INHIBITION STEPS IN SULFONAMIDE BACTERIOSTASIS OF *ESCHERICHIA COLI*

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The effectiveness of *p*-aminobenzoic acid (PABA) in overcoming sulfanilamide (SA) growth inhibition of *Escherichia coli* was explained on the basis that PABA was an essential metabolite and that SA interfered with its utilization by the cell (Woods, 1940). Reversal of sulfonamide action by structurally unrelated metabolites such as methionine, xanthine, serine, thymine and valine (Shive, 1950; Winkler and de Haan, 1948; Winkler *et al.*, 1949) was assumed to be due to their being products of PABA associated enzyme system(s). The activity of these metabolites in replacing the need for PABA in the nutrition of induced mutants of *E. coli* (Lampen *et al.*, 1946, 1949) lent support to this interpretation.

Pteroylglutamic acid (PGA) is known to overcome sulfonamide inhibition non-competitively and to replace the PABA requirement of certain organisms (Lampen and Jones, 1946, 1947). PGA is, however, inactive with *E. coli*, though the organism is known to elaborate a factor with folic acid (FA) activity, this synthesis being inhibited by sulfonamides (Lascelles and Woods, 1952).

Vitamin B_{12} has been reported (Shive, 1950) to potentiate PABA action as, in its presence, higher concentrations of SA are required to inhibit the biosynthesis of methionine, xanthine and serine. Also vitamin B_{12} is known to decrease the PABA requirement for a PABA auxotroph of *E. coli* (Davis, 1951; Gots and Chu, 1952). In combination with methionine, it further decreases the need for PABA (Gots and Chu, 1952).

The foregoing and other known relationships among PABA, FA and vitamin B_{12} suggested a study of their effects in the reversal of sulfonamide action by the antagonists reported by Shive (1950) and Winkler and de Hann (1948). Such a study was also indicated by our observation that, with sulfadiazine (SD) as inhibitor and at rather high concentrations of the drug, the non-competitive antagonists reported by Winkler *et al.* (1950) in *E. coli* were no longer sufficient to reverse growth inhibition. A preliminary report has appeared (Alimchandani and Sreenivasan, 1955*a*).

METHODS

E. coli Macleod strain was maintained by fortnightly transfer on peptone yeast extract agar slants. The organism was grown at 30 C for 24 hr.

The basal medium employed was that of Green and Sevag (1946). The glucose was freed from traces of PABA by adsorption on norite at pH 3.0. The pH of the medium was adjusted to 7.6. A 100 mg per cent solution of SD was prepared in the double strength medium, and the pH was readjusted to 7.6. In the growth experiments, concentrations of SD were varied by using appropriate amounts of this SD solution in a final volume of 5 ml of the double strength medium which, after other additions, if any, was made to 10 ml with distilled water. All other solutions were adjusted to pH 7.6. With optically active amino acids, the racemic forms were employed. The media in tubes were sterilized at 15 lb of steam pressure for 15 min. A 40-fold dilution of a 24-hr growth in the basal medium was used to inoculate the tubes dropwise. Growth was measured turbidimetrically on a Klett-Summerson photoelectric colorimeter at 660 mμ and expressed in terms of galvanometer deflections. All results reported are averages of at least three different sets of experiments.

RESULTS

In studies on the reversal of growth inhibition of *E. coli* by SD it was observed that, as the drug concentration was increased, sequential additions of methionine, xanthine, serine, thymine, and valine were necessary to bring about reversal. The observations were in conformity with those reported by Winkler and de Haan (1948) for SA and, at a concentration of 2 to 5 mg per cent,

almost complete reversal of inhibition was observable with all 5 metabolites. At a concentration of 30 mg per cent there was partial reversal. However, as the concentration of the drug was further increased to 50 mg per cent they were no longer effective even when added in larger concentrations (table 1). In fact, it was observed that increased concentrations of serine were inhibitory.

Effect of mixtures of amino acids, purines and pyrimidines and vitamins. The inability of these metabolites to overcome growth inhibition by SD suggested that the drug blocked biosynthesis of one or more other essential metabolites. In an attempt to ascertain its (their) nature, mixtures of purines and pyrimidines, amino acids and the B group of vitamins were tried (table 2) and it was found that the amino acid mixture and, to a lesser extent, the vitamins would reverse drug inhibition of growth considerably. Incidentally, the amino acid mixture was stimulatory to growth even in the absence of added SD.

