

## Defective Guanine Uptake in an 8-Azaguanine-resistant Mutant of *Salmonella typhimurium*

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An 8-azaguanine-resistant mutant, *azg-11*, derived from a guanine auxotroph, *gua-1*, of *Salmonella typhimurium* was isolated. This mutant was resistant to the analogue when grown on 2,6-diaminopurine, but showed greater susceptibility than the parent on guanine. Studies with the uptake of radioactive purines revealed that the mutant was defective in a mechanism for incorporation of guanine as well as of xanthine. Initial rates of uptake were determined for guanine at concentrations which were sufficiently low to make permeases limiting. The affinity constant  $K_m$  for the mutant was found to be  $2.5 \times 10^{-4}$  M; that of the parent was  $2.3 \times 10^{-5}$  M. Examination of cell-free extracts suggested that the purine nucleotide pyrophosphorylases, responsible for the conversion of free intracellular purines to the corresponding nucleotides, were present and unaltered. The results indicate that the mutant is defective in a mechanism for the active transport for guanine and possibly xanthine.

Genetic alteration leading to the loss of purine nucleotide pyrophosphorylases appears to be the most prevalent mechanism for resistance to purine analogues in bacteria and neoplastic cells (4, 19). A consequence of such a phenotypic change is the concomitant loss in the ability of the mutants to utilize purine bases for cellular growth (4, 15). These enzymes may play a primary role in the uptake of exogenous purines, or there may be other enzymes (for instance, permeases) which are important. Evidence for active transport of purines in bacteria is lacking, although it has been shown in yeast, fungi, and algae (8, 10, 21).

In the present paper, a mutant of *Salmonella typhimurium* is described in which the reduced uptake of guanine appears to be associated with the initial transport of the purine into the cells, with no alteration in purine nucleotide pyrophosphorylases.

### MATERIALS AND METHODS

**Bacterial strains.** *S. typhimurium* LT-2 and its guanine-requiring auxotroph have been described (14). 8-Azaguanine (AzG)-resistant mutant *azg-11* was isolated from *gua-1* as follows. The *gua-1* strain was grown overnight in minimal medium containing guanine (20  $\mu$ g/ml), washed, resuspended in saline to a cell concentration of  $10^9$  per ml, and irradiated with ultraviolet light at 2,537 Å (450 ergs/mm<sup>2</sup>). The culture was diluted 1:100 in fresh medium containing 20  $\mu$ g per ml of 2,6-diaminopurine (DAP) and grown with aeration for about 4 hr at 37 C. At this time,

AzG (50  $\mu$ g/ml) was added and the culture was incubated overnight. Samples were plated on minimal agar plates containing DAP (20  $\mu$ g/ml) and AzG (200  $\mu$ g/ml). Colonies appearing within 48 hr were tested for growth requirement and resistance. A revertant from this mutant was isolated by plating about  $10^8$  cells on minimal agar plates in the absence of any purine.

**Media.** Prototrophic strains LT-2 and *azg-11-R<sub>A</sub>* were routinely grown in minimal salts-glucose medium E (23). For growth of auxotrophic strains *gua-1* and *azg-11*, medium E was supplemented with guanine (20  $\mu$ g/ml). The E buffer used for washing the cells consisted of the above medium without glucose or purine.

**Growth studies.** Preparation of cultures, conditions of growth, and method of studying antagonism between guanine and its analogues were the same as reported earlier (14). Inocula consisted of freshly prepared cells grown to the logarithmic phase; they were then washed and resuspended in chilled E buffer. In all of the experiments, growth was stopped by chilling and adding 0.03% potassium cyanide to the sample. Growth was measured turbidimetrically at 540 m $\mu$  in a Spectronic-20 colorimeter.

**Incorporation of radioactive purines.** An overnight culture was diluted 1:20 in medium E and incubated with shaking for 2 hr at 37 C. Cells were harvested, washed twice in chilled E buffer, and transferred to fresh medium containing radioactive purines. In studies with auxotrophs, cells were starved for guanine (before transfer to the radioactive medium) by incubation without purine for 1 hr with constant agitation; 1-ml samples were removed at intervals during uptake for determination of the radioactivity in the whole cells and the trichloroacetic acid-insoluble fraction. Assays were carried by the membrane filtration technique (14, 15). Filter discs were placed in vials containing

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the scintillation fluid (10 ml of 0.4% 2,5 bis-[2-(5-*t*-butyl benzoxazolyl)] thiophene in toluene and 2 ml of ethyl alcohol). After 30 min at room temperature, the vials were counted in a liquid scintillation spectrometer (Packard Instrument Co., Inc., Downer's Grove, Ill.). Counting efficiency in these experiments was about 58 to 60%.

