequently converted to pantoate, the portion of the pantothenate molecule responsible for anti-adenine activity. The involvement of 5-methyl-tetrahydrofolate in the methylation of homocysteine to methionine is a well-established fact (13). The effect of lysine cannot be so readily explained as a product of single-carbon transfers. However, succinyl-coenzyme A (CoA) is involved in both lysine (5) and methionine (17) syntheses, and a deficiency in pantothenate might indirectly affect the syntheses of these amino acids by creating a deficiency in CoA; this could be partially relieved by exogenous lysine. A similar sparing

effect on the drain of CoA could explain the effect of homocysteine and cystathionine in the presence of lysine, since succinyl-CoA is involved in the conversion of homoserine to *o*-succinyl-homoserine (17) and thence to cystathionine and homocysteine. As homoserine is formed prior to the step involving succinyl-CoA action, it should not show any effect, and this was the case. It must be assumed that at no stage is there a complete absence of CoA, and hence relieving the shortage makes it available for other metabolic reactions. Of interest, in this respect, is the report by Brooke (Abstr. IV Intern. Congr. Biochem., p. 124) that succinic acid could relieve adenine inhibition in *Aerobacter*. Our own experience and that of Moyed (14) with succinic acid as a reversing agent has been erratic and nonreproducible.

The data can be interpreted by assuming that adenine, in some way, reduces the pool of available folic acid coenzymes. Under normal conditions, with the wild-type organism, there would be a sufficiently high level so that adenine could not reduce the level critically except at relatively high concentrations. In the adenine-sensitive mutants, although the genetic lesions occur at different sites, to decrease *p*-aminobenzoate synthesis in strain *ads-5* and perhaps to affect pteridine synthesis or folic acid metabolism in strains *ads-2* and *ads-4*, the net result in all cases would be a reduction in the available folate compounds to a level just sufficient to permit growth under prototrophic conditions. If adenine then lowers the level further, growth would be prevented, and any product of a folate-mediated reaction would relieve this inhibition by a sparing effect on the available single-carbon units. Sulfadiazine at subinhibitory concentrations would similarly lower the pool of available folate coenzymes in the wild type, and would sensitize it to acute inhibition by adenine.

Studies on sulfonamide inhibition in *E. coli* (8, 18) and *p*-aminobenzoic acid-requiring organisms (12) have indicated that methionine is the first limiting factor in a *p*-aminobenzoic acid-deficient system. In adenine inhibition, the major limiting factor seems to be thiamine. If sufficient thiamine is provided, there is a complete reversal of inhibition irrespective of adenine concentration. This is not so in the case of pantothenate, at least with a small initial inoculum. This greater effect of thiamine may simply be due to the fact that the reaction involving one-carbon transfer in the biosynthesis of the pyrimidine moiety has a low substrate affinity, thus requiring a large number of folate coenzymes for the formation of a relatively small amount of thiamine. This could serve as an efficient control mechanism, regulating the formation of the vitamin at the very

low concentration normally required by the organism. If this were so, an optimal supply of exogenous thiamine could make enough folate coenzymes available for the other reactions. It is also possible that adenine is involved at some stage in the complex series of reactions interconverting the tetrahydrofolate derivatives (2, 9), in some way inhibiting formation of a particular one-carbon tetrahydrofolate derivative specifically required for thiamine formation. A complete picture obviously cannot be obtained until the exact site of action of adenine is determined. At least, in the synthesis of pteridines from guanosine triphosphate, the *ads* mutants exhibit no apparent deficiencies (3). Further enzymatic studies on both the wild-type and the adenine-sensitive strains are in progress.

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