Using individual amino acids, it was observed that only glycine and, to a lesser extent, threonine were active in partially reversing SD growth inhibition. When added on a molar basis, glycine was found to be more active than threonine (table 3). A combination of these two did not improve the effect of glycine alone, suggesting that glycine was the product of the blocked enzyme reaction. The existence of an enzyme system

TABLE 1

Reversal of SD growth inhibition of Escherichia coli by methionine xanthine, serine, thymine and valine

SD per 10 ml of Medium	Additions to 10 Ml of Basal Medium					
	1 Nil	2 DL-Methionine, 0.5 mg.	3 As in (2) + xanthine, 0.25 mg.	4 As in (3) + serine, 0.2 mg	5 As in (4) + thymine, 0.25 mg	6 As in (5) + valine, 0.5 mg
	Growth at 48 hr (in terms of Klett readings)					
μg						
0	47	48	46	48	47	50
25	0	30	48	47	50	49
50	0	6	41	49	50	49
100	1	0	23	47	48	48
200	0	0	7	39	50	47
500	0	1	1	11	44	42
mg						
1	0	0	1	1	21	41
2	0	0	0	1	7	27
3	0	0	0	0	1	16
4	0	0	0	0	0	2
5	0	0	0	0	0	0

TABLE 2

Effect of amino acids, purines and pyrimidines and vitamins on SD growth inhibition of Escherichia coli

Additions to 10 ml of Medium*	Growth at 48 Hr	
	With SD, 5 mg per 10 ml	Without SD
None	0	51
Amino acid mixture†	54	75
Vitamin mixture‡	26	49
Purine-pyrimidine mixture§	0	50
PABA, 0.5 mg	49	48

* Basal medium + methionine (0.5 mg) + xanthine (0.25 mg) + serine (0.2 mg) + thymine (0.25 mg) + valine (0.5 mg).

† 0.2 mg each of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, norleucine, norvaline, phenylalanine, proline, threonine, tryptophan and tyrosine.

‡ Thiamin HCl, riboflavin, nicotinic acid, pantothenic acid and pyridoxin HCl (10 μg each), pteroylglutamic acid (3 μg), leucovorin [Lederle] (6 μg), biotin and vitamin B_{12} (10 mμg each).

§ 0.1 mg each of adenine, guanine and uracil.

TABLE 3

Comparative effects of glycine and threonine

Addition to Medium*	Growth at 48 Hr	
	With SD, 5 mg per 10 ml	Without SD
None	0	52
Glycine (M/6000)	23	51
Glycine (M/3000)	28	52
Glycine (M/1500)	27	52
DL-Threonine (M/6000)	15	49
DL-Threonine (M/3000)	19	51
DL-Threonine (M/1500)	19	49
Glycine + DL-Threonine (M/6000 each)	26	50
Glycine + DL-Threonine (M/3000 each)	29	49
Glycine + DL-Threonine (M/1500 each)	29	51

* As in table 2.

TABLE 4

Effect of glycine and vitamin B_{12}

Addition to 10 ml of Medium*	Growth at 48 Hr	
	With SD, 5 mg per 10 ml	Without SD
None	0	50
Glycine (0.25 mg)	27	49
Vitamin B_{12} (10 mμg)	21	49
Glycine (0.25 mg) + vitamin B_{12} (10 mμg)	41	51
PABA (0.5 mg)	50	52

* As in table 2.

TABLE 5

Effect of purine mixture

Additions to 10 ml of Medium*	Growth at 48 Hr	
	With SD, 5 mg per 10 ml	Without SD
None	0	46
PABA (0.5 mg)	46	47
Xanthine (0.25 mg)	40	48
Xanthine (0.5 mg)	38	47
Adenine + guanine + xanthine (0.1 mg each)	45	49

* Basal medium + methionine (0.5 mg) + serine (0.2 mg) + thymine (0.25 mg) + valine (0.5 mg) + glycine (0.25 mg) + vitamin B_{12} (10 mμg).

converting threonine to glycine in the organism is reported later.

The activity of the B vitamins in partially overcoming the growth inhibition was followed by using them individually under the same experimental condition as for the amino acids. Only vitamin B_{12} was observed to have an ability partially to reverse SD inhibition. Glycine and vitamin B_{12}, in combination, showed reversal effects which nearly approached that obtained with PABA (table 4).

In these experiments, the substitution of xanthine by a mixture of purines gave reversal of growth inhibition comparable to that obtained with PABA (table 5).

