STUDIES ON THE ACCUMULATION OF 4-AMINO-5-IMIDAZOLE CARBOXAMIDE IN *ESCHERICHIA COLI*

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When cultures of *Escherichia coli* are partially inhibited by sulfonamides they accumulate a diazotizable and nonacetylable amine in the medium (Fox, 1942). The amine was isolated by Stetten and Fox (1945) and characterized by Shive *et al.* (1947) as 4-amino-5-imidazole carboxamide. The latter authors suggested that the amine was a precursor of purines as it only required the addition of a one carbon fragment for completion of the purine skeleton. Its transformation to purines is mediated by *p*-aminobenzoic acid (PABA) and sulfonamides interfere in this conversion.

The nature of this amine for purine synthesis in *E. coli* has since been amply confirmed. Some purine requiring mutants of this organism accumulate it (Gots, 1950). Ben-Ishai *et al.* (1951) have reported that certain other purine auxotrophs of *E. coli* can utilize the amine for growth, though higher concentrations of it are needed.

Woolley and Pringle (1950) observed that the amine accumulated during growth inhibition of *E. coli* by aminopterin, suggesting that PABA acted through folic acid (FA) in the conversion of the amine to purine. The implication of FA in purine synthesis has been suggested by other lines of investigation. Purines have been shown to be active in decreasing or replacing the FA requirement for growth (Mitchell and Snell, 1941; Snell and Mitchell, 1941; Stokstad, 1941; Stokes, 1944; Krueger and Peterson, 1945) and in overcoming growth inhibition of a variety of microorganisms caused by FA analogue antagonists (Rogers and Shive, 1948).

Vitamin B_{12} has also been implicated in purine biosynthesis. Shive (1950) has shown that in the presence of vitamin B_{12} high concentrations of sulfanilamide are required to inhibit the synthesis of purines. Purine auxotrophs of *E. coli* utilize the amine for growth better in the presence of vitamin B_{12} (Ben-Ishae *et al.*, 1951).

The involvement of vitamin B_{12} and FA in purine synthesis discussed above suggested a study of the effects of pteroylglutamic acid (PGA), leucovorin (5-formyl,5-6-7-8-tetrahydrofolic acid; LV) and vitamin B_{12} on amine accumulation under cultural conditions of restricted growth in *E. coli*.

Methionine has also been included in the experiments for a study of its effects in view of the replaceability (Davis and Mingioli, 1950) of methionine and B_{12} for growth of certain induced mutants of *E. coli*.

METHODS

Escherichia coli (MacLeod strain) and the mutant strain of *E. coli* requiring vitamin B_{12} (NCIB 8134) were both maintained by fortnightly transfer in peptone yeast extract agar slants (Alimchandani and Sreenivasan, 1957*a*, 1957*b*).

The basal medium employed for the studies reported here was that of Green and Sevag (1946) supplemented with glycine which is known to stimulate the production of the amine (Ravel *et al.*, 1948). In case of the mutant, vitamin B_{12} or methionine were used additionally as supplements. The pH of the medium was adjusted to 7.

An aqueous solution of 10 mg per cent of sulfadiazine (SD) was prepared and pH adjusted to 7.

To 5 ml of double strength basal medium in test tubes the other additions were made and the volume adjusted to 10 ml. The tubes were capped and sterilized for 15 min at 121 C. A 40-fold dilution of a 24 hr growth in the basal medium was used to inoculate the tubes dropwise. Growth was measured in a Klett-Summerson photoelectric colorimeter at 660 mμ. The amine was estimated by the method of Bratton and Marshall (1939) after acetylation of interfering amines with acetic anhydride (Stetten and Fox, 1945). The concentrations of the amine were expressed in terms of galvanometer readings in the Klett-Summerson photoelectric colorimeter at 540 mμ.

RESULTS

The effects of PGA, LV, vitamin B_{12} and methionine on arylamine accumulation by *E. coli*

TABLE 1

Effect of various metabolites on amine accumulation in Escherichia coli

Additions	Sulfadiazine/ Medium	Growth at 24 Hr	Arylamine/ Medium
	μg/10 ml		*ml*
Nil	0	43	0
	5	22	27.5
	10	9	18.0
	15	7	16.0
	20	4	10.0
Pteroylglutamic acid (PGA) 3 μg/10 ml	0	42	0
	5	22	28
	10	10	18
	15	7	15
	20	3	8
Leucovorin (LV) 6 μg/10 ml	0	43	0
	5	23	27
	10	9	18
	15	8	15
	20	4	11
Vitamin B_{12} 20 mμg/10 ml	0	43.5	0
	5	43.0	3.0
	10	41.0	6.0
	20	40.0	17.5
	30	38.5	23.0
	50	33.0	39.5
Methionine 0.5 mg/ 10 ml	0	43.0	0
	5	42.0	14.0
	10	38.0	28.5
	20	35.0	42.0
	30	30.5	61.0
	50	21.0	46.0

in the presence of varying concentrations of SD are reported in table 1.

