The Assimilation of Amino-acids by Bacteria

10. ACTION OF INHIBITORS ON THE ACCUMULATION OF FREE GLUTAMIC ACID IN *STAPHYLOCOCCUS AUREUS* AND *STREPTOCOCCUS FAECALIS*

BY E. F. GALE
Medical Research Council Unit for Chemical Microbiology, Biochemical Laboratory, University of Cambridge

(*Received* 21 *June* 1950)

Certain Gram-positive bacteria have the ability to concentrate certain amino-acids in the free state within the cell (Gale, 1947; Taylor, 1947). Amino-acids may pass across the cell wall either by a process of diffusion, as in the case of lysine which becomes concentrated within the cell by a form of Donnan effect (Najjar & Gale, 1950), or as a result of an active transfer which requires energy obtained from exergonic metabolism such as fermentation. Glutamic acid does not enter either *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) or *Streptococcus faecalis* unless these organisms are metabolizing glucose, but the mechanism of this 'energy-linked' transfer across the cell wall is not yet known. Gale & Mitchell (1947) showed that the concentration of the free amino-acid within the cell is determined by the balance between the rate of passage into the cell and the rate of metabolism within the cell; thus a substance such as crystal violet which inhibits the internal metabolism of glutamic acid in *Strep. faecalis* increases the steady state concentration of free glutamic acid within the cell, while treatment of the cell with penicillin (Gale & Taylor, 1947) inhibits the passage of glutamic acid into *Staph. aureus* so that the internal concentration decreases until exhausted by internal metabolic processes. The passage of glutamic acid into *Staph. aureus* is also inhibited by 8-hydroxyquinoline which appears to act by chelation of a metal essential to the process; deprivation experiments show that the transfer is activated by manganese, although this can be replaced by magnesium in higher concentration (Gale, 1949).

Clifton (1946) has shown that oxidative assimilation of carbon substrates in Gram-negative organisms is inhibited by sodium azide or 2:4-dinitrophenol. These, together with arsenate, appear to act by uncoupling the generation of energy-rich phosphate bonds from oxidative processes (Needham & Pillai, 1937; Loomis & Lipmann, 1948; Cross, Taggart, Covo & Green, 1949). Spiegelman, Kamen & Sussman (1948) have shown that sodium azide prevents the transfer of inorganic phosphate to the organic fraction accompanying the coupled oxidation of glyceraldehyde phosphate. The present communication reports the action of these inhibitors on the accumulation of free glutamic acid within *Staph. aureus* and *Strep. faecalis*. During the course of the work it became clear that the process involved in the passage of glutamic acid into *Staph. aureus* has properties and sensitivities to inhibitors different from those of the similar process in *Strep. faecalis*. This can be correlated with differences previously noted in the quantitative aspect of the internal metabolism of glutamic acid (Gale & Rodwell, 1949) and in the conditions controlling the diffusion of free glutamic acid out of the cells (Gale, 1948).

METHODS

Organisms. Three organisms were used in the course of this work: *Staph. aureus* Duncan (Gale & Taylor, 1947); *Strep. faecalis* ST (National Collection of Type Cultures (N.C.T.C.) no. 6782) and *Strep. faecalis* Dunn (N.C.T.C. no. 6783).

Growth media. The organisms were grown for 16 hr. at 30° in the 'deficient' medium B previously described (Gale, 1947). This consists of a salt medium containing 1% glucose, 0·1% marmite and 0·1% (w/v) arginine; for the growth of *Strep. faecalis* strains pyridoxin, riboflavin and pantothenic acid were also added. The medium was made up in flasks for *Strep. faecalis*, and in 150 ml. quantities in Roux bottles to give good aeration for the growth of *Staph. aureus*. The cells were harvested on the centrifuge and washed once with distilled water. Suspensions were prepared containing approx. 20 mg. dry wt. of cells/ml. in water and these were diluted 1:10 in the reaction solutions for the experiments described below.

Estimation of glutamic acid and its accumulation within the cells. Free glutamic acid was estimated manometrically with glutamic acid decarboxylase preparations (Gale, 1945). The accumulation of free glutamic acid by the washed cells was studied as previously described (Gale, 1947) by estimation of the glutamic acid content of cell suspensions before and after rupture of the cells by boiling.

