The Assimilation of Amino-acids by Bacteria

12. THE ACTION OF INHIBITORS AND ANTIBIOTICS ON THE ACCUMULATION OF FREE GLUTAMIC ACID AND THE FORMATION OF COMBINED GLUTAMATE IN *STAPHYLOCOCCUS AUREUS*

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The passage of glutamic acid into *Staphylococcus aureus* takes place only when energy is supplied by some exergonic metabolism such as fermentation or respiration (Gale, 1947). It is possible to prevent the passage of glutamic acid into the cell without affecting fermentation by the addition of inhibitors such as sodium azide, 2:4-dinitrophenol and 8-hydroxyquinoline (Gale, 1949, 1951*a*). Penicillin has no action on the passage when this takes place in washed cells, but if added in bactericidal concentrations to the growth medium 60–90 min. before harvesting, it causes a complete inhibition of the entry of glutamic acid into the harvested cells, although their rate of fermentation is normal (Gale & Taylor, 1947). When washed cells of *Staph. aureus* are incubated with a mixture of amino-acids and glucose they synthesize cell protein (Hotchkiss, 1947), but the accumulation of free glutamic acid is greatly decreased (Gale, 1951*b*). The present communication deals with the action of a number of inhibitors and antibiotics on the processes of accumulation of free glutamic acid on the one hand, and of the increase in combined glutamate of the cells occurring when incubation takes place in the presence of a balanced mixture of amino-acids on the other.

METHODS

The methods used in these investigations were the same as those previously described (Gale, 1947, 1951*b*). The organism used throughout was *Staph. aureus* Duncan (Gale & Taylor, 1947). The authors are indebted to the Lederle laboratories for a sample of crystalline aureomycin, to Dr L. A. Sweetman for synthetic chloramphenicol (chloromycetin) and to Merck and Co. for streptomycin. Penicillin solutions were made from a commercial sample of crystalline benzylpenicillin assayed by the makers at 1650 Oxford units/mg.

RESULTS

Inhibitors effective on the processes in washed suspensions. Table 1 shows that sodium azide and 2:4-dinitrophenol affect the accumulation of free glutamic acid and the rate of increase in combined glutamate of the cells to significantly the same extent. 8-Hydroxyquinoline, which appears to inhibit the accumulation of free glutamic acid by combining with and inactivating an essential metal replaceable by manganese (Gale, 1949), is a more effective inhibitor of the accumulation of the free amino-acid than of its combination within the cell.

Aureomycin and chloramphenicol are alike in that they inhibit the formation of combined glutamate more effectively than the accumulation of glutamic acid in the free state. Loomis (1950) has shown that aureomycin, in concentrations greater than 100 μg./ml. (0·2 mM), will uncouple phosphorylation in mitochondrial preparations and should be grouped with azide and dinitrophenol in this respect. It is interesting to note that the accumulation of free glutamic acid is also inhibited by aureomycin at this concentration, although the growth of *Staph. aureus* is sensitive to much smaller concentrations. Chloramphenicol produces 10 % inhibition of free glutamic acid accumulation at a concentration of 1·5 mM (500 μg./ml.), and Loomis (1950) states that this antibiotic is not effective in uncoupling phosphorylation in his experiments. Chloramphenicol inhibits growth of *Staph. aureus* at a concentration of 1–5 μg./ml., and these concentrations cause 77–96 % inhibition of the formation of combined glutamate.

Streptomycin is a weak inhibitor of both processes, being ineffective against either at concentrations less than 0·1 mM, whereas growth is inhibited at a concentration of 0·001 mM. In the higher range of concentrations, streptomycin is a more effective inhibitor of combined glutamate formation than of the accumulation of the free amino-acid. Sulphathiazole in saturated solution had no significant inhibitory action on either process.

In all cases tested, the inhibitors were effective against glutamic acid metabolism at concentrations ineffective against glucose fermentation.

