Metabolic Abnormalities of 2,6-Diaminopurine-Resistant Mutants of *Salmonella typhimurium.** (27177)

GURUDUTT P. KALLE AND JOSEPH S. GOTS
Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia

During our studies on resistance to 2,6-diaminopurine (DAP), 2 mutants of *Salmonella typhimurium*, both resistant to DAP but differing phenotypically, were isolated. The striking difference between the 2 mutants was the ability of the one (*dap-r-3*), as distinguished from the other (*dap-r-6*), to allow satellite growth of the sensitive parent strain in presence of DAP. The mutants also differed in their cross-resistance to other purine analogues. The wild type strain *LT-2*, from which the mutants were derived, was easily inhibited by high concentrations of adenine. The mutation of *dap-r-6* type showed an increased sensitivity to adenine inhibition when compared with the parent. The *dap-r-3*, on the other hand, was resistant to adenine inhibition and allowed the satellite growth of the parent strain in presence of adenine. These mutants were, therefore, found to be of particular interest, since their obvious phenotypic differences implied the possibility of 2 different mechanisms of resistance to the same inhibitor. The present paper describes the isolation, characterization and properties of the mutants, and identification of the products excreted by *dap-r-3*.

Methods. *Salmonella typhimurium*, strain *LT-2*, was grown in a minimal medium(1) containing 0.2% glucose. The plating medium was the same with the addition of 1.5% agar. Mutants resistant to DAP were isolated by the gradient plate technic described by Szybalski(2), with upper layer containing 1 mg/ml of DAP. Two types of resistant colonies were isolated; one type, *dap-r-3*, showed distinct satellite growth around the colony while the other type, *dap-r-6*, did not. The resistant mutants were maintained on nutrient agar slants containing 200 μg/ml of DAP.

DAP-resistant mutants were isolated also from auxotrophic strains. Strain *adeE-11* of *S. typhimurium* requires a purine for growth and can be grown on adenine, xanthine, hypoxanthine, or guanine. The DAP-resistant mutants of this strain were isolated on plates containing 40 μg of xanthine, and 500 μg of DAP per ml.

A uracil requiring mutant of *Escherichia coli*, strain *BU⁻*, was kindly supplied by Dr. S. S. Cohen. This mutant can grow on either uracil or cytosine. A DAP-resistant mutant of this strain was isolated in presence of 40 μg of cytosine and 500 μg of DAP per ml.

An adenine specific mutant, *adeB-12*, of *S. typhimurium* and a uracil requiring mutant, *E. coli B 204*, were used for auxanographic analyses.

Inoculum for growth studies was obtained from cultures grown in absence of DAP. The size of the inoculum was found to be critical and was kept constant at 10^5 bacteria per ml for all strains studied. All cultures were incubated at 37°C for 24 hours and growth was measured turbidimetrically with a Klett-Summerson Photoelectric colorimeter using a green filter (No. 54).

Chromatographic procedure. The following solvents were used: (a) isoamyl alcohol and 5% Na_2HPO_4 in the ratio 1:1(3); (b) butanol-acetic acid-water in ratio of 2:1:1 (4); (c) 17 ml of isopropanol, 41 ml conc. hydrochloric acid (sp. gr. 1.19) and water to make 250 ml(5); (d) 85 ml of isopropanol, 15 ml of water and 1.3 ml concentrated (30%) ammonia solution(6); (e) 70 ml secondary butanol, 10 ml formic acid and 20 ml water(7).

Chromatography was performed by the ascending method on Whatman #1 paper at room temperature for 18 hours.

Results. *Cross-resistance to other purine analogues.* Phenotypically, the DAP-resistant mutant, *dap-r-3*, was easily distinguished from *dap-r-6* in its ability to allow satellite growth of the sensitive parent strain in the

* This investigation was supported by research grants from Nat. Inst. Health.