RESULTS

Preliminary investigations. Investigations of the inhibitory action of sodium azide on the passage of glutamic acid into bacterial cells were first carried out in parallel on *Staph. aureus* and *Strep. faecalis* strains. It was then found that concentrations of sodium azide which caused an inhibition of the

passage of glutamic acid into *Staph. aureus* produced a marked increase in the amount of this amino-acid which accumulated under similar experimental conditions inside *Strep. faecalis*. It became clear that there are at least two processes, first, the passage of glutamic acid from outside to the inside of the cell and, secondly, the metabolism inside the cell after the first process has taken place. It seemed that sodium azide affects the first of these two processes in *Staph. aureus* whereas, in *Strep. faecalis*, the second process is more sensitive to the action of azide than the first; consequently, an enhanced accumulation of free glutamic acid is found in the latter case but not in the former. Later investigations showed that many of the inhibitors studied have this differential action. Consequently, it is necessary to present separately the results obtained with the two organisms.

Inhibition of the accumulation of glutamic acid in Staphylococcus aureus

Sodium azide and 2:4-dinitrophenol. Fig. 1 shows the degree of inhibition of the rate of accumulation of free glutamic acid within *Staph. aureus*. The passage

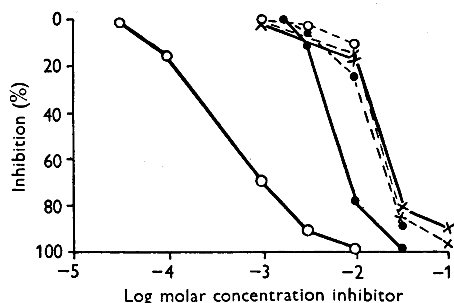

Fig. 1. Effect of inhibitors on glutamate accumulation and glucose fermentation in *Staph. aureus*. Organisms grown in medium B (Gale, 1947); prepared in washed suspension and incubated at 37° in buffered salt solution containing 1% glucose and 10 μmol. sodium glutamate/ml. Free glutamate within cells determined at intervals and rate of accumulation determined with and without inhibitors. Fermentation investigated in Warburg manometers containing 1·0 ml. washed suspension (3–4 mg. dry wt./ml.), 1·0 ml. 0·03 M-$NaHCO_3$, 0·5 ml. 0·05 M-glucose and 0·5 ml. water or inhibitor solution; manometers gassed with N_2 containing 5% CO_2. ——, glutamate accumulation; ----, glucose fermentation. ○, 2:4-dinitrophenol; ●, sodium azide; ×, sodium fluoride.

of this amino-acid into the cell is an energy-linked reaction and only occurs, under the experimental conditions, when fermentation of glucose occurs as source of energy. The figure also shows the inhibition of the rate of glucose fermentation, and it can be seen that the passage of glutamate into the cell is markedly more sensitive to inhibition by sodium azide and 2:4-dinitrophenol than the energy-providing fermentation system. In the case of sodium azide, the concentration producing 50% inhibition is 0·006 M for the passage of glutamate and 0·014 M for fermentation; the corresponding concentrations for 2:4-dinitrophenol are 0·0004 and 0·02 M. The degree of inhibition varies with the pH of the reaction mixture; Fig. 2 shows that the sensitivity of the glutamate-transferring system to dinitrophenol increases 300 times if the pH falls from the usual experimental value of 7·0 to 5·0.

Fig. 1 also shows results obtained with sodium fluoride as inhibitor. In this case the accumulation of free glutamate is inhibited to the same extent as the fermentation system, suggesting that impairment of energy provision causes an equivalent inhibition of glutamate transfer. Other inhibitors, whose action is like that of fluoride in this respect, are iodoacetic acid and patulin.

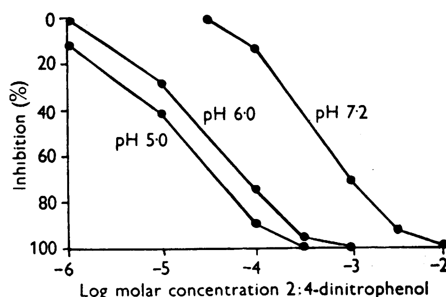

Fig. 2. Effect of pH on the inhibition of glutamate accumulation in *Staph. aureus* by 2:4-dinitrophenol. Conditions as for Fig. 1 with adjustment of buffered salt solution to pH values shown.

Sodium arsenate. The effect of sodium arsenate on the rates of glycolysis and of glutamate accumulation in *Staph. aureus* is shown in Table 1. 0·0001 M-Sodium arsenate causes 60% increase in the rate of fermentation, although concentrations higher than 0·01 M are inhibitory. The rate of glutamate accumulation is also increased by the presence of arsenate, although the highest increase recorded is 26% at a concentration of 0·001 M and no inhibitory action was found at the highest concentration (0·03 M) tested.