It may be seen that LV or PGA do not reverse growth inhibition by SD nor have they any effect on amine accumulation. Both vitamin B_{12} and methionine influence growth inhibition as well as amine formation in SD bacteriostasis. Reversal of growth inhibition is more pronounced with vitamin B_{12} than with methionine. The reverse is the case with amine formation. This latter is also brought out when amine accumulation per unit growth is plotted against SD concentration (figure 1). When amine accumulation per unit growth is plotted against growth (figure 2) it is found to be more with methionine than with vitamin B_{12}. It should be borne in mind that to get a certain inhibition varying concentrations of SD are required in presence of the different metabolites.

The above experiments indicated an association of vitamin B_{12} and methionine in the transformation of the amine to purine. To elucidate this, experiments were undertaken using the *E. coli* mutant requiring B_{12} or methionine, as well as their antagonists with the wild strain.

A PABA mutant grown in suboptimal amounts of vitamin B_{12} has been reported (Gots and Chu, 1952) to accumulate the amine. If vitamin B_{12} or methionine were involved in a common pathway directly then the amine should be expected to accumulate when the B_{12} auxotroph is grown in suboptimal concentrations of B_{12} or methionine. However, with the B_{12} auxotroph of *E. coli* it was observed that there was no amine accumulation whatsoever.

It was also ascertained that cell suspensions of the mutant (0.2 mg dry wt/ml) when incubated in 5 ml of the basal medium for 12 hr without any addition of vitamin B_{12} or methionine did not accumulate any amine.

Ethionine was studied as an antagonist of methionine (Jensen *et al.*, 1951; Levine and Tarver, 1951; Levine and Fopeano, 1953) and a B_{12} oxidation product as an antagonist of B_{12} (Beiler *et al.*, 1951; Rege and Sreenivasan, 1954). In the latter case it was ensured that, contrary to the report of Hendlin and Wall (1954), the oxidation product exerted an inhibition independently of any sodium chloride concentration (Alimchandani

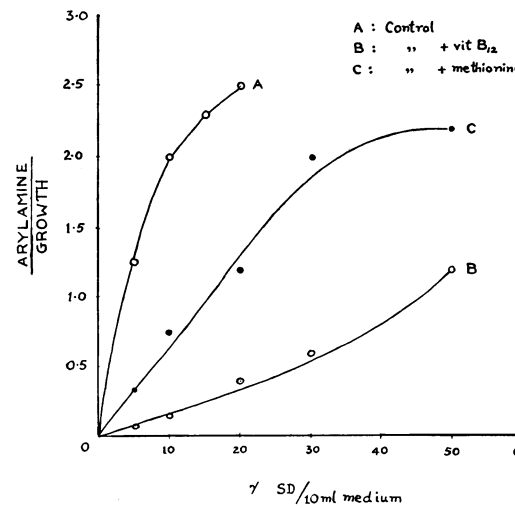

Figure 1. Influence of vitamin B_{12} (20 mμg/10 ml) and methionine (0.5 mg/10 ml) upon arylamine accumulation per unit growth of *Escherichia coli* (MacLeod strain) in the presence of increasing levels of sulfadiazine.

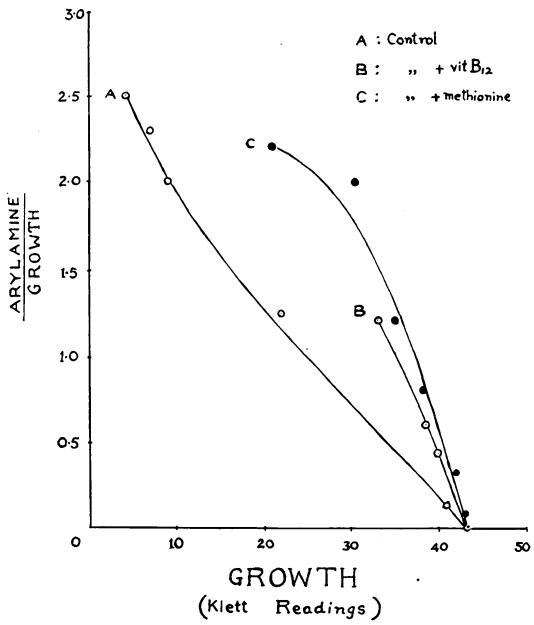

Figure 2. Influence of sulfadiazine, vitamin B_{12}, and methionine upon the accumulation of arylamine (per unit growth) as determined at different levels of growth of *Escherichia coli* (MacLeod strain).