Action of penicillin

Penicillin has no action on the accumulation of free glutamic acid in washed suspensions of *Staph. aureus*. If 10 units penicillin/ml. are added to the growth medium after 4 hr. growth at 37° and the

Table 1. *Inhibition of free glutamic acid accumulation and of the formation of combined glutamate in* Staphylococcus aureus

(*Growth:* growth (++) or inhibition (−) when *Staph. aureus* Duncan inoculated into casein-digest broth containing 1% glucose, 0·1% marmite and inhibitor as shown; size of inoculum = approx. 10^6 cells/5 ml. medium.

Glucose fermentation: tested in Warburg manometers containing washed cells of dry wt. = approx. 3 mg., 0·03 M-$NaHCO_3$, 0·05 M-glucose and water or inhibitor to 3·0 ml.; filled with N_2 gas containing 5% CO_2.

Glutamate accumulation: organisms harvested from deficient medium and incubated for 1 hr. at 37° in buffered salt solution containing 1% glucose and 1·34 μmol. sodium glutamate/ml.; increase in free internal glutamic acid determined and expressed as μmol. glutamic acid/100 mg. dry wt. of cells/hr.

Increase in combined glutamate: experiment carried out in parallel with accumulation but with addition of mixture of eighteen amino-acids each at a final concentration of 0·2 mg./ml. Free and total glutamic acid of cells determined before and after incubation; total glutamic acid determined after 18 hr. hydrolysis in boiling 5 N-HCl; results expressed as μmol. increase in combined glutamate/100 mg. dry wt. of cells/hr.)

Inhibitor	Concn. (mM)	Growth	Inhibition of glucose fermentation (%)	Glutamate accumulation (μmol./100 mg. dry wt. cells/hr.)	Glutamate accumulation Inhibition (%)	Increase in combined glutamate (μmol./100 mg. dry wt. cells/hr.)	Increase in combined glutamate Inhibition (%)
Sodium azide	0	++	.	27·0	.	6·7	.
	10·0	−	2	0	100	0	100
	3·3	++	0	16·2	43	1·56	65
2:4-Dinitrophenol	0	++	.	30·5	.	8·17	.
	1·0	−	0	3·8	87	1·66	80
	0·1	++	0	22·1	25	6·0	26
8-Hydroxyquinoline	0	++	.	39·5	.	6·98	.
	0·3	−	20	14·8	62	6·62	5
	0·03	++	0	28·6	27	6·8	2·5
Aureomycin	0	++	.	30·1	.	6·16	.
	0·2	−	19	0	100	0	100
	0·07	−	2	21·6	29	0	100
	0·014	−	0	29·2	3	3·08	50
Chloramphenicol (Chloromycetin)	0	++	.	43·6	.	7·64	.
	1·5	−	0	39·0	10	0	100
	0·15	−	0	43·4	0	0	100
	0·015	+	0	.	.	0·3	96
	0·003	++	0	.	.	1·84	77
	0·001	++	0	.	.	6·03	21
Streptomycin	0	++	.	33·0	.	9·15	.
	1·0	−	0	31·7	4	5·04	45
	0·1	−	0	33·0	0	6·72	26
	0·01	−	0	.	.	8·6	5
	0·001	++	0	.	.	9·4	0
Sulphathiazole	0	++	.	30·5	.	6·16	.
	Satd.	+	0	30·5	0	6·15	0

cells harvested after 90 min. further incubation, it is found that the washed cells can no longer accumulate free glutamic acid (Gale & Taylor, 1947). Hotchkiss (1950) states that washed *Staph. aureus*, aerated for 1 hr. and then incubated with glucose and casein hydrolysate, synthesizes cellular protein but that, if 50 units penicillin/mg. dry weight of cells are added, the removal of amino-acids from the medium continues but no increase in cell protein occurs, peptides accumulating in the medium instead. Washed suspensions of *Staph. aureus* Duncan, grown in deficient medium, were incubated with and without penicillin in the presence of glucose and a mixture of nineteen amino-acids including glutamic acid under conditions previously described (Gale, 1951*b*). Table 2 shows that the presence of penicillin has no effect on the rate of increase of combined glutamate of the cells and no accumulation of glutamyl peptides could be demonstrated in the external medium. If, however, penicillin is added to the growth medium and the cells harvested within 30–60 min. after the addition (before the passage of glutamic acid into the cell has been completely inhibited), it is found that the cells can no longer form combined glutamate (Table 3). Analysis of the changes in glutamic acid distribution during the incubation shows, however, that free glutamic acid has disappeared from the medium and that a proportion, which varies between 20 and 45%, can be recovered by acid hydrolysis of the supernatant

