THE GROWTH PROMOTING AND ANTISULFONAMIDE ACTIVITY OF PTEROYLGLUTAMIC ACID AND RELATED COMPOUNDS FOR ESCHERICHIA COLI AND AEROBACTER AEROGENES[1]

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A basis for understanding the mode of action of sulfonamide drugs in terms of the Woods-Fildes theory of antagonism (Woods, 1940; Fildes, 1940) was provided by the elucidation of the structure of folic acid (Stokstad *et al.*, 1946; Angier *et al.*, 1946; Mowat *et al.*, 1948). Since folic acid contains a *p*-aminobenzoic acid moiety, it was postulated that the structurally similar sulfonamides compete with *p*-aminobenzoic acid for a site in the folic acid synthesizing system (Jukes and Stokstad, 1948; Hotchkiss, 1948; Woods, 1950). The interaction of the sulfonamides and folic acid has since been studied by a number of workers in terms of the Woods-Fildes theory (Woods, 1950; Lampen and Jones, 1946*a*, *b*, 1947; Shive and Roberts, 1946). In most of the work on antagonism *Lactobacillus casei* and *Streptococcus faecalis*, strain R, were grown on a complex medium containing, apart from purines and pyrimidines, vitamins of the B complex and a casein hydrolyzate, which contains, in addition to most amino acids, large amounts of glutamic acid and various unidentified degradation products. Since *p*-aminobenzoic acid is concerned not only with the synthesis of folic acid but also indirectly via folic acid with the synthesis of the purines, thymine and methionine (Kohn and Harris, 1943; Shive and Roberts, 1946; Snell, 1946), it seemed advisable to the authors to investigate a medium free from possible precursors of folic acid or products of its synthetic enzymatic activity. Any growth in this medium then could be attributed to (1) the synthetic ability of the organism, (2) the addition of an added metabolite, (3) the antagonistic action of various compounds to an inhibitory agent.

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It is the purpose of this paper to present more quantitative data on the sulfonamide-*p*-aminobenzoic acid relationship by using *Escherichia coli* and *Aerobacter aerogenes*, which grow well on a synthetic medium and are known to synthesize folic acid (Thompson, 1942; Miller, 1944).

EXPERIMENTAL METHODS

Metabolites. The metabolites used singly and in combination were: *p*-aminobenzoic acid (PABA), *p*-aminobenzoyl-*l*(+)-glutamic acid, pteroic acid, folic acid, and "teropterin" (pteroyltriglutamic acid). Their growth promoting and antagonistic properties were compared on a molar basis. Solutions of the metabolites were prepared in 0.02 M buffer (K_2HPO_4, 0.016 M and KH_2PO_4, 0.004 M), and successive dilutions also made in this buffer. Sulfonamide was dissolved directly into the medium at 4×10^{-2} M. This concentration (2×10^{-2} M after dilution) gave an 80 per cent inhibition of growth after 24 hours of incubation. This degree of inhibition allowed for better evaluation of antagonistic activity than the 50 per cent usually employed (McGeady, 1950).

Medium. The synthetic medium of Kohn and Harris was used throughout these experiments. All ingredients were dissolved in double distilled water and the pH adjusted to 7.2. The medium was tubed into Coleman spectrophotometer cuvettes, which had been matched to 1 per cent transmission. Five ml of graded concentrations of metabolites were added in duplicates to 5 ml of medium with and without sulfonamide. All tubes were autoclaved then including those containing sulfonamide since autoclaving proved to have no effect on its inhibitory action (McGeady, 1950).

Cultures. Stock cultures were maintained on Kohn and Harris synthetic medium. All but the reference tubes were inoculated with 1 drop of a 16 hour culture of *A. aerogenes* or *E. coli*. The

results were unaffected by varying the size of inoculum from 1 to 4 drops which confirmed the results of Kohn and Harris (Harris and Kohn, 1941; Kohn and Harris, 1943).

