The Assimilation of Amino-acids by Bacteria

6. The Effect of Protein Synthesis on Glutamic Acid Accumulation and the Action thereon of Sulphathiazole

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SUMMARY: The level of free glutamic acid accumulating within cells of certain Gram-positive cocci is lower in growing cells than in 'resting' cells, other conditions being equal. Part of the glutamic acid assimilated by growing *Staphylococcus aureus* is condensed into peptides or proteins, thus accounting for this apparent drop in glutamic acid accumulation. Sulphathiazole interferes with this condensation of glutamic acid into peptide form.

When Gram-positive cocci are grown in a medium deficient in free glutamic acid and then suspended in a solution of glutamic acid and glucose, glutamic acid is assimilated and concentrated in the internal environment of the cells. As the amino-acid is assimilated a portion of it undergoes metabolism, and the level of free glutamic acid attained inside the cell represents a balance between the rate at which it enters the cell and the rate at which it is metabolized inside the cell. When assimilation is studied in washed suspensions of cells, the metabolism of glutamic acid inside the cell does not lead to peptide formation but consists of some other form of alteration of the free glutamic acid molecule (Gale & Mitchell, 1947). The results presented in this communication show that in growing cells there is a further metabolism which results in peptide and protein formation and that this form of metabolism is inhibited by sulphathiazole.

Methods and organisms. The methods of estimation of glutamic acid, growth and assimilation have been described in the previous papers of this series (Gale, 1947). Two organisms have been used: *Staphylococcus aureus* 6773 isolated by Dr E. Topley and found by her to be sensitive to sulphathiazole at 1 mg./100 ml. concentration, and *Streptococcus faecalis* ST, used in the previous studies. Three growth media have been employed:

Medium A: tryptic digest of casein + 0·1 % (w/v) Marmite + 1 % (w/v) glucose.

Medium B: Stephenson's inorganic salts (Stephenson, 1939) + 0·1 % (w/v) Marmite + 1 % (w/v) glucose.

Medium C: 1 % (w/v) peptone (Difco) + 1 % (w/v) glucose.

Assimilation of glutamic acid by growing and resting cells of Streptococcus faecalis

The curves of Fig. 1 represent the internal concentration of glutamic acid/100 mg. dry weight of *Strep. faecalis* cells when these cells are tested at different ages under the various conditions indicated. Gale & Mitchell (1947) have

shown that curve 3 represents the assimilation of glutamic acid in the absence of internal metabolism, and the curve suggests that the capacity to assimilate this amino-acid is roughly constant throughout the growth period but falls rapidly soon after growth ceases. Curve 2 represents the balance between the rate of assimilation (or entry into the cell) and the rate of metabolism of glutamic acid in washed non-growing cells, so that the difference between curves 2 and 3 for each culture represents the proportion of assimilated glutamic acid undergoing metabolism (other than peptide formation) inside the cell.

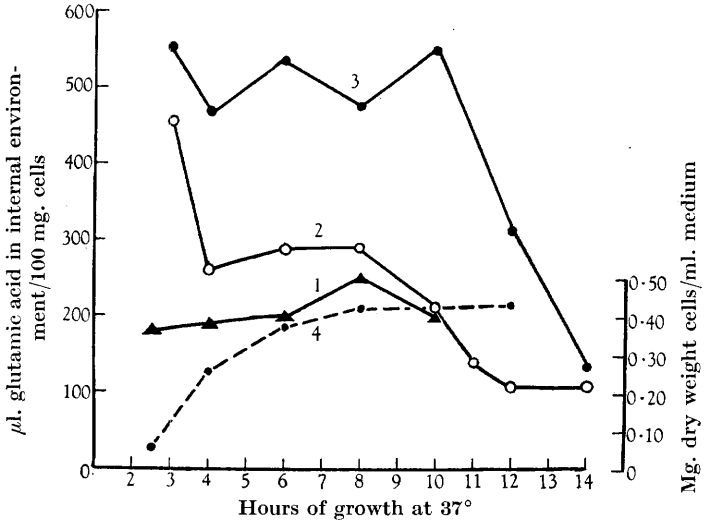

Fig. 1. Internal concentration of free glutamic acid in *S. faecalis* cells. Curve 1, cells grown in medium A (approx. 200 μl. free glutamic acid/ml.), harvested from the growing culture, washed and internal glutamic acid level assayed immediately. Curve 2, cells grown in medium B, harvested, washed, incubated for 1 hr. at 37° in glutamic acid (200 μl./ml.) and glucose (0·5%). Curve 3, cells grown and harvested as in 2 but incubation carried out in presence of crystal violet (1:10,000). Curve 4, growth curve in medium A.

