Utilization of Pteroylglutamic Acid Antagonists for Growth by a Strain of *Streptococcus faecalis.** (19838)

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The toxicity of certain pteroylglutamic acid (PGA) antagonists has been demonstrated in a wide variety of species including microorganisms, rats, mice, the chick embryo and man. In microorganisms, Jukes, *et al.*(1) observed the inhibitory effects of several of the PGA antagonists for *Streptococcus faecalis* (ATCC No. 8043) and it was also noted that 4-amino-PGA (aminopterin) was toxic for the growth of *Escherichia coli*(2). The inhibition of growth of *Lactobacillus casei* by PGA antagonists was studied by Hutchings, *et al.*(3) and as with *S. faecalis* the toxicity was reversed under limited conditions by PGA. Sauberlich and Baumann(4) found that *Leuconostoc citrovorum* (ATCC No. 8081) was inhibited by aminopterin and this toxicity could be reversed by the citrovorum factor (CF). Studies with rat liver slices have revealed that aminopterin was effective in blocking the enzymatic conversion of PGA to CF (5). Kidder, Dewey and Parks(6,7) and Tittler and Belsky(8) observed that one of the PGA antagonists, aminopterin, could replace PGA in the nutrition of *Tetrahymena geleii*. Kidder *et al.*(6) further reported that 10-methyl-PGA (methopterin) could also substitute for PGA but not as effectively as aminopterin. 4-amino-10-methyl-PGA (amethopterin) on the other hand, could not substitute for PGA and was an inhibitor even in the presence of PGA. The toxicity of 4-amino-9-methyl-PGA (aninopterin) and 4-amino-9, 10-dimethyl-PGA (adenopterin) could be reversed by aminopterin and methopterin if concentrations equivalent to PGA were used.

A strain of *S. faecalis* resistant to 4-amino-10-methyl-PGA (amethopterin) has been shown to be capable of utilizing certain PGA antagonists for growth in lieu of PGA(9,10). This strain of *S. faecalis* to which amethopterin resistance was induced, hereinafter designated as *S. faecalis*/A, has been the subject of the present study in which a number of PGA analogs have been investigated for their effect upon the growth of this organism.

Methods. The microorganisms used in this study were *S. faecalis* (ATCC No. 8043) and *S. faecalis*/A. Both organisms were maintained in agar stabs and were transferred once a month to fresh media. *S. faecalis* was carried on Difco micro inoculum agar and *S. faecalis* /A was carried on Difco folic acid assay broth plus amethopterin (20 γ/ml), PGA (10 mγ/ml) and agar (1.5%). The technic employed for all growth experiments was that customarily used in microbiological vitamin assays.

Results. The ability of *S. faecalis*/A to utilize certain PGA antagonists to meet its requirement for PGA has been reported(9,10). When this resistant strain was compared with the parent strain it was found that the PGA requirement for half maximum growth (HMG) was appreciably decreased, from .3 to .1 mγ/ml. This was also the case with 10-formyl-PGA (.1 compared with .5 mγ/ml), pteroyl-glutamyl-glutamyl-glutamic acid (teropterin) (25 compared with 50 mγ/ml) and even a

TABLE I. Utilization of PGA Antagonists for Growth by *S. faecalis*/A.

PGA analog	mγ/ml HMG	mγ/ml Growth range
"9-methyl-"*	3000	150 - 50000
10-methyl-	10	.5 ->500000
4-amino-	.25	.005->500000
-10-methyl-	25	5 - 100000
Pteroyl-aspartic-acid (PAA)	5	.05 - 250000
4-amino-PAA	2.5	.5 - 500000
-pteroyl-alanine	50	.01 - 25000
-teropterin	100	50 ->500000
10-methyl-pteroic acid	2.5	.25 - 500

* This preparation contained 2 components one of which did and one of which did not support growth of *S. faecalis*/A (see text).

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TABLE II. Inhibition* of *S. faecalis* (8043) and *S. faecalis*/A at Two Levels of PGA and CF.

PGA analog	*S. faecalis* (8043)				*S. faecalis*/A			
	PGA		CF		PGA		CF	
	10 mγ	100 mγ	10 mγ	100 mγ	10 mγ	100 mγ	10 mγ	100 mγ
"9-methyl-"	500	200	30	3000	9000	>10000	>100000	>10000
10-methyl-	.3	.3	.1	3	6000	600	6000	600
9-10-dimethyl-	1	1	3	3	800	800	80	20
4-amino-	.2	.2	.3	1	—	—	—	—
-9-methyl-	.3	.03	1	2	7500	1500	4500	1000
-10-methyl-	.1	.03	.5	.5	60000	8000	10000	20000
-9-10-di-methyl	.1	.01	.05	.08	2000	200	300	200

* Inhibition: Ratio of antagonist to PGA or CF for H.M.G.

greater decrease was noted with pteroic acid (.1 compared with 50 mγ/ml). The CF (leucovorin) requirement was not altered (.5 compared with .4 mγ/ml).