and Sreenivasan, 1957*a*, 1957*b*). Using *E. coli* it was observed that both these inhibited growth though no amine accumulated.

DISCUSSION

While increasing concentrations of SD result in the expected increase in inhibition of growth of *E. coli*, amine accumulation in general bears no relation to growth. Further, PGA or LV has no effect on either growth inhibition or amine accumulation in SD bacteriostasis. Both vitamin B_{12} and methionine in the concentrations used overcome to a considerable extent growth inhibition by SD; vitamin B_{12} is more effective in this respect than methionine. While there seems no relationship between growth and arylamine formation, the latter is less in the presence of vitamin B_{12} than with methionine. This superiority of vitamin B_{12} over methionine is also seen when amine formation per unit growth is plotted against SD concentration. The nearly linear relationship obtained in all cases between SD concentration and amine accumulation per unit cell mass suggests that SD blocks the conversion of the amine to purine in proportion to its concentration. Purine formation from the amine would itself appear to be favored more by vitamin B_{12} than by methionine. Bergmann *et al.* (1952) had also observed a similar effect of vitamin B_{12}. However, they could not get depression of amine formation with methionine unless catalytic amounts of PABA were also present. These authors explained that methionine served as "methyl" donor. Methionine activity could also be explained on the basis that it spares the PABA required for its synthesis. The effect of vitamin B_{12} may be explained as due to its potentiating action on PABA-associated enzymes (Shive, 1950).

In view of the fact that the concentration of SD required to bring about the same degree of inhibition varies with methionine and vitamin B_{12} it was of interest to compare amine accumulation per unit growth with growth itself. When this is done (figure 2) it is seen that amine accumulation is least in the control set and is increased by methionine and to a less extent by vitamin B_{12}. It would seem that in the absence of methionine or vitamin B_{12} the growth inhibition is primarily due to obstruction in methionine synthesis while in the presence of methionine the inhibition is solely due to a block in the conversion of the amine to purine. As in the latter case, purine synthesis is the main reaction blocked, amine accumulation should be higher for the same degree of growth inhibition (figure 2).

In presence of vitamin B_{12}, the amine accumulation per unit growth is increased but not to the same extent as with methionine. This may be because vitamin B_{12} potentiates methionine synthesis to a greater extent than that of purines.

From the foregoing, it would seem that vitamin B_{12} and methionine are not involved directly in the metabolism of the amine. This view is further borne out by the results obtained with the B_{12} auxotroph of *E. coli* and with the methionine and B_{12} antagonists using the wild strain. There is a possibility that in these cases reaction(s) other than purine formation is the limiting one.

SUMMARY

The effects of pteroylglutamic acid (PGA), leucovorin (LV), vitamin B_{12} and methionine on the accumulation of 4-amino-5-imidazole carboxamide by *Escherichia coli* (MacLeod strain) during sulfadiazine (SD) bacteriostasis were studied.

PGA and LV were ineffective in reversing SD growth inhibition or in influencing amine accumulation; vitamin B_{12} and methionine at identical concentrations depressed the amine accumulation per unit growth to different degrees, vitamin B_{12} being more effective than methionine.

Amine accumulation when expressed as a variable against growth was more in the presence of methionine and least in the control set without added vitamin B_{12} or methionine, vitamin B_{12} effect being intermediate.

These observations are interpreted to mean that the primary effects of vitamin B_{12} or methionine are on growth and other aspects of cell metabolism that are interfered with by sulfonamides. Their involvement in amine to purine conversion is only indirect.

Ethionine and a vitamin B_{12} oxidation product depressed growth but no amine accumulation was observed.

The inability of an *E. coli* mutant to accumulate the amine under conditions of deficiency of vitamin B_{12} or methionine also suggested that these two metabolites are involved only indirectly in purine synthesis.

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