INACTIVITY OF PTEROYLGLUTAMIC ACID AND LEUCOVORIN IN OVERCOMING SULFONAMIDE GROWTH INHIBITION OF *ESCHERICHIA COLI*

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It was reported earlier (Alimchandani and Sreenivasan, 1957*a*) that in *Escherichia coli* (MacLeod strain) pteroylglutamic acid (PGA) and leucovorin (5-formyl,5-6-7-8,tetrahydrofolic acid; LV) are inactive in overcoming growth inhibition by sulfonamides or in substituting for any of the other metabolites (methionine purines, serine, thymine, valine, and glycine) which are active in reversing the same in spite of the vitamin being known to be intimately connected with the synthesis of most of these metabolites. It was also observed (Alimchandani and Sreenivasan, 1957*b*) that PGA and LV are unable to decrease the accumulation of arylamine by *E. coli* during sulfonamide bacteriostasis, though in the presence of aminopterin the biosynthesis of purines is known to be blocked at this step (Woolley and Pringle, 1950).

The following three points must be examined before definite conclusions can be drawn: (1) Folic acid (FA) is not involved in these systems in *E. coli*, (2) the functionally active form of FA for *E. coli* is different from PGA and LV and these can not be utilized as intermediates in its synthesis by the organism, and (3) PGA and LV are not utilized by the cell.

The experiments reported here are an attempt to find an explanation for the observed inactivity of PGA derivatives in replacing *p*-aminobenzoic acid (PABA) in overcoming inhibition of *E. coli* by sulfonamides.

METHODS AND RESULTS

Characterization of folic acid active factor elaborated by E. coli (MacLeod strain). Growth studies:—The response of *Streptococcus faecalis* to graded doses of *E. coli* culture filtrate was compared with that of PGA and LV.

E. coli was grown in 100 ml quantities in the medium of Green and Sevag (1946) dispensed in 250 ml Erlenmeyer flasks. Incubation was for 24 hr. Following this the contents were adjusted to pH 7, steamed for 5 min and the cells spun down in a centrifuge. Aliquots of the supernatant were used for the assay. Assays were done with *S. faecalis* using the method of Luckey *et al.* (1944) as modified by Mitbander and Sreenivasan (1954).

Growth curves with PGA, LV, and a suitably diluted culture filtrate concentrate of *E. coli* are shown in figure 1. The FA synthesised by the organism gave a response curve which differed from that for PGA and LV in that heavier growth was produced.

Since *E. coli* culture filtrate could support growth of both *S. faecalis* strain ATCC 8043 and *Leuconostoc citrovorum* strain ATCC 8081 (now identified as *Pediococcus cerevisiae* by Felton and Niven, 1953), a differential assay was carried out. The total *S. faecalis* activity was measured in terms of PGA using *S. faecalis* as assay organism. The citrovorum factor activity, expressed in terms of LV, was assayed by the method of Sauberlich and Baumann (1948) using *Leuconostoc citrovorum.*

In a typical experiment, *S. faecalis* activity was 1.2 mμg PGA/ml and CF activity was 0.97 mμg LV/ml. As LV is only half as active as natural CF (Keresztesy and Silverman, 1951; Cosulich *et al.*, 1952) the CF activity is in fact only 0.48 mμg/ml.

Inhibition analysis:—The activity of *E. coli* culture filtrate was compared with that of PGA and LV in overcoming growth inhibition of *S. faecalis* by the PGA analogue antagonists methyl folic acid (1 μg/ml) and aminopterin (0.03 μg/ml). Other details of the method were as above. Using a series of concentrations of the three substances, the inhibition indices were determined from the concentration of the compounds required to effect reversal. The inhibition index represents the ratio of the concentration of the inhibitor to that of the metabolite (McIlwain, 1942; Shive and Macow, 1946).