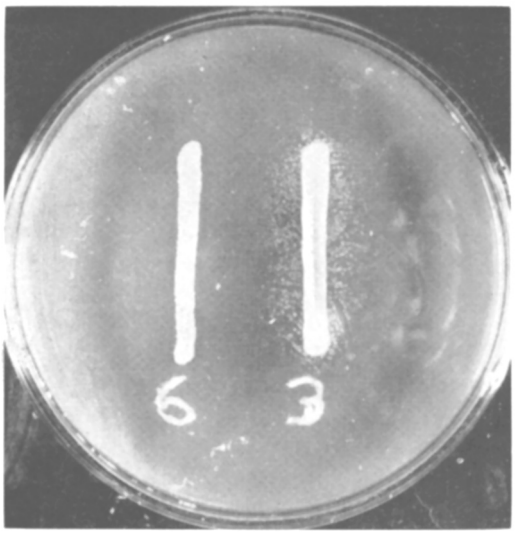

FIG. 1. Phenotypic difference between mutants *dap-r-3* and *dap-r-6*. Mutants were streaked on minimal salts-glucose-agar plates containing 500 μg/ml of 2,6-diaminopurine. Plates were uniformly seeded with the wild type strain *LT-2*, which is inhibited at this concentration of inhibitor. Satellite growth is evident only around *dap-r-3* streak.

presence of DAP (Fig. 1). An additional difference was revealed in their cross-resistance patterns with respect to other purine analogues (Table I). The *dap-r-3* mutant was also resistant to other adenine-like analogues, but was easily inhibited to about the same extent as the parent strain *LT-2* by other analogues. The *dap-r-6* mutant showed cross-resistance only to thioadenine, thioguanine, and 6-mercaptopurine. Unlike other natural purines, adenine can inhibit the growth of *S. typhimurium*. In contrast to the resistance to adenine exhibited by *dap-r-3*, the *dap-r-6* mutant was about 10 times more sensitive to adenine than the original parent strain.

Excretion products of dap-r-3. When streaked against the background of inhibited sensitive parent strain, satellite growth was obtained around *dap-r-3* not only with DAP, but also with adenine and 8-azaadenine as the inhibitors. This phenomenon was not observed with any of the other analogues to which this mutant was resistant. The supernatant fluids obtained from an overnight culture of *dap-r-3* grown in salts-glucose medium showed neutralizing action against inhibition by DAP or adenine. Similar cultures of *dap-r-6* and *LT-2* parent strain did not show this effect. The possibility that the inhibitor was being destroyed by an enzyme elaborated by *dap-r-3* was unlikely since boiling of supernatant fluids did not alter their neutralizing activity.

Antagonists excreted by analogue-resistant organisms are usually the corresponding metabolites capable of competitively antagonizing the bacteriostatic action of the analogues. On preliminary studies, adenine was found to be the only natural purine capable of competitively preventing the action of both DAP and 8-azaadenine, the 2 antipurines which were antagonized by the excreted products of *dap-r-3*. Adenine was identified tentatively as one of the products elaborated by this mutant by its ability to feed the adenine specific auxotroph. A disturbing incongruity had yet to be explained; the fact that *dap-r-3* excreted something which could also prevent the bacteriostasis with adenine as the inhibitor. The data to follow will show that

TABLE I. Resistance of *Salmonella typhimurium*, Strains *LT-2*, *dap-r-3*, and *dap-r-6* to Purine Analogues.

Purines	Purine substituents			Conc. for 50% inhibition, μg/ml		
	2	6	8	*LT-2*	*dap-r-3*	*dap-r-6*
2-Aminopurine	NH_2	—	—	278	142	107
2,6-Diaminopurine	NH_2	NH_2		100	>1000	>1000
Adenine	—	NH_2	—	110	>1000	10
8-Azaadenine	—	NH_2	N=	47	370	65
8-Aza-2,6-diaminopurine	NH_2	NH_2	N=	21	> 500	73
Thioadenine	SH	NH_2	—	162	335	315
Thioguanine	NH_2	SH		35	42	102
6-Mercaptopurine	—	SH		150	146	396
8-Azahypoxanthine	—	OH	N=	135	100	115
8-Azaguanine	NH_2	OH	N=	<2	<2	<2
2-Thio-6-oxypurine	SH	OH	—	163	270	186

TABLE II. Comparison of Compounds Excreted by *dap-r-3* with Adenine and Uracil.