**Enzyme assays.** Preparation of cell-free extracts and the assay of purine nucleotide pyrophosphorylase were the same as reported earlier (13). Extracts were incubated in 0.66  $\mu$  purine, 0.6  $\mu$ M 5-phosphoribosyl-1-pyrophosphate (PRPP), 10  $\mu$ M phosphate (pH 7.5), and 6.4  $\mu$ M MgCl<sub>2</sub>·7H<sub>2</sub>O for 30 min at 37 C. The reaction was stopped by heating, and purine-dependent disappearance of PRPP was estimated in the reaction mixture after adsorbing the nucleotide on charcoal. To facilitate sedimentation of charcoal particles during washing, 0.1 ml of 95% ethyl alcohol was added per 2 ml of sample (7). All assays were checked by isolating and identifying the corresponding nucleotide by paper chromatography with Carter's solvent (5). Activity with AzG and 6-mercaptapurine (MP) was measured in 0.03 M sodium acetate buffer (pH 7.2) instead of phosphates (6). Protein in the extracts was estimated by the method of Lowry et al. (17).

**Chemicals.** All chemicals were obtained from commercial sources. Adenine-8-<sup>14</sup>C, guanine-8-<sup>14</sup>C, and xanthine-8-<sup>14</sup>C were prepared by the Isotope Division of Bhabha Atomic Research Centre, Bombay, India. Hypoxanthine-8-<sup>14</sup>C was purchased from the New England Nuclear Corp., Boston, Mass.

## RESULTS

### Effect of AzG on the growth of mutant *azg-11*.

Parent strain *gua-1* was inhibited by AzG only when grown on DAP. The mutant under similar conditions showed more than 200-fold higher resistance to the inhibitor (Table 1); it was inhibited by MP, but showed a small degree of resistance to 6-thioguanine (TG). When the same mutant was grown on guanine as a source of

TABLE 1. Cross-resistance to structural analogues of guanine and hypoxanthine<sup>a</sup>

Strain	Purine in medium	Concn $\mu$ g/ml	Analogue concn for 50% inhibition ( $\mu$ mole/ml)		
			AzG	MP	TG
<i>gua-1</i>	DAP	20	0.016	0.184	1.50
	Guanine	5	>3.3		
<i>azg-11</i>	DAP	20	>3.3	0.160	2.27
	Guanine	5	0.650		
LT-2			0.013	0.200	0.93
	DAP	40	0.065		
<i>azg-11-R<sub>A</sub></i>	Guanine	2	>3.3		
			>3.3	0.212	1.59
	DAP	20	>3.3		
	Guanine	2	>3.3		

<sup>a</sup> Growth was determined after 48 hr.

purine, its growth was inhibited by AzG. This was a surprising finding since the parent strain was not inhibited under these conditions (Fig. 1). Thus, the mutant showed resistance or hypersensitivity to the same inhibitor depending on the source of purine used for growth. DAP, which served as a growth factor for *gua-1*, was actually an inhibitor of growth for the prototrophic strain LT-2. Under these conditions, it did not reverse the inhibition by AzG (Table 1). A slight increase in the concentration of the analogue required for 50% inhibition may be due to the partial reversal of inhibition caused by guanine formed from DAP during growth (14). The prototrophic revertant *azg-11-R<sub>A</sub>*, derived from *azg-11*, showed resistance even when grown in the presence of DAP, suggesting that the mechanism of resistance to AzG in the auxotroph as well as in the revertant is independent of DAP. Guanine, on the other hand, completely reversed the inhibition by the analogue.

To elucidate further the nature of inhibition by AzG in *azg-11* grown on guanine, growth was studied under various concentrations of both of these purines. Addition of the inhibitor to the medium increased the amount of guanine required for supporting the same amount of growth, suggesting a competitive nature of antagonism between the purine and its analogue (Fig. 2).