Curve 1 shows a further decrease in the balance of free glutamic acid during the early stages of growth. Since the cells used to obtain curve 1 were growing but those for curve 2 were 'resting', it is possible that the difference between curves 1 and 2 represents, for each culture, the proportion of assimilated glutamic acid which is undergoing condensation into protein. It can be seen that curves 1 and 2 meet at that age of culture when active cell growth ceased, as judged turbidimetrically, and it has already been shown that peptide formation does not take place in non-growing cells of this nature (Gale & Mitchell, 1947). In the growing cells (curve 1) the level of free glutamic acid on balance rose as growth proceeded, suggesting that the proportion of assimilated glutamic acid entering into combination was greater in the early stages of growth. This aspect of assimilation can be studied more easily in *Staph. aureus*, since this organism accomplishes a higher concentration of glutamic acid in the internal environment than *Strep. faecalis* and consequently effects of this nature are of greater magnitude.

Accumulation of free glutamic acid inside growing Staphylococcus aureus *cells*

Fig. 2 shows the levels of glutamic acid attained inside cells of two strains of *Staph. aureus* at various times during growth in medium A. The curves are of the same nature as curve 1 in Fig. 1 and show the same general increase in concentration during growth. It can be seen that when growth was taking place most rapidly, between 5 and 6 hr., the accumulation of free glutamic acid was temporarily checked, again suggesting that this level within the cell is a balance between the rate of entry and the rate of combination into protein, etc. The

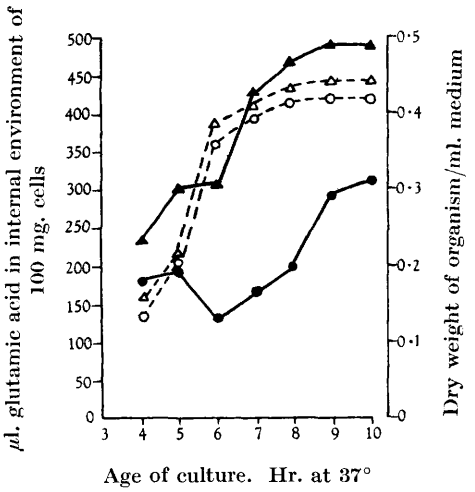

Fig. 2. Growth and accumulation of free glutamic acid in internal environment of *Staph. aureus*. Medium: casein digest+2% glucose+0·1% Marmite. Growth: strain A, O---O, strain D △---△; glutamic acid: strain A ●—●, strain D, ▲—▲.

same type of curve is given when growth and assimilation are followed in medium B and, for reasons explained below, this medium has been used for many of these studies. Fig. 1 shows that, in the case of *Strep. faecalis*, the amount of glutamic acid assimilated (i.e. withdrawn from the external environment) was approximately constant during the period of growth, and if the suggestion be true that the difference between curves 1 and 2 (Fig. 1) is due to condensation of free glutamic acid into protein or peptides, then it should be possible to show a variation in the ratio of combined/free glutamic acid at various stages of the growth period.