Studies with other PGA analogs have led to the observation that certain of the PGA antagonists may also be substituted for PGA in a PGA deficient medium. The data in Table I give the HMG requirements and the growth range of *S. faecalis*/A on a series of PGA antagonists. The fact that 4-aminoteropterin did not support growth at a low concentration was to be expected since the unsubstituted triglutamate was not a particularly effective substitute for PGA.

The high concentration of "9-methyl-PGA" required for HMG and the rather narrow range over which the compound supported growth suggested the possibility that the compound was impure. By means of paper strip chromatography using an acetic acid-aqueous butanol system followed by bioautography of the chromatogram on a medium containing 1 mγ/ml of PGA and inoculated with *S. faecalis* (8043) two zones of inhibition were observed. One of the components had an Rf value of 0.63-0.68 which was comparable to other 9-methyl analogs, and a second component had an Rf value of 0.21-0.28. The identity of the slower moving component is not known. In another similar experiment it has been shown that the slower moving compound was capable of supporting the growth of *S. faecalis*/A, but that the faster moving compound did not support growth and was in fact inhibitory.

All the antagonists tested with the exception of aminopterin, methopterin and 4-aminoteropterin inhibited *S. faecalis*/A at various concentrations of or below 500 γ/ml. (Table I). It was also noted (Table I) that the 4-amino group, the 10-methyl group, or the presence or absence of a substitute for the glutamic acid moiety slightly affected the response of *S. faecalis*/A. The addition of a methyl group in the 10 position of pteroic acid made that compound ineffective as a growth factor above 500 mγ/ml, while pteroic acid was able to support maximum growth at more than 1000 times this concentration.

The PGA analogs that had a 9-methyl radical did not support the growth of *S. faecalis*/A. It was of interest then to test the effects of some of these compounds on *S. faecalis/A* in the presence of PGA, CF, and aminopterin. The data presented in Table II show the inhibition ratios of a series of these and other PGA antagonists with PGA and CF for both *S. faecalis* and *S. faecalis*/A. With *S. faecalis* it was evident that CF exerted a more competitive action on these antagonists than did PGA. With *S. faecalis*/A the reversal was not competitive except in the case of PGA with "9-methyl-PGA" and 9, 10-dimethyl-PGA, and CF with amethopterin and 4-amino-9, 10-dimethyl-PGA; once a limiting concentration of antagonist was reached the addition of more growth factor did not reverse the toxicity.

In a separate experiment with 10-methylpteroic acid it was found that the toxicity for *S. faecalis/A* was reversed by PGA; the inhibition ratio for PGA at 1 mγ was 500 and for PGA at 5 mγ was 800. This represents a 100-fold increase in resistance by comparison with *S. faecalis*.

The observation that aminopterin was almost as effective as PGA in supporting the growth of *S. faecalis/A* led to an experiment in which a comparison was made between

these two compounds in regard to their relative effectiveness in reversing the inhibitory action of aninopterin and adenopterin. In the presence of 10 mγ of either PGA or aminopterin it was found that half maximum growth was obtained with 50 γ of aninopterin and 20 γ of adenopterin, with inhibition ratios of 5000 and 2000 respectively.

Comparative studies of morphological, cultural, and physiological characteristics show no change in the resistant culture other than its altered PGA metabolism.

Discussion. *S. faecalis*/A differed from *S. faecalis* (ATCC 8043) in respect to the quantitative utilization of PGA and some PGA analogs, as well as having developed a pathway through which several PGA antagonists could be used for growth. The same CF requirements for both cultures indicated that the alteration in the resistant strain was in the enzyme system which formed CF from PGA(11,12). The PGA analogs that did not support the growth of *S. faecalis*/A were those that had a methyl group in the 9 position. There was a definite increase in resistance to 9-methyl compounds, however, when the amethopterin resistant culture was compared with the parent strain. This appeared to be similar to the situation that exists in mouse leukemia(13,14), and possibly in human leukemia. In mouse leukemia it has been shown that after resistance develops to amethopterin there is a cross resistance to 4-amino-9-methyl PGA, and 4-amino 9-10-dimethyl PGA(13,14).