Effect of inhibitors on the accumulation of glutamic acid by Streptococcus faecalis

Sodium azide. If washed cells of *Strep. faecalis*, rendered deficient in free glutamic acid by growth in an amino-acid deficient medium (Gale, 1947), are incubated for 1 hr. in a buffered salt solution containing 10 μmol. sodium glutamate/ml. and glucose (1·0%) with and without 0·01 M-sodium azide, it is found that the content of free glutamic acid at the end of the incubation may be 300–700% higher in the cells treated in the presence of azide than in those

Table 1. *Effect of sodium arsenate on fermentation and glutamic acid accumulation in* Staphylococcus aureus

(Organism grown for 16 hr. at 30° in medium B (Gale, 1947). Accumulation of internal free glutamate determined before and after incubation for 45 min. at 37° in buffered salt solution containing 1% glucose and 10 μmol. sodium glutamate/ml. Results expressed as μmol. increase in free glutamate/hr./100 mg. dry wt. of cells. Fermentation determined in manometers containing 1·0 ml. washed suspension (approx. 3 mg. dry wt. of cells/ml.), 1·0 ml. 0·03 M-$NaHCO_3$, 0·5 ml. 0·05 M-glucose and 0·5 ml. water or sodium arsenate solution; manometers filled with N_2 containing 5% CO_2.)

	Fermentation		Accumulation of free glutamate	
Concentration of sodium arsenate (mM)	Q_{CO_2} (μl./mg. dry wt./ hr.)	Effect (%)	μmol. glutamate/ hr./100 mg. dry wt. cells	Effect (%)
0	113	—	34·0	—
0·1	184	+62	34·2	0
1·0	175	+54	42·9	+26
3·0	144	+27	38·6	+14
10·0	105	− 7	36·9	+ 8
30·0	48	−49	36·0	+ 6

treated in its absence. Fig. 3 shows the rate of appearance of free glutamic acid in the cells under these conditions; it can be seen that azide has increased the rate at which the free glutamate accumulates and also the final internal concentration attained. Fig. 4 shows the effect of the concentration of sodium azide on the level attained after 1 hr. incubation, in comparison with the level attained in control cells without azide; there is apparently a double response. At low concentrations, the presence of azide results in a lowering of the concentration of glutamate below that attained in the control; at high concentrations, the presence of azide produces an internal concentration of glutamic acid markedly greater than that attained in the control. Parallel experiments on the rate of fermentation show that concentrations of azide which produce a fall in the internal glutamate concentration produce a significant acceleration of fermentation.

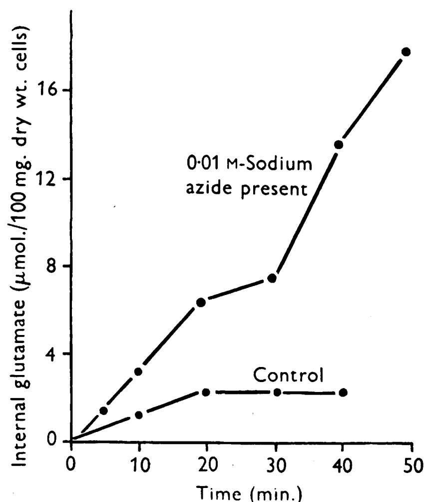

Fig. 3. Effect of sodium azide on the accumulation of free glutamate within *Strep. faecalis* cells. Deficient cells incubated at 37° in buffered salt solution containing 1% glucose and 10 μmol. sodium glutamate/ml. with and without 0·01 M-sodium azide. Cells removed at intervals, rapidly cooled, washed and internal free glutamate determined.

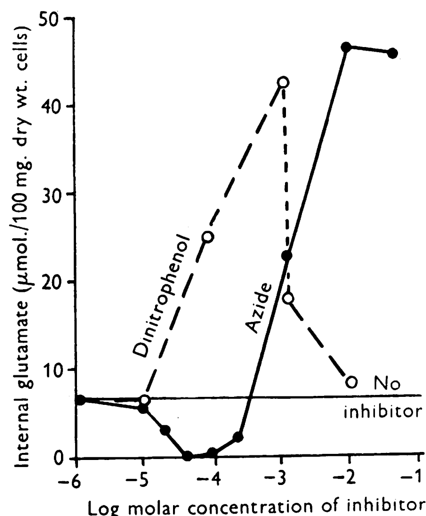

Fig. 4. Effect of 2:4-dinitrophenol and sodium azide on the accumulation of free glutamate in *Strep. faecalis*. Conditions as for Fig. 3; samples taken at end of 45 min. at 37° for determination of internal free glutamate.