Table 2. *Effect of penicillin on distribution of glutamic acid during incubation of* Staphylococcus aureus *with glucose and amino-acid mixtures*

(*Staph. aureus* Duncan grown for 16 hr. at 30° in deficient medium, washed once in water and made into suspension in water, dry wt. of cells = 21·2 mg./ml. 5·0 ml. suspension taken for each experiment and incubated for 1 hr. at 37° in buffered salt solution containing 1% glucose, 1·34 μmol. sodium glutamate/ml., mixture of eighteen amino-acids each at final concentration 0·2 mg./ml. (Gale, 1951*b*) with and without 100 units penicillin/ml. as below. Final suspension density = 2·12 mg. dry wt. of cells/ml. Free and combined glutamate of cells and supernatant determined before and after incubation; changes expressed as μmol. glutamate/106 mg. dry wt. of cells.)

Incubation mixture	Glucose	Glucose + glutamate	Glucose + glutamate + penicillin	Glucose + glutamate + amino-acid mixture	Glucose + glutamate + amino-acid mixture + penicillin
Internal free glutamate of cells	+0·2	+29·8	+27·5	−6·91	−7·82
External free glutamate of medium	+1·48	−48·1	−42·7	−7·4	−2·8
Combined glutamate of cells	−0·54	− 1·5	−13·8	+8·8	+6·6
Combined glutamate of medium	+1·1	+ 2·7	+ 1·3	+2·0	0·0
Balance	+2·37	−17·2	−27·6	−3·44	−4·15

Conditions as above with samples removed for estimation at times indicated.

Time (min.)	Increase in combined glutamate of cells (μmol./79 mg. dry wt. cells)		Glutamic acid balance expressed as % initial total	
	Normal	Penicillin present	Normal	Penicillin present
Initial	(27·2)	(27·2)	—	—
60	5·6	7·2	99·0	103·1
90	11·1	11·7	95·8	99·2
120	15·4	13·9	94·3	85·0
150	18·8	18·8	88·7	88·3

Table 3. *Glutamic acid metabolism of penicillin-treated* Staphylococcus aureus

(Cells grown in deficient medium at 37° for 4 hr.; 10 units penicillin/ml. medium added and cells harvested 1 hr. after addition. Washed suspensions made and treated as in Table 2. Changes in glutamate distribution during 1 hr. at 37° expressed as μmol. glutamate/100 mg. dry wt. of cells.)

Incubation mixture	Glucose	Glucose + glutamate	Glucose + glutamate + amino-acid mixture
Internal free glutamate of cells	− 1·57	+ 0·9	−2·01
External free glutamate of medium	+ 1·03	−41·5	−1·6
Combined glutamate of cells	−13·8	− 8·6	−6·3
Combined glutamate of medium	—	+19·4	0
Balance	—	−29·8	−9·9

medium. It seems probable that conditions have arisen in this case which have results similar to those reported by Hotchkiss (1950).

SUMMARY

1. The action of inhibitors has been studied on the growth, glucose fermentation, accumulation of free glutamic acid, and formation of cellular combined glutamate in *Staphylococcus aureus*. In all cases quoted, the inhibitors suppressed the accumulation of free glutamic acid or its combination in the cell at concentrations ineffective against fermentation.

2. Of the two processes (*a*) accumulation of free glutamic acid, (*b*) formation of cellular combined glutamate, sodium azide and 2:4-dinitrophenol are equally effective against both; 8-hydroxyquinoline is more effective against (*a*) than (*b*); aureomycin and chloramphenicol are markedly more effective as inhibitors of (*b*) than (*a*); streptomycin affects (*b*) at concentrations markedly higher than those required to prevent growth.