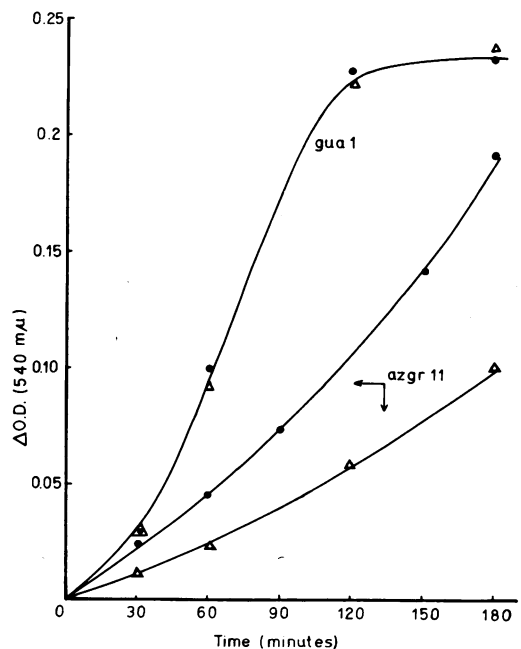


FIG. 1. Effect of 8-azaguanine (AzG) on growth rates of *gua-1* and *azg-11*. Both strains were grown in the presence of guanine (20  $\mu$ g/ml). (●) Without AzG; (▲) with AzG (250  $\mu$ g/ml). (The designation *azgr 11* in the figures is synonymous with *azg 11* in the text.)

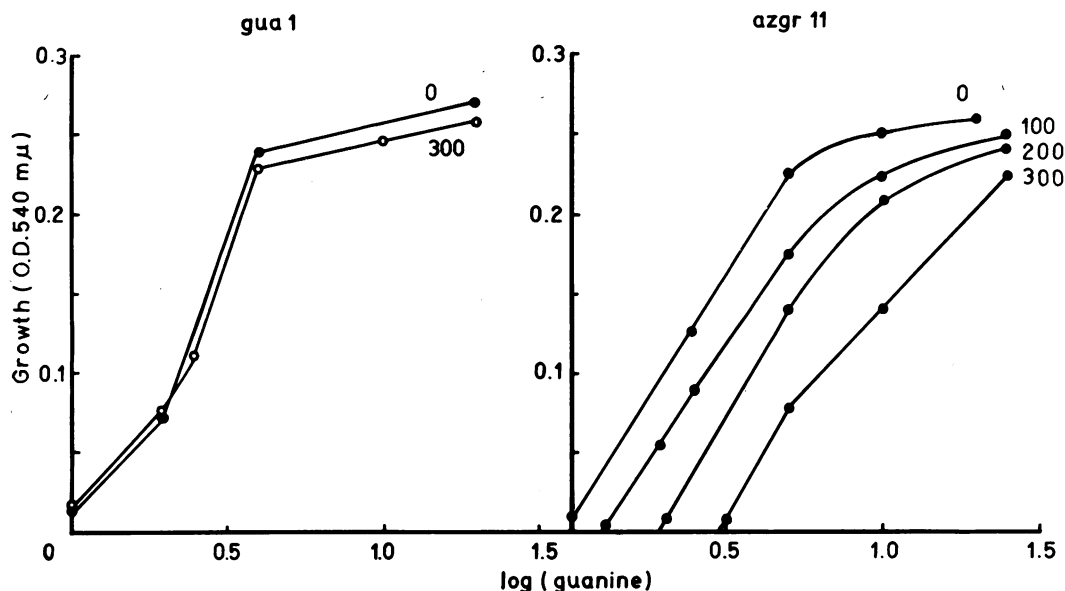


FIG. 2. Effect of AzG on the growth response of strains *gua-1* and *azg-11*. Concentrations of AzG and guanine are given in micrograms per milliliter. The numbers on the curves represent the concentrations of AzG.

This effect was not observed with the parent strain with the concentrations of the inhibitor used in the experiment.

**Growth response of *azg-11*, and *gua-1*.** The most striking difference between *azg-11* and *gua-1* was the varying growth response of the mutant to the increasing concentrations of exogenously supplied guanine (Fig. 3). This was not observed with the original parent strain, in which growth rates were the same irrespective of the purine concentration used in the experiment. Furthermore, the growth rate of the mutant at any given concentration was lower than in *gua-1*, though the final yield of cells reached at the end of the growth period was the same in both cases. Thus, it would appear *a priori* that the mutant was defective in a mechanism which determined its rate of incorporation of guanine during growth. This was subsequently confirmed by studies on the uptake of radioactive purines.