Tests			Compound I	Adenine	Compound II	Uracil
Chromatography (Rf values)		Solvent a*	.37	.38	.68	.68
		b	.63	.66	.70	.68
		c	.35	.38	.67	.69
		d	.32	.32	.38	.37
		e	.40	.40	.62	.62
Spectral properties ($m\mu$)	pH = 2.0	λ max	263	263	260	259
		λ min	230	230	232	232
		O.D. 250/260	.78	.80	.80	.82
		O.D. 280/260	.40	.40	.24	.19
	pH = 9.0	λ max	268	267	285	284
		λ min	236	237	243	241
		O.D. 250/260	.59	.60	.71	.71
		O.D. 280/260	.56	.57	1.90	1.40
Auxanography†		*adeB-12*	+	+	—	—
		B 204	—	—	+	+

* Description of solvents is given under *Methods*.
† Ability (+) or inability (—) of compounds to support growth of test strains.

a second substance is excreted by *dap-r-3*, which specifically antagonizes the inhibitory action of adenine. This has been identified as uracil.

The mutant *dap-r-3* was grown in salts-glucose medium in absence of DAP. After removing the cells by centrifugation, the supernate was filtered through a Seitz filter and then passed through a column of Norit A. The charcoal was washed several times with water to remove all the salts, then eluted with 50% ethanol containing 6% ammonium hydroxide. The eluate was dried *in vacuo* and the residue dissolved in a minimum of water. This was placed on Whatman #1 paper and the ascending chromatograms were developed in the butanol-acetic acid solvent. Two ultraviolet absorbing bands were strongly visible at Rf values of 0.63 and 0.70. The bands were eluted with water and rechromatogrammed several times in the isoamyl-phosphate solvent until a sharp resolution was obtained with Rf values of 0.37 (compound I) and 0.68 (compound II) respectively. The final eluates were reabsorbed on charcoal to remove phosphates from the solvent, and eluted with ammoniacal ethanol as before.

Identification was established by means of chromatographic, spectrophotometric and auxanographic analyses. The identity of compound I with adenine and compound II with uracil is evident from the data presented in Table II. Table III shows the ability of compound I and adenine to prevent inhibition of growth of strain *LT-2* by DAP, and that of compound II and uracil (but not cytosine) to prevent inhibitory effects of adenine. The inhibitory effects of adenine were most effectively prevented by thiamine or its pyrimidine moiety and histidine. As little as 0.02 μg/ml of thiamine was sufficient to nullify inhibition by adenine. Much higher concentrations of uracil or uridine were required to achieve similar effects.

Studies with auxotrophs. It has been postulated often that resistance to an antimetabolite may be a direct result of over-production of the corresponding natural metabolite. To determine whether the resistance in *dap-r-3* mutant was a direct consequence of neutralizing action of the excreted adenine,

TABLE III. Reversal of Adenine and 2,6-Diaminopurine Inhibition of *Salmonella typhimurium*, Strain *LT-2*.

Compounds	Amt, μg/ml	Growth (turbidity) 2,6-Diaminopurine*	Adenine*
No additions	0	10	13
Compound I	5	70	10
Adenine	5	85	0
Compound II	5	0	50
Uracil	5	10	65
Thiamin	1	13	135
B_1-Pyrimidine	1	13	120
Histidine	10	13	95
Cytosine	10	13	13

* Concentrations of 2,6-diaminopurine and adenine were 500 μg/ml in all tubes.