Experimental procedures. The amount of growth was measured turbidimetrically with a Coleman spectrophotometer. Readings were taken at 6 and 24 hours because antagonistic or growth promoting effects could best be evaluated at these two periods. Where results warranted a closer study, readings were taken every 2 hours over a range of 12 hours, with a final reading made at 24 hours (figure 4). Forty-eight hour readings were abandoned because little increase in growth was found to take place after 24 hours. A PC4 filter generally was employed and readings taken at 450 mμ to correct for the strong yellow coloring of folic acid and "teropterin"; a PC5 filter was used and readings taken at 550 mμ.

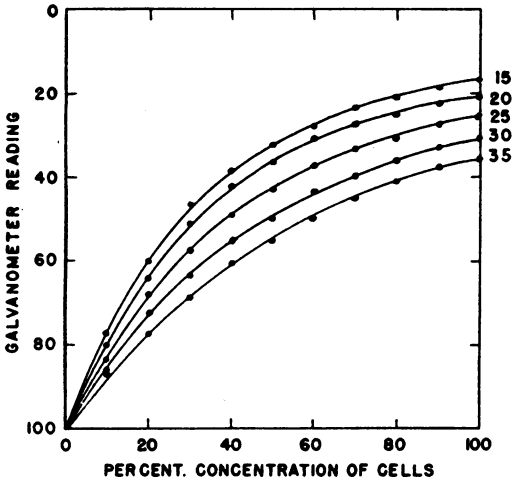

Figure 1. Relationship between bacterial concentration of *Escherichia coli* and galvanometer readings.

Dilutions of a 24 hr culture of *E. coli* giving readings from 15 to 35 galvanometer deflection units. Final concentration was taken as 100 per cent and diluted stepwise by 10 per cent to correlate turbidity with actual cell concentration.

In order to interpret galvanometer readings in terms of percentage of inhibition of growth, a 24 hour culture of *E. coli* was diluted to give final readings of 15 to 35 galvanometer deflection units (figure 1). The concentrations were decreased stepwise by 10 per cent, and galvanometer readings taken for each dilution. The graph obtained permitted direct conversion of any given galvanometer reading into concentration of cells.

RESULTS

Growth promoting and antisulfonamide activity of p-aminobenzoic acid, p-aminobenzoylglutamic acid, and glutamic acid. The growth of *E. coli* and *A. aerogenes* in the synthetic medium demonstrates the ability of the two organisms to synthesize their own requirements of folic acid. *p*-Aminobenzoic acid therefore should prove to be an effective antagonist of sulfonamide, in a medium devoid of folic acid and precursors or end

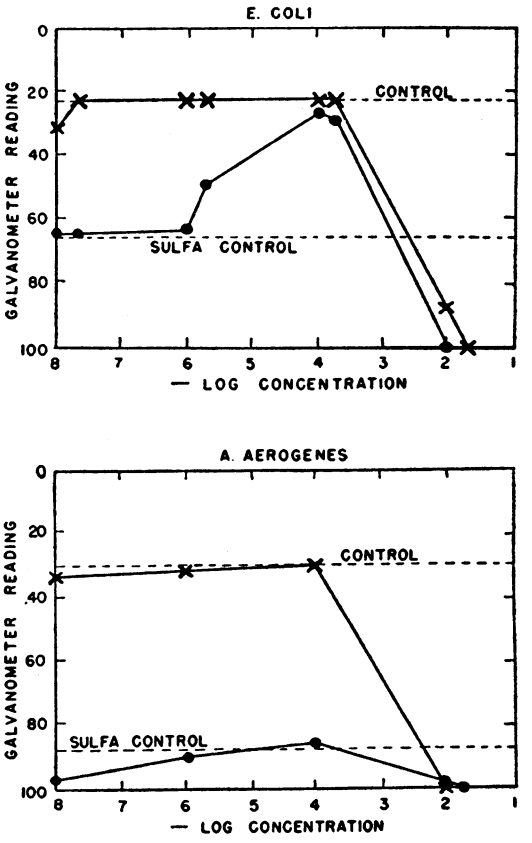

Figure 2. The growth promoting and antagonistic effect of *p*-aminobenzoic acid on *Escherichia coli* and *Aerobacter aerogenes*.