**Incorporation of radioactive purines by *gua-1* and *azg-11*.** Incorporation of guanine in the auxotrophic strains was studied at a concentration of guanine sufficiently low to make the initial enzymatic step limiting. The method used in determining the affinity constant,  $K_m$ , was essentially the same as described by Ames (2), with a few variations. The uptake was determined in whole cells, since preliminary experiments indicated that practically all of the radioactivity in the whole cells appeared in the trichloroacetic acid-insoluble fraction under the conditions of

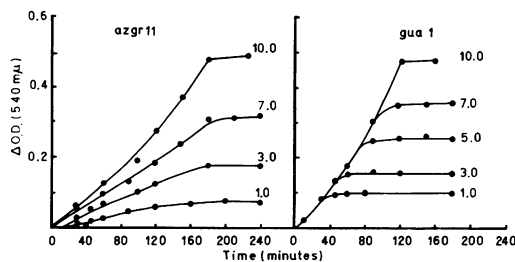


FIG. 3. Effect of various concentrations of guanine on growth rates of *azg-11* and *gua-1*. Numbers on the curves represent the concentrations of guanine in micrograms per milliliter.

our experiments. Logarithmically growing cells were harvested, washed, and starved for 1 hr. The initial rate of uptake was measured during the first 30 min immediately following the addition of the radioactive purine. During this period, the uptake was linear with time and there was no measurable increase in cell mass.

Guanine uptake by the mutant was considerably reduced, compared with the parent strain (Fig. 4). In one experiment the rate of uptake by the mutant was measured at higher concentrations of the exogenous purine and it was found to reach a saturation value at about 150  $\mu$ moles/liter. Furthermore, the maximal rates attained in the mutant were half those obtained with the parent strain. Apparent  $K_m$  values for guanine uptake in the two strains were calculated from

Lineweaver-Burke plots (Fig. 5) and found to be  $2.5 \times 10^{-4}$  M for *azg-11* and  $2.3 \times 10^{-5}$  M for *gua-1*. Thus, the affinity for guanine in the mutant was about one-tenth that in *gua-1*. To determine whether the reduced affinity for guanine was primarily responsible for the inhibitory effects of AzG in the mutant, as indicated in growth experiments, the uptake was studied in the presence of the inhibitor. AzG competitively blocked the incorporation of guanine in both strains (Fig. 6). Inhibitor constant ( $K_i$ ) for the mutant was  $3.5 \times 10^{-3}$  M, as compared to  $7.7 \times 10^{-3}$  M

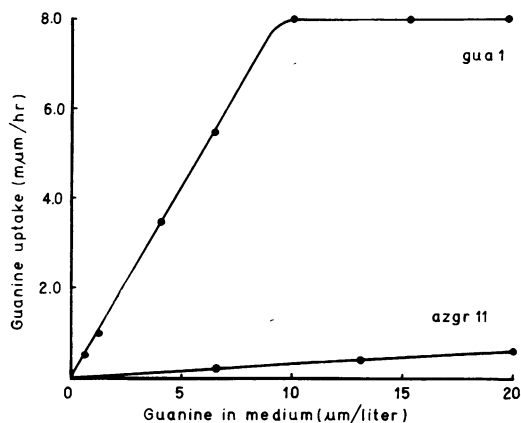


FIG. 4. Effect of concentration of exogenous guanine on its rate of incorporation into cells. Specific activity of guanine- $8^{14}\text{C}$  in the medium was  $1.3 \mu\text{C}/\mu\text{mole}$  with *azg-11* and  $0.13 \mu\text{C}/\mu\text{mole}$  with *gua-1*.