To test this *Staph. aureus* 6773 was grown in medium B in Roux bottles lying flat, all inoculated with a standard inoculum, and suitable amounts of the culture were harvested at various times during growth. The harvested cells were washed and the external, total and internal free glutamic acid assayed on samples as usual (Gale, 1947); the rest of the cells were then subjected to 20 hr. hydrolysis in boiling 5 N-HCl, the excess acid removed *in vacuo* and the total glutamic acid (free+that released from protein by hydrolysis) assayed. The ratio of the combined/free glutamic acid in the internal environment could

then be calculated. Table 1 shows a series of results obtained in such a manner. It can be seen that: (1) the free glutamic acid content of the cells rose during growth, as shown before; (2) the combined glutamic acid/100 mg. dry weight of cells fell during growth; (3) the ratio of combined/free glutamic acid fell from 5·6 for 3 hr. culture to 1·5 for 8 hr. culture; (4) the total (free + combined)

Table 1. *Free and combined glutamic acid content of growing* Staphylococcus aureus *cells*

Age of culture (hr.)	Growth (mg. dry wt. cells/ml.)	Glutamic acid (μl./100 mg. dry wt. cells) Free	Total	Combined	Ratio combined/free
3	0·07	160	1050	890	5·58
4	0·127	188	981	793	4·23
4½	0·164	215	1083	868	4·04
5	0·179	289	1116	827	2·86
6	0·232	371	1147	776	2·09
7	0·264	405	1134	729	1·80
8½	0·302	405	1003	598	1·48

glutamic acid/100 mg. cells was approximately constant throughout the growth period. It follows from these results that the difference between curves 1 and 2 Fig. 1 (or their equivalent for *Staph. aureus*) was due to protein or peptide synthesis and that glutamic acid after assimilation into the growing cells was partly incorporated into protein, etc.

Effect of sulphathiazole on protein formation

Sensitivity to sulphathiazole. Staph. aureus 6773 was stated by Dr E. Topley to be sensitive to sulphathiazole at 1 mg./100 ml. When tested in medium C sulphathiazole at 10 mg./100 ml. was necessary to prevent the growth of an inoculum of 10^6 cells/ml. Since it was desirable to deal with reasonably large amounts of cells, the effect of adding sulphathiazole to the medium 1 hr. after inoculation was tested. In medium C 100 mg. sulphathiazole/100 ml. brought the growth to a stop after 5 hr., when the crop was approximately 60 % of that in the control culture; in medium B 100 mg. sulphathiazole/100 ml. slowed the growth so that the final crop was 80 % of the control, but the two cultures ceased active growth together; in medium A it was not possible to demonstrate any significant action of sulphathiazole under these conditions. The three media differ mainly in their free amino-acid content; the internal concentrations of free glutamic acid attained in 100 mg. of cells at the end of growth in the three media (sulphonamide-free) were: A, 500 μl.; B, 405 μl.; C, 195 μl.

Absence of effect of sulphathiazole on glutamic acid assimilation by washed cells. Cells harvested from medium B were incubated in the presence of glutamic acid (200 μl./ml.) and glucose (0·5 %) as usual, and the increase in the internal level of free glutamic acid assayed as previously described (Gale, 1947). Sulphathiazole even in saturated solution had no effect on the assimilation process under these conditions.

Effect of sulphathiazole on glutamic acid accumulation in growing cells. The organism was cultivated in medium B and sulphathiazole added to portions of the culture after inoculation; later the cells were harvested and their internal glutamic acid level assayed. Table 2 shows that the presence of sulphathiazole in the growth medium increased the amount of glutamic acid accumulating in the cells at the time of harvesting. Previous experience with the action of triphenylmethane dyes on assimilation (Gale & Mitchell, 1947) showed that an increase in the level of glutamic acid inside the cell might be attributable to an inhibition of some metabolic process involving glutamic acid inside the cell. Since sulphathiazole had no effect on assimilation in washed cells, it does not affect the metabolic processes blocked by crystal violet under similar circumstances. However, the results given in Table 2 are shown only by growing cells and the work described here has shown that there is a further form of metabolism in such cells which results in condensation of free glutamic acid into a combination, presumably of peptide nature. The question arises whether the increased levels of free glutamic acid in the presence of sulphathiazole are attributable to disorganization of this protein synthesis.

Table 2. *Effect of sulphathiazole on accumulation of glutamic acid in internal environment of growing* Staphylococcus aureus

All cultures inoculated with *Staph. aureus* 6773 at time 0 and incubated at 37°. Sulphathiazole added as below at 1 hr., all cells harvested at 4½ hr. and washed before assay.