Gale & Mitchell (1947) showed that the increase in the internal concentration of free glutamic acid in *Strep. faecalis*, produced by the presence of crystal violet and other dyes of the triphenylmethane series, was due to an inhibition of the internal metabolism of glutamic acid within the cells. Experiments have been carried out with sodium azide similar to those reported with crystal violet, and Table 2 shows that the same inhibition of internal metabolism can

Table 2. *Internal metabolism of glutamic acid in* Streptococcus faecalis

(*Strep. faecalis* grown in deficient medium, washed and incubated at final suspension density of 1–2 mg. dry wt. of cells/ml. in buffered salt solution containing 1 % glucose and approx. 70 μmol. sodium glutamate. After 1 hr. at 37°, cells removed and free glutamate content determined. Content of cells and supernatant before and after incubation determined and net loss of glutamic acid calculated = glutamic acid metabolized (Gale & Mitchell, 1947).)

Inhibitor	Concn. (mM)	Glutamate metabolized/100 mg. dry wt. cells: μmol.		Metabolism in control (%)
None	—	14·9, 15·0, 12·5, 13·0	13·8	100
Sodium azide	10·0	1·3, 2·3, 1·6, 3·4	2·15	15·6
	0·1	19·9		144
2:4-Dinitrophenol	0·1	10·0		70

be demonstrated with 0·01 M-sodium azide. 0·0001 M-Sodium azide, which produces a decrease in the internal concentration of glutamate, accelerates the disappearance of free glutamate from within the cells. No increase in internal free glutamate can be shown in cells incubated with or without azide in the absence of external glutamate, so the increased concentration of internal free glutamate found in some cases cannot be due to any autolytic process. Consequently, it is probable that the variations in the internal level in the presence of azide can be explained, as previously postulated (Gale & Mitchell, 1947), by variations in the rate of internal metabolism of the free glutamic acid.

2:4-*Dinitrophenol*. The addition of dinitrophenol to the external medium during the accumulation of glutamic acid in the presence of glucose by *Strep. faecalis* produces an increase in the internal free glutamate concentration attained (Fig. 4). At concentrations greater than 0·001 M, dinitrophenol appears to inhibit the passage of the amino-acid into the cell so that the internal concentration again falls. The concentration 0·001 M is critical; in some cases it produces an increase in the internal concentration to a value equal to that attained in the presence of 0·01 M-sodium azide, but in other experiments under similar conditions the effect is much less marked, suggesting that partial inhibition of the passage of the amino-acid into the cell is taking place. Table 2 shows that 0·0001 M-dinitrophenol, which gives an increase in internal glutamate concentration equal to about 50 % of that produced by 0·01 M-sodium azide, also effects approximately 70 % inhibition of the internal metabolism of glutamic acid.

Sodium arsenate. 0·0001 M-Sodium arsenate produces 25 % increase in the internal concentration of glutamate in *Strep. faecalis*; higher concentrations inhibit the appearance of free glutamate within the cells.

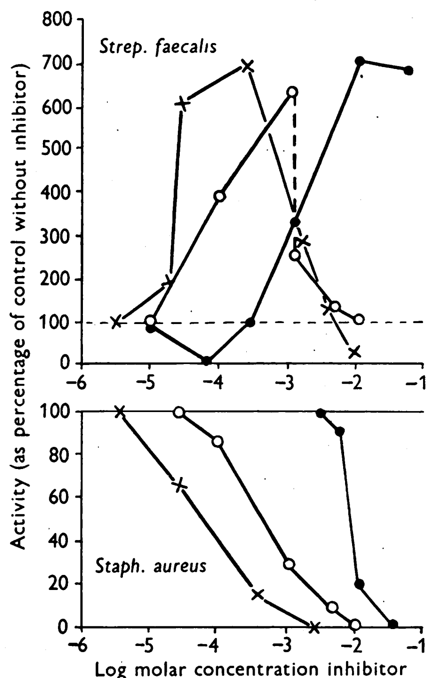

Fig. 5. Effect of inhibitors on the accumulation of glutamate in *Staph. aureus* and *Strep. faecalis*. Conditions as for Figs. 1 and 4. ×—×, crystal violet; ○—○, 2:4-dinitrophenol; ●—●, sodium azide.