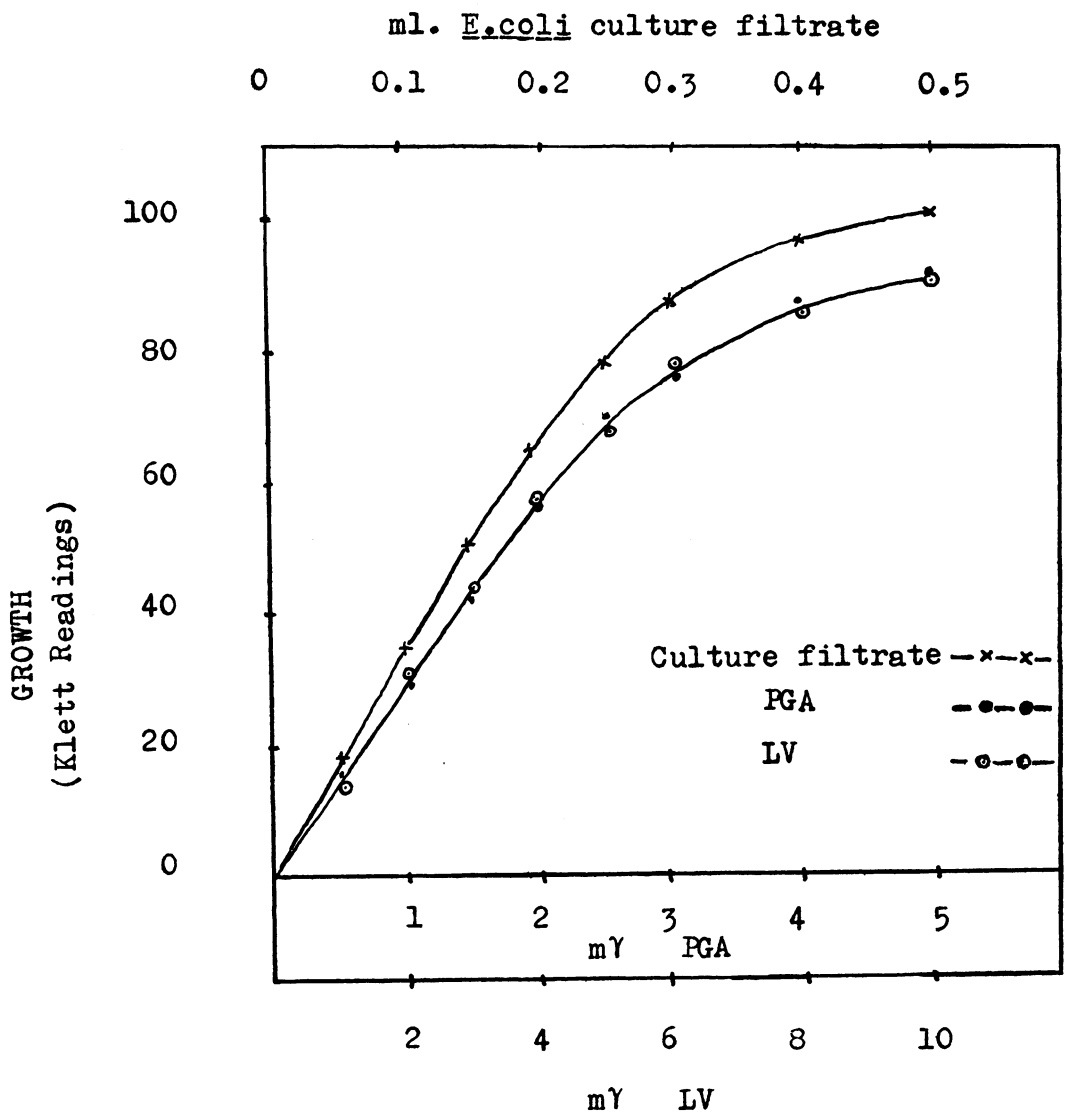

Figure 1. Growth response of *Streptococcus faecalis* strain R to culture filtrate of *Escherichia coli*

TABLE 1

Activity of Escherichia coli culture filtrate in overcoming growth inhibiton of Streptococcus faecalis by pteroylglutamic acid (PGA) antagonists

	Inhibition Index With:	
	Aminopterin 0.03 μg /ml	7-methyl PGA 1 μg/ml
Pteroylglutamic acid (PGA).....	0.2	120
Leucovorin (LV).....	3.1	400
E. coli culture filtrate.....	2.7	430

It was found that the factor elaborated by *E. coli* was more active than PGA in overcoming growth inhibition by both the antagonists. The activity of the factor approaches that of LV though it is slightly less with aminopterin and a little higher with 7-methyl folic acid (table 1).

Bioautography:—The technique followed was based on that of Winsten and Eigen (1948). A 100 fold concentrate of *E. coli* culture filtrate was prepared after neutralization by freeze-drying and was spotted on paper strips along with PGA and LV. Chromatograms were developed for 4 to 5 hr at room temperature in 0.1 M phosphate

TABLE 2

Bioautography of folic acid (FA) active factors in Escherichia coli culture filtrate

Sample	Rf Value
1. Pteroylglutamic acid (PGA).....	0.51
2. Leucovorin (LV).....	0.81
3. *Escherichia coli* culture filtrate concentrate	
(1).....	0.59
(2).....	0.74

The *E. coli* culture filtrate concentrate gave two spots with Rf values between those for PGA and LV. Thus it would seem that *E. coli* elaborates two FA active factors different from PGA and LV.

buffer of pH 7 (Zakrzewski and Nichol, 1953) by descending chromatography. Strips were allowed to dry and laid on agar basal medium seeded with *S. faecalis*. The use of the dye triphenyl-tetrazolium bromide in the medium was of value because of its reduction of red color at the region of growth, thus making spotting easy. The results (Rf values) of the bioautographic analysis are given in table 2.