A 24 hr culture grown in presence and absence of sulfonamide with concentration of *p*-aminobenzoic acid varying from 10^{-2} M to 10^{-8} M.

x—x *p*-aminobenzoic acid.

●—● *p*-aminobenzoic acid and sulfonamide.

products of its synthesizing enzyme system. As can be seen from figure 2, *p*-aminobenzoic acid had no growth promoting effect for either *E. coli* or *A. aerogenes*. At 1×10^{-2} M *p*-aminobenzoic acid inhibited the growth of the two organisms

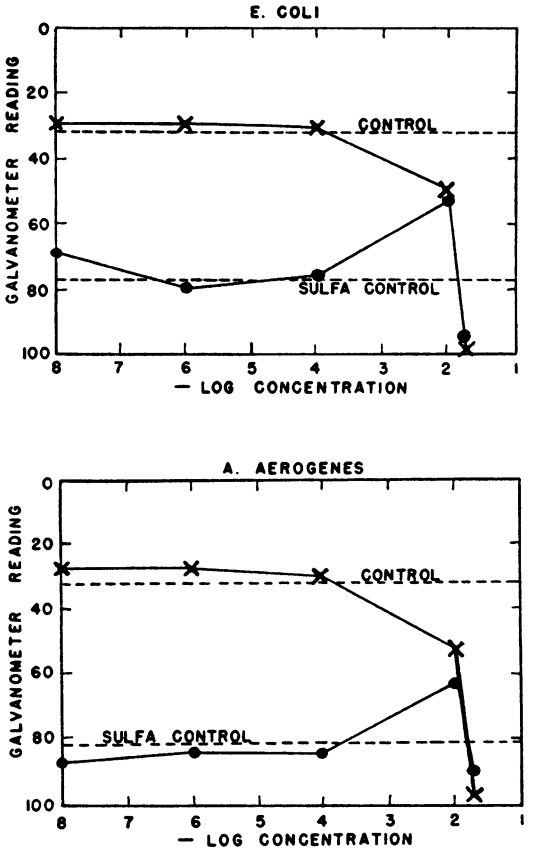

Figure 3. Effect of *p*-aminobenzoyl-*l*(+)-glutamic acid on *Escherichia coli* and *Aerobacter aerogenes*.

A 24 hr culture grown in presence and absence of sulfonamide with concentration of *p*-aminobenzoylglutamic acid varying from 2×10^{-2} to 10^{-8} M.

x—x *p*-aminobenzoylglutamic acid

●—● *p*-aminobenzoylglutamic acid and sulfonamide.

both with and without sulfonamide. At lower concentrations it markedly overcame the inhibitory effect of sulfonamide for *E. coli* but was completely ineffective for *A. aerogenes*.

An interesting effect was observed (figure 3) with varying concentrations of *p*-aminobenzoylglutamic acid. At 2×10^{-2} M it was inhibitory for both organisms in the presence and absence of sulfonamide. At the concentration of 1×10^{-2} M, however, it was inhibitory in the absence of sulfonamide but antagonistic in its presence. In the absence of sulfonamide, *p*-aminobenzoylglutamic acid probably is hydrolyzed completely by enzymes of both organisms into glutamic acid and *p*-aminobenzoic acid, the latter being inhibitory at 1×10^{-2} M. The presence of sulfanilamide might block this hydrolysis, and *p*-aminobenzoylglutamic acid then could be antagonistic either directly or indirectly through a gradual release of *p*-aminobenzoic acid.