Glycine-serine relationship. The requirement for glycine in the presence of serine by the organism was rather unexpected in view of the known interchangeability of the two amino acids in several systems (Roepke *et al.*, 1944; Tatum, 1949). Whether this effect of glycine was due to an insufficient amount of serine in the medium was ascertained by using increased concentration of the latter. However, serine at concentrations higher than 0.2 mg per ml was found to be toxic. Increased concentrations of glycine could not also substitute for serine (table 6). It would seem therefore that in this organism there does not exist the mechanism for serine to glycine conversion. Alternatively, this step may be blocked by SD.

Enzymic conversion of threonine to glycine in E. coli. The activity of threonine in replacing glycine observed in the above experiments (table 3) suggested the existence of an enzymic mechanism in the organism capable of bringing about the transformation of threonine to glycine and acetaldehyde similar to the one reported in liver and in yeast (16, 17). This was ascertained by the following experiment.

Cells of *E. coli* grown in 500-ml lots of basal medium in Roux bottles were harvested by centrifugation and washed twice with ice water. The cells were crushed thoroughly with sand and freed from the latter by decantation. Aliquots of the crushed cell suspension (approximately 3 mg dry wt) were incubated with threonine in phosphate buffer at pH 7.2 (final conc. of buffer adjusted to 0.1 M). After incubation at 30 C

TABLE 6

Non-interchangeability of serine and glycine

Addition to 10 ml of Medium*	Growth at 48 Hr	
	With SD, 5 mg per 10 ml	Without SD
None	0	49
Serine (0.1 mg)	19	50
Serine (0.15 mg)	24	48
Serine (0.2 mg)	24	49
Serine (0.3 mg)	22	47
Serine (0.4 mg)	15	44
Glycine (0.25 mg)	0	48
Glycine (0.5 mg)	0	47
Serine (0.2 mg) + glycine (0.25 mg)	47	49

* Basal medium + methionine (0.5 mg) + thymine (0.25 mg) + adenine, guanine and xanthin (0.1 mg each) + valine (0.5 mg) + vitamin B_{12} (10 mμg).

TABLE 7

Conversion of threonine to glycine in Escherichia coli

System	Glycine Formed in 2 Hr	
	Without threonine	With threonine 0.6 mmole
	μg/10 mg dry wt cells	
Crushed cells	150	195
Crushed cells, heated	135	132

for 2 hr the reaction was stopped by addition of sodium tungstate and sulfuric acid, and glycine formed was estimated by the method of Alexander *et al.* (1945). It was observed (table 7) that threonine gives rise to glycine under the conditions of these experiments. In one experiment, when hydroxylamine (1 mmole final concentration) was included to trap the acetaldehyde, there was a 20 per cent increase in glycine formed.

Inactivity of PGA and leucovorin. The relationship disclosed earlier between PABA, FA and vitamin B_{12} suggested testing of their reversal effect on SD growth inhibition.

Employing the basal medium with additions of methionine, serine, thymine, valine, glycine, vitamin B_{12} and purine mixture (as in table 5) it was observed that none of these metabolites could be replaced by PGA (3 μg) or leucovorin (LV) (6 μg). This was so even when the concentration of B_{12} was increased to 100 mμg which was above that for reversal of SD inhibition.

Use of methionine precursors. The ability of methionine but not homocysteine or homocystine to replace vitamin B_{12} for growth of B_{12} auxotrophs of *E. coli* implicated vitamin B_{12} in *E. coli* metabolism in the conversion of homocysteine to methionine (Davis and Mingioli, 1950). However, when with *E. coli* methionine in the composite medium with purine mixture (table 5) was replaced by homocystine or homocysteine (0.5 mg each) no reversal of SD growth inhibition occurred. Increasing vitamin B_{12} concentration to 100 mμg also had no effect. This would suggest that, if homocystine or homocysteine were to be the precursors of methionine in *E. coli*, vitamin B_{12} and the other additions present in the medium are not sufficient for the biosynthesis of the methyl moiety.

DISCUSSION

The present experiments have shown that, in addition to the other metabolites discovered by Shive (1950) and Winkler and de Haan (1948), the biosynthesis of glycine is yet another reaction mediated by PABA and blocked by SD. The activity, to a lesser extent, of threonine at the stage where glycine is involved in overcoming SD inhibition, and the absence of any increase in antisulfonamide activity when these two amino acids are used in combination, suggests that it is the synthesis of glycine that is blocked. The activity of threonine thus appears to be due to an ability of the organism to convert it to glycine. The existence of such a mechanism in *E. coli* cells was confirmed experimentally. Such a possibility has been indicated by Ravel *et al.* (1948) who showed that accumulation of 4-amino-5-imidazole carboxamide was increased by glycine and to a lesser extent by threonine during sulfonamide bacteriostasis. Other workers also have shown the presence of an enzyme, in tissues of various animals and in plasmolysed yeast cells, capable of converting threonine to glycine (Braunshtein and Vilenkina, 1949; Meltzer and Sprinson, 1952).