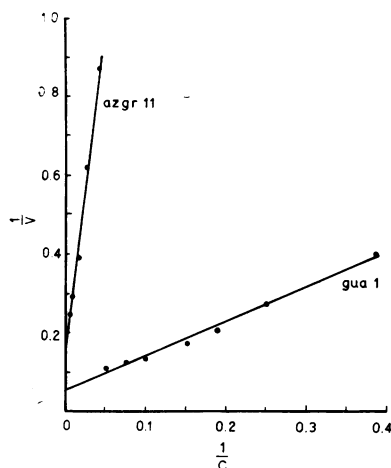


FIG. 5. Lineweaver-Burk plot for guanine uptake in *gua-1* and *azg-11*.  $V$  = rate of incorporation of guanine- $8^{14}\text{C}$ , expressed as millimicromoles per hour;  $C$  = concentration of guanine in the medium in micromoles per liter.

for *gua-1*. These results, therefore, imply that the affinity of guanine for the active site at which the competition is taking place is reduced significantly in relation to that for the inhibitor and could account for the competitive inhibition of the mutant by AzG.

*Incorporation of other purines by a revertant from azg-11.* It is very difficult to study the incorporation of other purines in the guanine-requiring auxotroph, since it is easily inhibited by adenine and does not grow on any of the other purines (14). Prototrophic strains, on the other hand, provide a better system for studying the uptake of purines under the conditions of active growth (15). The prototrophic revertant *azg-11-R<sub>A</sub>* was, therefore, isolated from the mutant *azg-11*. This strain resembled its parent in the pattern of resistance to guanine analogues (Table 1) and in purine nucleotide pyrophosphorylase activity (Table 3). Growth rates of the revertant were similar to that of its parent in the presence of guanine or to the prototrophic wild-type strain LT-2 in the absence of guanine.

Incorporation of guanine by the revertant during growth was about one-fifth of that by LT-2 (Fig. 7). In addition to guanine uptake, the revertant was also defective in the incorporation of xanthine (Fig. 8). Xanthine uptake was not linear with the increase in cell mass, as with other purines, and its rate generally decreased with growth. This appears to be the characteristic of xanthine uptake in LT-2 (15); the reason for this is not yet understood. The mutant, however, showed a lower rate of incorporation during growth and reached a saturation level much earlier than did LT-2. Since *gua-1* is blocked at the step involving xanthyl aminase (14), one could postulate that a reversion at this locus might lead to a reduced synthesis of this enzyme. This would, in turn, limit nucleic acid synthesis and therefore growth owing to the accumulation of xanthyl acid. However, the growth rates of LT-2 and the revertant were found to be the same in the presence and absence of xanthine. It is, therefore, likely that the reduced uptake of xanthine is also associated with the defective incorporation of this purine. Studies with radioactive adenine and hypoxanthine indicated that their uptake was relatively unaffected in the mutant (Table 2).

*Enzymatic studies.* Previous studies have shown that decreased incorporation of purines in *S. typhimurium* is generally accompanied by a corresponding reduction in the activity of specific purine nucleotide pyrophosphorylases (13, 15). However, cell-free extracts of both auxotrophic and prototrophic strains did not show any significant difference in the specific activity of any