The exact pathway through which aminopterin, amethopterin, amino-an-fol, and other PGA antagonists pass in the process of becoming growth factors for *S. faecalis*/A is not known. It appears likely, however, that the compounds are in some way converted to PGA which in turn is utilized as a substrate for the formation of CF. The marked decrease in the amount of pteroic acid required for HMG further indicates the increased efficiency of this culture, and suggests that the activity of a constitutive enzyme(s) has also been increased in the development of amethopterin resistance.

Present evidence suggests that PGA antagonists such as methopterin and aminopterin function as inhibitors by blocking the conversion of PGA to CF(5,11), and that aminopterin interferes with the action of CF in metabolic reactions(15). It appears that *S. faecalis*/A has the ability to remove the 4-amino group without difficulty as shown by the fact that aminopterin is an especially effective metabolite. The 10-methyl group is only slightly less easily removed in methopterin and amethopterin but the removal of a 9-methyl group in 9-methyl-PGA or 4-amino-9-methyl-PGA apparently cannot be accomplished by this organism. This may be due to the greater strength of the carbon-carbon bond as contrasted to the carbon-nitrogen linkage in the other compounds.

The enzyme pattern found in the resistant leukemia may be similar to that of *S. faecalis*/A. It seems likely that the resistant cells in both cases are capable of utilizing certain concentrations of the antagonists or have developed modified enzymes which are not as vulnerable to the antagonists. Toxicity may occur when these enzymes are saturated, and therefore due to conversion of the anti-metabolite to a metabolite a much higher concentration of antagonist would be required to exhibit toxicity.

Summary. 1. *S. faecalis*/A, an organism made resistant to amethopterin, grew well on a series of PGA antagonists except those with a methyl group in the 9 position. 2. This culture was also more efficient than the parent strain of *S. faecalis* in the utilization of PGA analogs that served as growth factors for both organisms, with the notable exception of CF. The amount of CF (leucovorin) required for HMG was approximately the same for each strain. 3. Aminopterin was as effective as PGA in reversing the toxicity of aninopterin and adenopterin in the growth of *S. faecalis*/A.

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Thermal Shock in a New Vibrio Phage.*† (19839)

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The effect of exposure to cold on bacteriophage was studied by d'Herelle(1) who found that young phase resists exposure to −180°C for 10 minutes while older phage does not. Rivers(2) demonstrated that a bacteriophage lytic for colon bacilli was inactivated by repeated freezing (−185°C) and thawing, and that the type of diluent influences the percentage of phase inactivated in this manner.

During the course of studies on heat inactivation of a newly isolated Vibrio phage, a sample of lysate was heated for 0.5 hour at 60°C then plunged into an ice bath (2°C) for 1 hour. There was a pronounced drop in titre after exposure to cold; more than 90% of the particles which survived heating at 60°C for 0.5 hour were inactivated during the period at 2°C. Experiments undertaken to investigate this phenomenon are described below.

Materials and methods. Materials and methods were the same as those described by Smith and Krueger(3). An ice bath main-

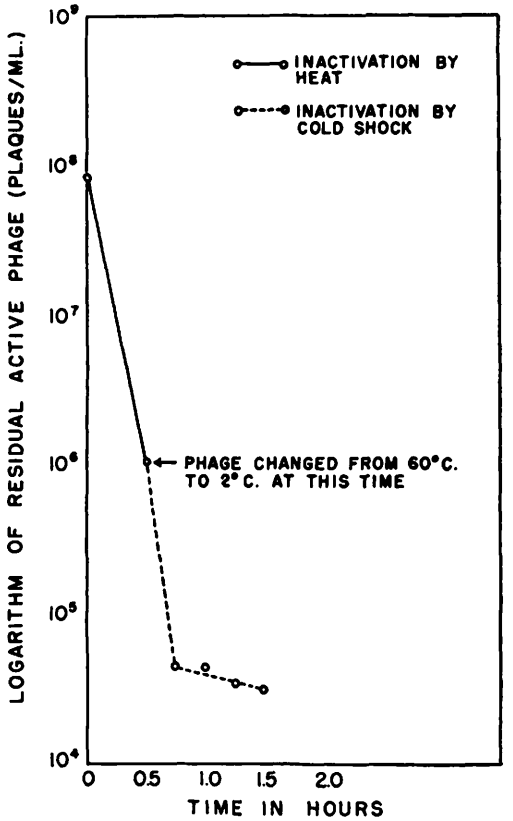

FIG. 1. Course of inactivation due to "Thermal Shock." Samples were titrated after various times at 2°C.

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