The interchangeability of glycine and serine in several systems (Roepke *et al.*, 1944; Tatum, 1949) suggested that the need for glycine could be due to an insufficiency of serine and *vice versa.* However, using increased concentrations of both these metabolites, it has been demonstrated that they cannot replace each other as antisulfonamide agents, which suggests that the biosynthesis of both these metabolites is blocked independently by the drug.

The potentiating action of vitamin B_{12} on the reversal of SD growth inhibition by the various metabolites, and its sparing effect on PABA requirement of *E. coli* mutants (Davis, 1951), could be explained as due to a function for PABA in B_{12} synthesis or for B_{12} in the biosynthesis of PABA or of its conversion to its active coenzyme form. Davis (1951) had suggested that probably PABA is the precursor of the benzimidazole moiety of B_{12}. However, pseudovitamin B_{12}, which contains adenine in place of the benzimidazole moiety, possesses the activity of the vitamin for microorganisms (Davis, 1952).

In the case of the B_{12} auxotroph of *E. coli*, methionine replaces the vitamin for growth

(Davis and Mingioli, 1950). But during sulfanilamide bacteriostasis higher concentrations of the drug are required to inhibit growth in the presence of B_{12} than in the presence of methionine (Alimchandani and Sreenivasan, 1955*b*). This suggests that B_{12} has other functions than the biosynthesis of methionine in the *E. coli* mutant, and that these additional functions become evident or are assumed during sulfonamide growth inhibition. This latent role(s) of vitamin B_{12} appears to be identical to that exhibited by the vitamin with the wild strain during inhibition of growth by sulfanilamide (Shive, 1950).

Growth studies with B_{12} auxotroph have implicated B_{12} in the synthesis of methionine from homocysteine (Davis and Mingioli, 1950). The inability, however, of homocysteine and B_{12} to replace methionine in overcoming SD bacteriostasis in the wild strain suggests that PABA also is involved in methionine synthesis of this stage. Gibson and Woods (1952) have indicated the role of these two vitamins in this conversion. Cell-suspensions of an *E. coli* mutant requiring PABA have been shown by these workers to require PABA for this transformation; the further addition of B_{12} is stimulatory though B_{12} alone is inactive.

The greater activity of the purine mixture over xanthine alone suggests either that xanthine is not a normal intermediate in the biosynthesis of other purines or that SD interferes with the interconversion of purines as well.

The inability of PGA or LV to replace PABA for the growth of a PABA auxotroph has also been reported by Davis (1951). It has further been observed by us that both PGA and LV, even in the presence of large concentrations of B_{12}, could not substitute for any of the other metabolites in overcoming SD inhibition. This could be due to any of the following reasons: (1) PABA is involved in these syntheses not through FA. (2) PGA or LV are not intermediates in the biosynthetic pathway of *E. coli* for the formation of the functionally active form of FA required by the organism. (3) Preformed PGA and LV cannot be absorbed by this organism. These points are further discussed in a subsequent communication.

Contrary to the observations of Winkler and de Haan (1948) and Shive (1950), PGA or LV could not substitute for thymine in the combination of metabolites effective in reversing sulfonamide growth inhibition in *E. coli*. In our experience even LV was ineffective. A similar inactivity of PGA is reported by Davis (1951) with an *E. coli* PABA auxotroph.

SUMMARY

Reversal of sulfadiazine (SD) growth inhibition in *Escherichia coli* Macleod strain has been examined with several metabolites functionally related to *p*-aminobenzoic acid (PABA).

It was observed that vitamin B_{12} and glycine or to a lesser extent threonine were essential (in addition to the known combination of methionine, xanthine, serine, thymine and valine) to obtain reversal at high concentrations of SD.

Substitution of xanthine by a mixture of purines together with additions of the above mentioned metabolites gave reversal of growth inhibition comparable to that obtained with PABA.

The activity of threonine was shown to be due to an ability of the organism to convert it to glycine.

From studies with varying concentrations of glycine and serine, in SD inhibited organisms, it was concluded that sulfonamide blocks the biosynthetic mechanism(s) for glycine serine interconversion.

Folic acid or leucovorin were inactive in reversing SD growth inhibition or in replacing any of the SD reversing metabolites even in the presence of excess B_{12}.

Homocysteine or homocystine could not replace methionine even in combination with B_{12}.

The observations are discussed in relation to the inhibition steps in sulfonamide bacteriostasis.

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