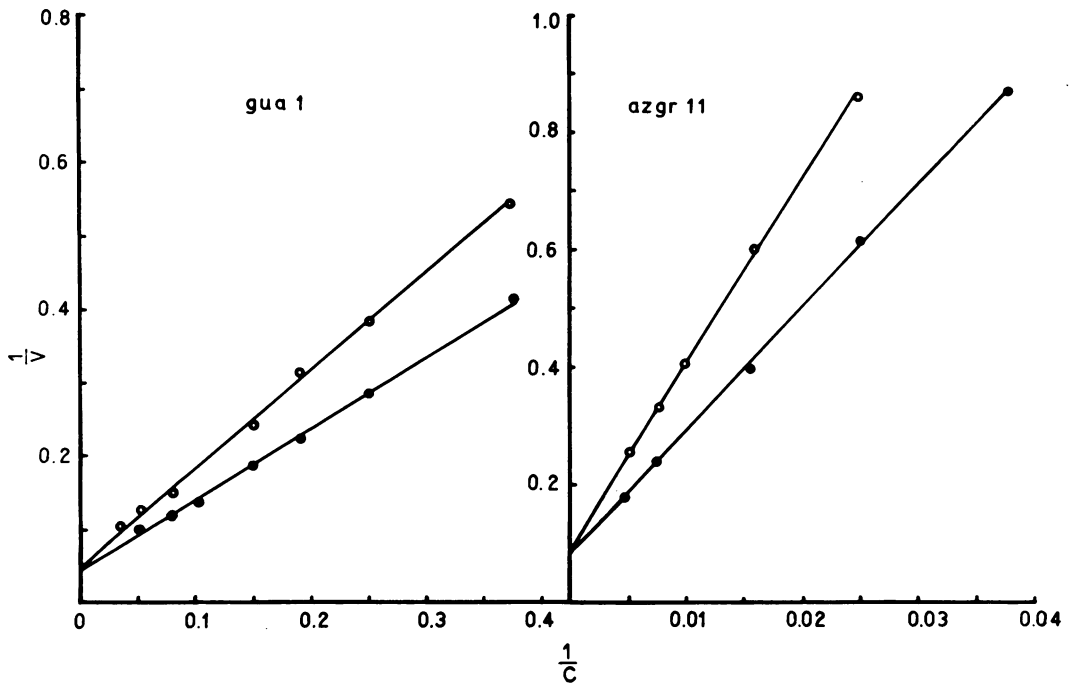


FIG. 6. Lineweaver-Burk plot for the inhibition by AzG of guanine uptake in *gua-1* and *azg-11*. Units for  $V$  and  $C$  are the same as in Fig. 5. Concentration of AzG was  $1.6 \times 10^{-3}M$ . (○) With AzG; (●) without AzG.

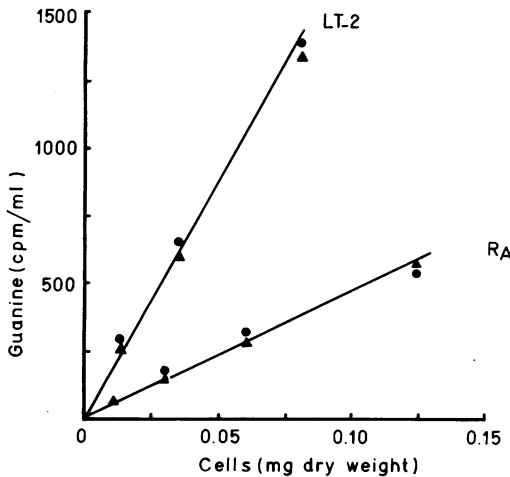


FIG. 7. Incorporation of guanine-8- $^{14}C$  ( $0.13 \mu\text{C}/\mu\text{mole}$ ) by strains LT-2 and *azg-11*  $R_A$  during logarithmic growth. Concentration of guanine in the medium  $5 \mu\text{g}/\text{ml}$ . (●) Incorporation in whole cells; (▲) incorporation in the trichloroacetic acid-insoluble fraction.

of these enzymes (Table 3). The reduced uptake of guanine and xanthine appears, therefore, to be due to some enzyme functioning at a level of transport.

#### DISCUSSION

Evidence for active transport for purines in bacteria has been indirect and generally based on the competition between different purines and their derivatives in supporting growth of the auxotrophic strains (12, 20). This is probably due to the difficulty in demonstrating the relatively small pool size of free purines inside the cells. Recently, Berlin and Stadtman (3), using a dual isotope technique, provided direct evidence for the intracellular compartmentalization of the free adenine pool, as distinct from the subsequent biochemical steps in its utilization. The mutant described here provides additional evidence for the existence of a transport system for guanine, as distinct from the purine nucleotide pyrophosphorylases which convert free intracellular purines to the corresponding nucleotides.

Several types of evidence suggest the existence of a separate mechanism for the transport of guanine into the cells. The  $K_m$  values determined for initial uptake are different from those obtained for guanylic pyrophosphorylase in *S. typhimurium* (1). These values were determined at concentration of guanine much lower than those required for supporting growth. Under these conditions, the permeation system becomes rate-limiting. Ames (2) used this technique to determine