Permeability of cells of E. coli to PGA and LV: —Cells of *E. coli* were harvested after 24 hr growth in the medium of Green and Sevag (1946) and washed twice with sterile saline. The cells were incubated aseptically with PGA and LV in presence of phosphate buffer of pH 7 in a final concentration of 0.1 M at 30 C. At the end of 2 hr, the cells were spun down in a centrifuge and suitable aliquots of the supernatant were used for assay. It was found that the cells excrete an active compound of *S. faecalis* into the medium; however, the difference between the experimental and control values was equivalent to the PGA or LV added as the case may be.

TABLE 3

Permeability of Escherichia coli cells to pteroylglutamic acid (PGA) and leucovorin (LV)

System Total Volume 5 ml	*Streptococcus faecalis* Activity/ml in Terms of PGA
1. Cells*.....	8
2. Cells + PGA 10 mμg/ml.....	17
3. Cells + LV 20 mμg/ml.....	18.5
4. PGA 10 mμg/ml.....	9.5
5. LV 20 mμg/ml.....	10.8

* Dry weight 3.2 mg in a final volume of 5 ml.

DISCUSSION

The FA active compound elaborated by *E. coli* gives higher growth response than PGA or LV with *S. faecalis*, indicating perhaps that this factor is different and more active than PGA or LV for this organism. The possibility exists that the culture filtrate contains activators. In a study of synthesis of FA activity by *Lactobacillus arabinosus*, Mitbander and Sreenivasan (1951) also reported on the differential response to growth of *S. faecalis* to the *L. arabinosus* culture factor. While the present work was in progress, Lascelles and Woods (1952) reported on the FA activity of *E. coli* culture filtrate with similar conclusions. The observations reported here show that the compound(s) elaborated by *E. coli* possesses citrovorum factor activity. However, the CF activity can not account for all the *S. faecalis* activity. This might be due to the fact that the compound has a low CF activity or that it is a mixture of two compounds, one lacking in CF activity.

The activity of the culture filtrate in reversing growth inhibition due to PGA antagonists is comparable to that of LV. Considering however that natural CF is twice as active as LV (Keresztesy and Silverman, 1951; Cosulich *et al.*, 1952) the factor can not be identical to the natural L-form of LV.

Bioautographic studies reveal the existence of two active factors distinct from PGA and LV though it may be possible that one is derived from the other.

The *S. faecalis* activity of these factors rules out the possibility that the factors are glutamic acid conjugates, as all known conjugates of PGA are inactive for this organism (Hutchings and Mowat, 1948).

Recent work (Blakley, 1954; Kisliuk and Sakami, 1955; Alexander and Greenberg, 1955) has indicated that tetrahydrofolic acid and its hydroxymethyl derivative are more closely related to the functionally active form of FA.

In the permeability experiment it was observed that there was an excretion of FA active compound into the medium. Nevertheless, the whole of the added PGA or LV is recoverable quantitatively from the medium indicating no diffusion of added compounds into the cell.

The FA active factors synthesized by *E. coli*

are different from PGA and LV as indicated by *S. faecalis* growth response curve, inhibition indices and bioautography. The possibility that PGA or LV may be intermediates in, or are concerned with, the biosynthesis of these factors can not be excluded. The inability of PGA and LV to diffuse into the cells may account for their inactivity in overcoming sulfonamide growth inhibition.

SUMMARY

Culture filtrate of *Escherichia coli* shows folic acid and citrovorum factor activities.

With *Streptococcus faecalis* strain R a dose response curve different from those for pteroylglutamic acid and leucovorin (5-formyl,5-6-7-8,tetrahydrofolic acid) is obtained.

Using methyl folic acid and aminopterin as growth inhibitors of *S. faecalis* it is found that the inhibition index of the culture filtrate is different from those of pteroylglutamic acid and Leucovorin (5-formyl,5-6-7-8,tetrahydrofolic acid).

Bioautography of the culture filtrate shows the existence of at least two factors distinct from both pteroylglutamic acid and leucovorin (5-formyl,-5-6-7-8,tetrahydrofolic acid).

Evidence is presented which indicates that cells of *E. coli* are impermeable to PGA or LV.

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