TABLE 1

Effect of glutamic and pteroic acid on growth and sulfonamide inhibition of Escherichia coli and Aerobacter aerogenes

	METABOLITE, MOLES/L	SULFONAMIDE, MOLES/L	GALVANOMETER READING			
			E. coli		*A. aerogenes*	
			6 hr	24 hr	6 hr	24 hr
Glutamic acid	—	—	63	30	84	26
	—	2×10^{-2}	88	73	100	88
	2×10^{-2}	—	50	30	99	33
	1×10^{-2}	—	55	30	95	25
	1×10^{-4}	—	51	26	90	30
	1×10^{-6}	—	59	25	90	33
	1×10^{-8}	—		25	89	32
	2×10^{-2}	2×10^{-2}	73	55	100	90
	1×10^{-2}	2×10^{-2}	88	69	97	88
	1×10^{-4}	2×10^{-2}	88	71	92	79
	1×10^{-6}	2×10^{-2}	84	75	92	80
	1×10^{-8}	2×10^{-2}	88	77	93	87
Pteroic acid	—	—	41	28	79	26
	—	2×10^{-2}	86	68	88	75
	1×10^{-4}	—	45	28	80	25
	1×10^{-6}	—	50	30	79	22
	1×10^{-8}	—	51	29	81	23
	1×10^{-4}	2×10^{-2}	68	35	81	61
	1×10^{-6}	2×10^{-2}	88	70	90	79
	1×10^{-8}	2×10^{-2}	90	70	92	81

Glutamic acid was found to have no growth promoting effect at any concentration used for either *A. aerogenes* or *E. coli* (table 1). For *E. coli* it showed an antagonistic effect at a concentration of 2×10^{-2} M; the turbidity, however, never approached that of the control containing no sulfonamide. Glutamic acid did not overcome

sulfonamide inhibition at any concentration for *A. aerogenes* (table 1).

Effect of pteroic acid, pteroylglutamic acid, and "teropterin". The slight solubility of pteroic acid necessitated its use at an initial concentration of

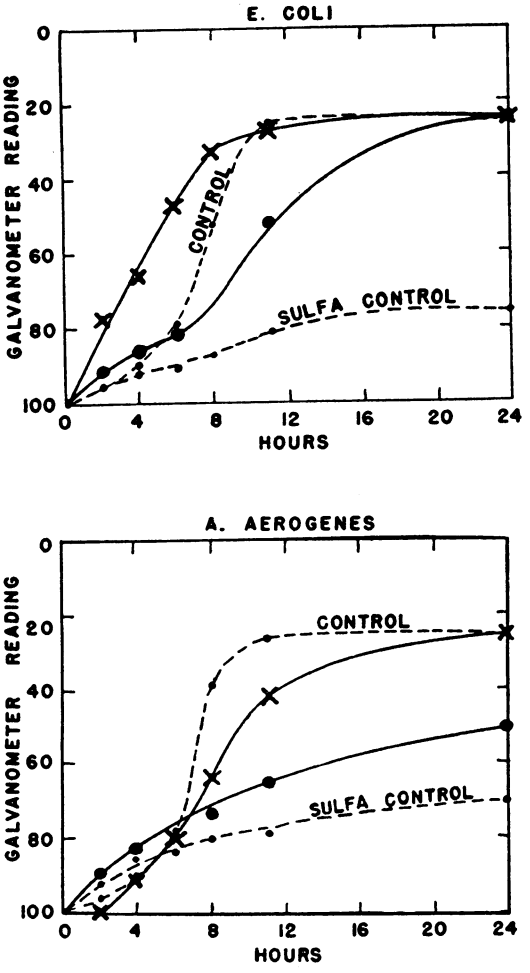

Figure 4. The effect of combinations of metabolites on *Escherichia coli* and *Aerobacter aerogenes* 2×10^{-2} M.

●—● pteroic acid 1×10^{-4} M and sulfonamide.

x—x pteroic acid 1×10^{-4} M, glutamic acid 1×10^{-2} M and sulfonamide 2×10^{-2} M.