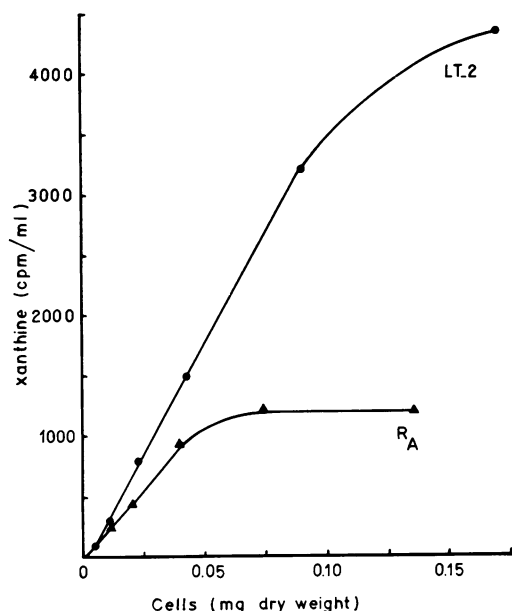


FIG. 8. Uptake of xanthine-8-<sup>14</sup>C (0.125  $\mu\text{C}/\mu\text{mole}$ ) by whole cells of LT-2 and *azg-11* R<sub>A</sub> during growth. The concentration of xanthine was 20  $\mu\text{g}/\text{ml}$ .

TABLE 2. Incorporation of radioactive purines by *Salmonella* prototrophs

Purine (20 $\mu\text{g}/\text{ml}$ )	Concn of purine <sup>a</sup>	
	LT-2	<i>azg-11</i> -R <sub>A</sub>
Guanine-8- <sup>14</sup> C	165	40
Xanthine-8- <sup>14</sup> C	390	217
Hypoxanthine-8- <sup>14</sup> C	450	472
Adenine-8- <sup>14</sup> C	610	620

<sup>a</sup> Expressed as millimicromoles per milligram (dry weight) of cells.

TABLE 3. Purine nucleotide pyrophosphorylase activity in *Salmonella* mutants

Strain	Enzyme activity <sup>a</sup>				
	Guanine	Aza-guanine <sup>b</sup>	Hypo-xanthine	6-Mer-capto-purine <sup>b</sup>	Xanthine
<i>gua-1</i>	116	137	109	93	96
<i>azg-11</i>	109	128	120	95	105
LT-2	94	104	89	108	125
<i>azg-11</i> -R <sub>A</sub>	112	110	90	93	116

<sup>a</sup> Expressed as millimicromoles per hour per milligram of protein.

<sup>b</sup> Assayed in 0.03 M acetate buffer (pH 7.2).

affinity constants for amino acid permeases in *Salmonella* and found that they were in good agreement with those obtained with conventional assays of starved cells in a resting system. Mutation to *azg-11* has resulted in the decreased incorporation of guanine as well as xanthine, suggesting overlapping active sites for both of these purines. Furthermore, it also distinguishes the uptake system from the biochemical step involving guanylic and xanthylic pyrophosphorylases, which can be separated chemically by fractionation procedures and genetically by mutation (13). These conclusions differ from those of other workers, who studied antagonism between different purines and suggested a common mechanism for incorporation of adenine, guanine, and hypoxanthine, distinct from that responsible for xanthine (9, 12).

Studies with *azg-11* throw an interesting side-light on the site of action of AzG. This analogue is known to inhibit only after being converted to phosphoribosylated derivatives (4, 19). Results presented here show that AzG can, under certain conditions, inhibit competitively by interfering with guanine at the site of incorporation. It is generally difficult to observe the effect in growth experiments with auxotrophic strains such as *gua-1* because of relatively high affinity of guanine for the site at which the competition is taking place. Thus, guanine can effectively overcome the inhibition by the analogue at the concentrations which normally support growth of the auxotrophs. On the other hand, it is easily observed in the mutant *azg-11*, since the affinity for guanine is selectively reduced without significant alterations in  $K_i$  for the inhibitor.

Selection for resistance to the structural analogues of metabolites as a means of obtaining mutants deficient in specific permeases have been used widely, especially in studying amino acid transport (2, 16, 18, 22). Such mutants often provide useful information regarding the specificity of transport mechanisms and their genetic control. Among the mutants selected for resistance to purine analogues in *S. typhimurium*, the most commonly encountered phenotypic change was the synthesis of altered purine nucleotide pyrophosphorylases (13). In our studies with *gua-1*, we found that many of the mutants selected for resistance to AzG, by using DAP as a growth factor, were of the type described in this paper. The reason for the prevalence of such mutants in this system is not clear. Previous studies with *gua-1* suggested that AzG inhibits noncompetitively the conversion of DAP to guanine, involving deamination of carbon 6 of the purine ring (11, 14). If AzG by itself is the effective inhibitor of this reaction before it is converted to

its ribosylated derivatives, then it is conceivable that the majority of the mutants selected for resistance would be those blocked in the entry of this inhibitor to the site of inhibition.

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