3. There is a close correlation between the inhibitory action of chloramphenicol on (*b*) and its action as growth inhibitor.

4. Penicillin has no action on the processes in washed suspensions; addition of penicillin to the medium 30–60 min. before harvesting results in complete inhibition of (*a*) and (*b*).

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The Differential Determination of Mixtures of *p*-Aminosalicylic Acid and Sulphetrone or Sulphonamides in Body Fluids

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Since the introduction of *p*-aminosalicylic acid (PAS) to the chemotherapy of tuberculosis, several methods for its estimation have been described. These methods have depended on the presence of the free aryl amino group in the molecule, and have the disadvantage of a lack of specificity since free aryl amino groups are of frequent occurrence in synthetic drugs. Difficulties may therefore arise in estimating one in the presence of another. This can occur for instance in the combined use of PAS and diaminodiphenylsulphone derivatives, such as sulphetrone, (tetrasodium 4:4′-bis(γ-phenyl-*n*-propylamino)diphenylsulphone-αγα′γ′-tetrasulphonate), or PAS, and sulphonamide derivatives. All the drugs may be estimated singly by the standard method described by Bratton & Marshall (1939) of diazotizing and coupling with *N*-1-naphthylethylenediamine, and an examination of the visible absorption spectra of the coloured solutions (Fig. 1) shows that no differentiation is possible for PAS, sulphetrone, sulphanilamide and sulphathiazole, since the peak absorptions all lie in the same spectral area. This was also found to be true of other sulphonamides examined, namely, sulphadiazine, sulphaguanidine and sulphamezathine. To enable differentiation to be made, alternative methods of estimating the PAS alone have been examined. These have included the use of the reaction with ferric iron, coupling with *p*-dimethylaminobenzaldehyde, and coupling with diazonium salts of various amines in alkaline solution.

Reaction with ferric iron. PAS gives the characteristic salicylate reaction in neutral or faintly acid solution, producing a coloured complex with solutions of ferric salts. Sulphetrone and the sulphonamides do not give this reaction. It has been used for the estimation of PAS in urine (Venkatamaran, Venkatamaran & Lewis, 1948) and may be adapted to the estimation of high concentrations in blood filtrates. Unfortunately the colour produced is not intense, making the method somewhat insensitive and of little value for general purposes.

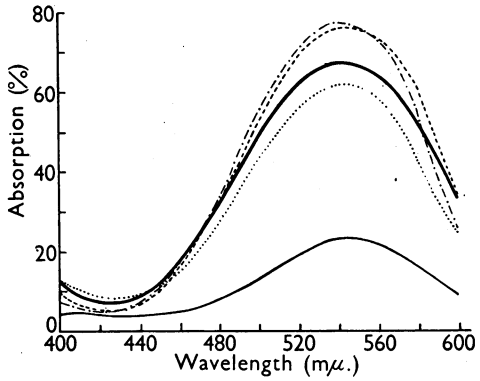

Fig. 1. Visible absorption spectra after diazotizing and coupling with *N*-1-naphthylethylenediamine. Sulphetrone and PAS (1·0 mg./100 ml. of each), ——; PAS (1·0 mg./100 ml.), ——; sulphathiazole (0·2 mg./100 ml.), - - - - -; sulphanilamide (0·2 mg./100 ml.), -·-·-; sulphetrone (1·0 mg./100 ml.), ...

Coupling with p-*dimethylaminobenzaldehyde.* *p*-Dimethylaminobenzaldehyde reacts with free aryl amino groups yielding characteristic lemon-yellow compounds. This has been applied by Venkatamaran *et al.* (1948) and others to the estimation of PAS. The sulphonamides give a similar reaction, and comparison of the visible absorption spectra of the coloured solutions at equivalent concentration shows the intensities of the colours to be approximately equal (Fig. 2). Sulphetrone also gives a colour with *p*-dimethylaminobenzaldehyde, but it is much less intense, and concentrations in the coloured solution of less than 0·2 mg./ml. do not differ significantly from the blank. At the dilutions