1×10^{-4} M. This concentration had a strong antagonistic effect for *E. coli* but was less pronounced for *A. aerogenes* (table 1). Since *p*-aminobenzoic acid has a strong antagonistic effect at 1×10^{-4} M for *E. coli*, this effect might be due to the *p*-aminobenzoic acid moiety of pteroic acid. Neither pteroic acid, folic acid, nor "teropterin" had any growth promoting effect for *E. coli* and *A. aerogenes*. Neither folic acid nor "teropterin" antagonized the inhibition of sulfonamide. Apparently both organisms cannot utilize the preformed molecule to avoid the interference in the folic acid synthesis by sulfonamide (Havas, 1950).

Effect of combinations of parts of the folic acid molecule. The combinations compared in this study were as follows: (1) *p*-aminobenzoic acid and glutamic acid versus *p*-aminobenzoylglutamic acid, (2) pteroic acid and glutamic acid versus folic acid, (3) folic acid and glutamic acid versus "teropterin".

TABLE 2

Effect of combinations of glutamic acid and p-aminobenzoic acid (PABA) on sulfonamide inhibition of Escherichia coli and Aerobacter aerogenes

GLUTAMIC ACID, MOLES/L	PABA, MOLES/L	SULFONAMIDE, MOLES/L	GALVANOMETER READINGS			
			E. coli		*A. aerogenes*	
			6 hr	24 hr	6 hr	24 hr
—	—	—	81	24	78	26
—	—	2×10^{-2}	91	76	84	70
2×10^{-2}	—	2×10^{-2}	88	76	84	66
1×10^{-2}	—	2×10^{-2}	94	47	95	79
—	1×10^{-4}	2×10^{-2}	80	31	88	68
1×10^{-2}	1×10^{-4}	—	63	33	75	27
1×10^{-2}	1×10^{-4}	2×10^{-2}	73	32	73	33

For *E. coli* it can be seen from table 2 that glutamic acid and *p*-aminobenzoic acid have a strong antagonistic effect which, however, is no more pronounced than when *p*-aminobenzoic acid is used alone. For *A. aerogenes* this same combination is also strongly antagonistic. It will be recalled that for this organism neither *p*-aminobenzoic acid nor glutamic acid has any antagonistic effect. The combination of *p*-aminobenzoic acid and glutamic acid is more effective for both organisms than the preformed molecule of *p*-aminobenzoylglutamic acid (figure 3).

Pteroic acid and glutamic acid also prove more effective in overcoming sulfonamide inhibition than when either metabolite is used alone (figure 4). The most effective combination used was glutamic acid and pteroic acid at concentrations of 1×10^{-2} M and 1×10^{-4}, respectively. Although pteroic acid overcomes sulfonamide in-

hibition at the end of 24 hours for *E. coli*, this effect is much more pronounced in the early hours when it is used in combination with glutamic acid. For *A. aerogenes* pteroic acid alone has very little effect in overcoming sulfonamide inhibition, while glutamic acid has none at all. The combination of glutamic acid and pteroic acid is strikingly effective, and growth approaches the control containing no sulfonamide (figure 4).

The third combination, glutamic acid and folic acid, has no effect for either *E. coli* or *A. aerogenes* at concentrations ranging from 1×10^{-2} M to 1×10^{-6} M (Havas, 1950). This is to be expected since neither folic acid nor "teropterin" is growth promoting or antagonistic for either organism.

DISCUSSION

Lampen and Jones (1946*a*, *b*, 1947) report that for organisms requiring the preformed molecule of folic acid, high levels of pteroylglutamic acid or pterolytriglutamic acid are effective in overcoming sulfonamide inhibition. Strains capable of synthesizing their own folic acid are sensitive to sulfonamide inhibition when dependent upon this synthesis, but they are resistant in the presence of the preformed molecule.

In our experiments with *E. coli* and *A. aerogenes*, which also synthesize their own requirements, pteroylglutamic and pteroyltriglutamic acid proved entirely ineffective in overcoming sulfonamide inhibition, even when they were used at the highest possible levels.