Sulphathiazole content of growth medium (mg./100 ml.)	Growth at harvesting (mg. dry weight of cells/ml.)	Glutamic acid in internal environment (μl./100 mg. dry wt. cells)
0	0·146	198
1	0·135	280
10	0·121	306
100	0·102	325

Fig. 3 shows the accumulation of free glutamic acid within cells growing in medium C in the presence and absence of sulphathiazole. It can be seen that whereas the curve has the normal shape for the accumulation of glutamic acid in cells growing in the absence of sulphathiazole, the curve obtained for the cells growing in the presence of sulphathiazole is approximately a straight line coming at a level slightly below that attained by the normal cells at the end of growth.

Table 3 shows the ratio of free and combined glutamic acid determined during growth of the organism in the presence of sulphathiazole in medium B for purposes of comparison with Table 1. The ratio is approximately constant over the period studied. It has been shown in the early part of this paper that the variation of the level of free glutamic acid in growing cells is due to protein formation such that the lower the level, the greater the rate of protein synthesis. The data presented in Fig. 3 and Table 3 show that the normal condensation of glutamic acid into protein becomes disorganized in the presence of sulphathiazole. It does not, of course, follow that sulphathiazole interferes directly

with the condensation of glutamic acid into peptide, as any interference or inhibition of protein synthesis would be expected to produce the results described

Table 3. *Free and combined glutamic acid content of* Staphylococcus aureus *growing in medium B+100 mg. % sulphathiazole*

Age of culture (hr.)	Growth (mg./ml.)	Glutamic acid (μl./100 mg. dry wt. cells) Free	Glutamic acid (μl./100 mg. dry wt. cells) Combined	Ratio combined/free	Ratio for cells of same age in control culture without sulphathiazole
3	—	—	—	—	5·58
4	0·09	284	821	2·89	4·23
4½	0·110	379	834	2·20	4·04
5	0·119	332	842	2·54	2·86
6	0·170	297	822	2·76	2·09
7	—	—	—	—	1·80

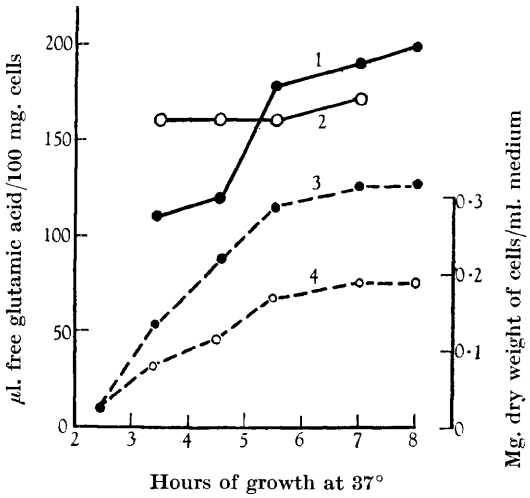

Fig. 3. Effect of sulphathiazole on accumulation of free glutamic acid in growing cells (*Staph. aureus*). Medium, 1% peptose+1% glucose. Curve 1, glutamic acid accumulation in normal medium. Curve 2, glutamic acid accumulation in medium containing 100 mg. sulphathiazole % added at 1 hr. Curve 3, normal growth curve. Curve 4, growth curve in medium containing 100 mg. sulphathiazole %.

DISCUSSION

The work described in this series of papers can be summarized as follows:

(1) The passage of glutamic acid across the cell wall of certain Gram-positive bacteria requires energy and this can be supplied by exergonic metabolism such as glycolysis (Gale, 1947).

(2) The Gram-positive bacteria examined assimilate glutamic acid and concentrate it in the free state in the internal environment so that, at equilibrium, the internal concentration is greater than that in the external environment (Gale, 1947; Taylor, 1947).

(3) This ability to concentrate glutamic acid in the free state within the cell is apparently confined to Gram-positive species (Taylor, 1947).

(4) Within the cells, glutamic acid undergoes metabolic change. In growing cells, part of the assimilated glutamic acid is condensed into peptides or proteins. In non-growing as well as in growing cells, other forms of metabolism take place during assimilation (Gale & Mitchell, 1947).