Comparison of effects of inhibitors on Staphylococcus aureus *and* Streptococcus faecalis. Fig. 5

shows the actions of sodium azide, dinitrophenol and crystal violet on the accumulation of free glutamate in *Staph. aureus* and *Strep. faecalis* exposed to glutamate and glucose. The three substances produce an inhibition of the passage of the amino-acid into *Staph. aureus* but an increase in the internal concentration attained in *Strep. faecalis* although, in high concentrations, they appear to have an inhibitory action on the passage of the amino-acid into the cells in this case as well. The latter inhibition can, in the case of crystal violet, be correlated with interference with the energy-supplying fermentation system in *Strep. faecalis* (Gale & Mitchell, 1947), although this is not the explanation for the inhibition of the passage into *Staph. aureus*. The increase in the internal concentration of free glutamate in *Strep. faecalis* can be ascribed to an inhibition of metabolism of the amino-acid within the cell, and it is interesting to note that concentrations of the three substances which produce optimal inhibition of internal metabolism in *Strep. faecalis* are approximately the same as those producing almost complete inhibition of passage into *Staph. aureus*.

SUMMARY

1. The accumulation of free glutamic acid within *Staphylococcus aureus* requires energy supplied, under the experimental conditions used, by the fermentation of glucose. Sodium azide and 2:4-dinitrophenol inhibit the accumulation of free glutamate without inhibiting the fermentation reactions.

2. Sodium arsenate accelerates the rates of fermentation and of glutamate accumulation.

3. Sodium azide and 2:4-dinitrophenol increase the amount of free glutamate accumulating within *Streptococcus faecalis* incubated in the presence of glutamic acid and glucose. The effect can be correlated with the inhibition, by these substances, of internal metabolism of glutamic acid in these cells.

4. Concentrations of sodium azide and 2:4-dinitrophenol producing optimal increase in the internal glutamate concentration in *Strep. faecalis* are approximately the same as those producing complete inhibition of glutamate accumulation in *Staph. aureus*.

REFERENCES

Clifton, C. E. (1946). *Advanc. Enzymol.* **6**, 269.
Cross, R. S., Taggart, J. V., Covo, G. A. & Green, D. A. (1949). *J. biol. Chem.* **177**, 655.
Gale, E. F. (1945). *Biochem. J.* **39**, 46.
Gale, E. F. (1947). *J. gen. Microbiol.* **1**, 53.
Gale, E. F. (1948). *Bull. Johns Hopk. Hosp.* **83**, 119.
Gale, E. F. (1949). *J. gen. Microbiol.* **3**, 369.
Gale, E. F. & Mitchell, P. D. (1947). *J. gen. Microbiol.* **1**, 299.
Gale, E. F. & Rodwell, A. W. (1949). *J. gen. Microbiol.* **3**, 127.
Gale, E. F. & Taylor, E. S. (1947). *J. gen. Microbiol.* **1**, 77.
Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.
Najjar, V. A. & Gale, E. F. (1950). *Biochem. J.* **46**, 91.
Needham, D. M. & Pillai, R. K. (1937). *Biochem. J.* **31**, 1837.
Spiegelman, S., Kamen, M. D. & Sussman, M. (1948). *Arch. Biochem.* **18**, 409.
Taylor, E. S. (1947). *J. gen. Microbiol.* **1**, 86.

The Assimilation of Amino-acids by Bacteria

11. THE RELATIONSHIP BETWEEN ACCUMULATION OF FREE GLUTAMIC ACID AND THE FORMATION OF COMBINED GLUTAMIC ACID IN *STAPHYLOCOCCUS AUREUS*

BY E. F. GALE
Medical Research Council Unit for Chemical Microbiology, Biochemical Laboratory, University of Cambridge

(*Received* 21 *June* 1950)

Staphylococcus aureus (*Micrococcus pyogenes* var. *aureus*), in common with certain other Gram-positive bacteria, possesses the ability to concentrate certain amino-acids in the internal environment (Gale, 1947*a*, *b*; Taylor, 1947). In the case of glutamic acid, passage into the cell only occurs when some exergonic metabolism such as glycolysis is also taking place. If cells containing little free glutamic acid are incubated in a medium consisting of buffered saline, glucose and glutamic acid, the amino-acid enters the cell until the internal concentration is many times that in the external medium. The passage of the amino-acid into the cell is prevented by any substance which inhibits glycolysis and is also inhibited, at concentrations ineffective against glycolysis, by sodium azide, 2:4-dinitrophenol and 8-hydroxyquinoline (Gale, 1949, 1951). All the studies so far reported on the passage of glutamic acid into *Staph. aureus* and other Gram-positive cells have referred to the process which takes