An explanation for the inability of some organisms to utilize the preformed molecule was offered by Woods (1950, 1951) who postulated that there might be several higher forms of folic acid into which the commercial folic acid cannot be converted by some microorganisms. The recently isolated citrovorum factor might be one of these forms (Bond *et al.*, 1949; Bardos *et al.*, 1949).

As there are species specific proteins, there might be species specific folic acids which differ but slightly, i.e., in their mode of linkage, active methyl groups, or number of glutamic acid residues. The various organisms might differ in their ability to synthesize the final form of folic acid from the preformed molecule. This would explain the apparent inability of *A. aerogenes* and *E. coli* to utilize folic acid or "teropterin".

Pteroic acid was found (Lampen and Jones, 1946*b*, 1947) to be ineffective in overcoming sulfonamide inhibition for *Lactobacillus arabinosus* which synthesizes its own supply of folic acid, but was effective for *S. faecalis*, strain R, and *L. casei* which require the preformed molecule for growth.

Experiments conducted in this laboratory showed that for both *E. coli* and *A. aerogenes* which synthesize their own requirements, pteroic acid is an effective antagonist. This antagonism by pteroic acid for *A. aerogenes* is even more pronounced when it is used in combination with glutamic acid. For *E. coli* the antagonistic effect might be due to the *p*-aminobenzoic acid moiety of the molecule. This cannot be the case for *A. aerogenes* for which a similar concentration of *p*-aminobenzoic acid is entirely ineffective. It can be assumed, therefore, that for *A. aerogenes*, at least, pteroic acid is antagonistic, possibly because it is a naturally occurring intermediate in the synthesis of a folic acid, folinic acid, or higher forms of folic acid.

p-Aminobenzoic acid completely reverses the inhibition by sulfonamides for *E. coli* but not for *A. aerogenes*. It is suggested, therefore, that *p*-aminobenzoic acid is a normal intermediate for *E. coli* but not for *A. aerogenes*, which might use another path of synthesis leading to another form of this growth factor.

In accordance with the findings of other workers (Lampen and Jones, 1946*b*, 1947; Williams, 1944), *p*-aminobenzoylglutamic acid was found to be less effective than *p*-aminobenzoic acid as an antagonist. The optimum effective concentration, which is 100 times that required of *p*-aminobenzoic acid for *E. coli*, does not overcome sulfonamide inhibition as completely as does *p*-aminobenzoic acid. It is of interest to note that it is also antagonistic for *A. aerogenes* for which *p*-aminobenzoic acid has no effect. *p*-Aminobenzoylglutamic acid was inhibitory when used alone, i.e., without sulfonamide, possibly because in the absence of sulfonamide it undergoes complete hydrolysis to *p*-aminobenzoic acid and glutamic acid and *p*-aminobenzoic acid is inhibitory at certain concentrations. In the presence of sulfonamide this hydrolysis may take place to a lesser degree, or possibly not at all.

In a sulfonamide containing medium the synthesis of folic acid or a higher form thereof is aided by supplying *p*-aminobenzoic acid, pteroic acid, or pteroic acid and glutamic acid but not by supplying the preformed molecule of folic

acid. It is suggested, therefore, that *p*-aminobenzoic acid, pteroic acid, and glutamic acid are naturally occurring stepping stones in the synthesis of the final form of folic acid, while the synthetic folic acid cannot be transformed into the specific molecule used by *E. coli*.

For *A. aerogenes*, *p*-aminobenzoic acid seems not to be a naturally occurring intermediate, but the synthesis might involve *p*-aminobenzoylglutamic acid and possibly proceed via pteroic acid to its specific molecule of folic acid.

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SUMMARY

A quantitative study on the metabolite-antimetabolite relationship of folic acid and related compounds was carried out by growing *Escherichia coli* and *Aerobacter aerogenes* on a chemically defined medium. Studying the effect of folic acid and parts thereof on microorganisms with apparently similar nutritional requirements, it was shown that the various metabolites showed a marked difference in their antagonistic effects on two microorganisms with apparently similar growth requirements.

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