(5) Tyrocidin and detergent substances release the glutamic acid from inside the cells by modification of the permeability of the cell wall (Gale & Taylor, 1947*a*), Tyrocidin causes an actual rupture or partial solution of the cell wall of *Strep. faecalis* (Mitchell & Crowe, 1947).

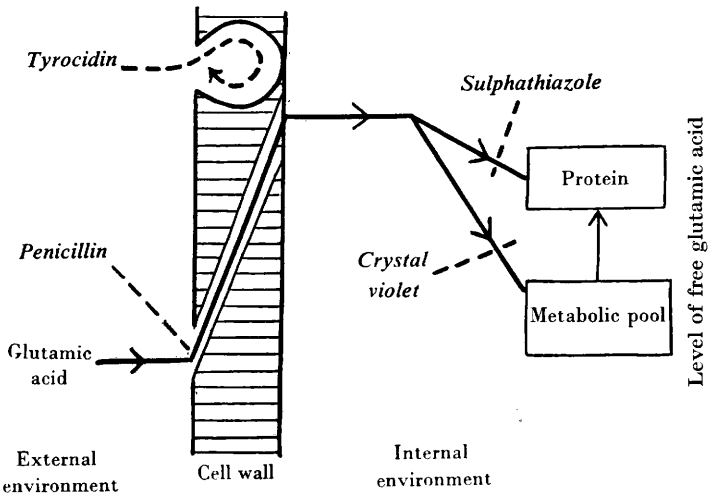

Fig. 4. Assimilation of glutamic acid by Gram-positive bacterial cell and action thereon of chemotherapeutic agents.

(6) The level of free glutamic acid attained within the cell depends upon (*a*) the external concentration and (*b*) the balance between the rate of entry into the cell and rate of metabolism within the cell. Consequently, any substance inhibiting the internal metabolism will give rise to an increased concentration of free glutamic acid within the cell while any substance interfering with the passage of the amino-acid across the cell wall will give rise to a decreased concentration within the cell.

(7) Penicillin prevents the passage of glutamic acid across the cell wall but does not interfere with the internal metabolism of glutamic acid. Penicillin acts in this way only if the cells concerned are grown for a short period in its presence (Gale & Taylor, 1947*b*).

(8) Triphenylmethane dyes inhibit the internal metabolism of glutamic acid in *Strep. faecalis* (Gale & Mitchell, 1947).

(9) Sulphathiazole interferes with the formation of protein, etc., from glutamic acid in growing cells.

These findings are represented diagrammatically in Fig. 4. The question arises as to how far the effects described for the action of various substances

can explain the antibacterial action of these substances. All the substances tested so far are markedly more effective against the Gram-positive bacteria than the Gram-negative species but some of them, e.g. the dyes and sulphathiazole, are effective in high concentration against the latter. Taylor (1947) has shown that the concentration of free glutamic acid within the cells was a property only of the Gram-positive organisms examined, but it may be that the later stages of protein synthesis, etc., within the cell are common to all bacteria. In general Gram-negative bacteria are able to synthesize glutamic acid and consequently do not need to assimilate the free amino-acid, so that they would not be expected to be sensitive to the action of a substance whose only function was to prevent such assimilation. This may be the case with penicillin (see Gale & Taylor, 1947*b*). If the later stages of the assimilation process—peptide condensation and amino-acid interchange—are essentially similar in all bacterial cells then one would expect their sensitivity to inhibitors of these processes to depend upon the importance of the rates of the relevant reactions and the concentrations of relevant reactants in the cells. To understand the importance of assimilation processes with regard to dye and sulphonamide action, it will be necessary to determine how the anabolic processes in Gram-negative organisms are related to these disclosed for Gram-positive species. The relation between these processes and resistance to penicillin, the sulphonamides and dyestuffs also needs investigation. The action of tyrocidin and the detergents lies in a rupture of the cell membrane, and the release of glutamic acid etc. from the internal environment is therefore merely symptomatic of the release of all soluble cell-constituents (Hotchkiss, 1944).

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