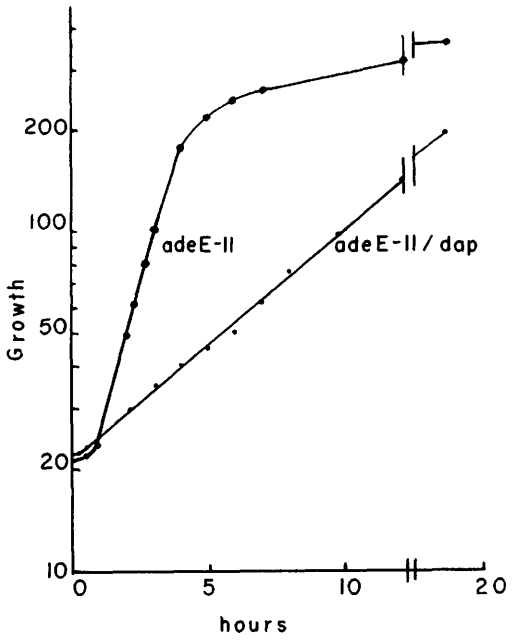

FIG. 2. Growth rates of *adeE-11* and *adeE-11/dap* in presence of 40 μg/ml of adenine.

a search was made for the *dap-r-3* type of mutation in purine requiring auxotrophs incapable of synthesizing adenine *de novo*. Such mutants, *e.g., strain adeE-11,* are not inhibited by DAP when adenine serves as a growth-promoting purine. Indeed, under these conditions, DAP actually has a sparing effect on the requirement for adenine(8). However, growth on other purines, *e.g.*, xanthine, is easily inhibited by DAP. Mutants resistant to DAP were readily isolated in the presence of its inhibitory concentrations by plating strain *adeE-11* with xanthine as a growth factor. Since these mutants would not be expected to produce adenine, excretion of uracil was used as an index to detect *dap-r-3* mutants. Auxanographic analysis revealed existence of uracil excreters among the resistant mutants; these were designated as *adeE-11/dap*. Thus, the existence of *dap-r-3* type of mutation in strains unable to synthesize adenine indicated that the excretion of adenine *per se* was not responsible for resistance in *dap-r-3,* but rather a dispensable consequence of it.

Fig. 2 reveals another defect created by the resistance to DAP. The growth rate of the DAP-resistant mutant was greatly reduced compared to that of the parent strain *adeE-11*. The generation time for the mutant was 260 minutes as compared to 69 minutes for the parent strain, when grown in the synthetic medium with adenine as a source of purine. A similar type of limited growth response was observed with other growth-promoting purines (hypoxanthine, xanthine or guanine) or their nucleosides.

It was also possible to demonstrate the *dap-r-3* type of mutation in a pyrimidine requiring mutant of *E. coli strain BU*$^-$ by plating it in presence of inhibitory concentrations of DAP with cytosine as the growth-promoting pyrimidine. Auxanographic analysis, using the adenine specific strain *adeB-12* as an indicator, revealed the presence of adenine excretors in the population of resistant colonies. This mutant did not show any defect in its growth response to uracil, cytosine or its ribonucleosides when compared with the parent DAP-sensitive strain *BU*$^-$.

Since the *dap-r-3* type of mutation can be demonstrated in both purine and pyrimidine requiring auxotrophs, it is reasonable to conclude that the overproduction of adenine or uracil *per se* is not responsible for the resistance to DAP, but rather may be a consequence of it.

Discussion. The mutant *dap-r-3* is unique in several respects. As a consequence of its resistance to DAP, it not only excretes adenine, the natural antagonist of DAP, but also uracil. It is of particular interest that the products excreted are aglycones, since purines and pyrimidines are usually excreted as nucleosides. The excretion of adenine or uracil by this mutant does not seem to be directly responsible for resistance to either DAP or adenine, since the *dap-r-3* type of mutation can also be demonstrated in strains unable to synthesize adenine or uracil *de novo*. However, one must consider the possibility that increased internal concentrations of adenine may contribute to the general level of resistance. Studies with auxotrophic strains, further, indicate that resistance to DAP imposes a specific alteration in purine rather than pyrimidine metabolism. Thus, DAP-resistant mutants derived from purine requiring auxo-

trophs are limited in their growth response to all purines. The pyrimidine requirement, on the other hand, is not affected in mutants derived from pyrimidine requiring auxotrophs.

A close interrelationship between adenine and uracil metabolism is indicated by the fact that both compounds are excreted as a result of resistance to DAP. One possible explanation of this phenomenon would be the possibility of a disturbance in a common regulatory control for both purine and uracil biosynthesis. The reactions involved in formation of a common precursor leading to the synthesis of both adenine and uracil could be altered so that feed-back inhibition is prevented. However, the present knowledge concerning purine and pyrimidine biosynthesis suggests that the regulatory control mechanisms for the two are independent of each other(9,10). Studies to resolve this possibility are described in the succeeding paper (11).

Summary. Resistance to 2,6-diaminopurine in *Salmonella typhimurium* resulted in 2 phenotypically distinguishable mutants. The mutant *dap-r-3* differed from the mutant *dap-r-6* in its cross-resistance to other purine analogues and also in its ability to allow the satellite growth of the parent sensitive strain *LT-2* when plated in presence of inhibitory concentrations of adenine or DAP. The satellite phenomenon could be explained by the fact that adenine and uracil, which were excreted by the mutant *dap-r-3,* prevented the inhibition of the wild type by DAP and adenine respectively. Excretions of these compounds were not directly responsible for the resistance to DAP, since the *dap-r-3* type mutation could also be demonstrated in auxotrophic strains incapable of synthesizing adenine or uracil *de novo.* The mutation to a *dap-r-3* type in a purine requiring auxotroph resulted in an additional defect characterized by poor growth response to all the purines.

1. Vogel, H. J., Bonner, D. M., *J. Biol. Chem.,* 1956, v218, 97.

2. Szybalski, W., *Science,* 1953, v116, 46.

3. Carter, C. E., *J. Am. Chem. Soc.,* 1950, v72, 1466.

4. Goldthwait, D. A., Greenberg, G. R., *Methods in Enzymology,* S. P. Colowick and N. O. Kaplan, eds. Academic Press, N. Y., 1955, vII, p516.

5. Wyatt, G. R., *Biochem. J.,* 1951, v48, 584.

6. Hershey, A. D., Dixon, J., Chase, M., *J. Gen. Physiol.,* 1953, v36, 777.

7. Roberts, R. B., Cowie, D. B., Philip, H. A., Bolton, E. T., Britten, R. J., *Studies in Biosynthesis in* Escherichia coli, Carnegie Inst. Wash. Pub. No. 607, 1955, p33.

8. Kalle, G. P., Gots, J. S., *J. Bact.,* 1961, v81, 331.

9. Yates, R. A., Pardee, A. B., *J. Biol. Chem.,* 1956, v221, 757.

10. Gots, J. S., *ibid.,* 1957, v228, 57.

11. Kalle, G. P., Gots, J. S., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 281.

Received September 5, 1961. P.S.E.B.M., 1962, v109.

Feedback Inhibition of Purine Biosynthesis in Adenine-Excreting Mutant of *Salmonella typhimurium.** (27178)

GURUDUTT P. KALLE AND JOSEPH S. GOTS

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia

In the preceding article(1) we reported a mutant of *Salmonella typhimurium* which was resistant to 2,6-diaminopurine (DAP) and which excreted adenine into the medium during growth. This phenomenon, whereby resistance to an antimetabolite leads to overproduction of its corresponding competitive metabolite, has been reported for other inhibitory analogues(2-6). Evidence has been accumulated to indicate that the excretion of the metabolites, as well as resistance to the antimetabolites, is the result of faulty feedback control of biosynthesis. Moyed(5) found that a bacterial mutant resistant to 5-

* This investigation was supported by research grants from Nat. Inst. Health.