The Assimilation of Amino-acids by Bacteria

2. The Action of Tyrocidin and some Detergent Substances in Releasing Amino-acids from the Internal Environment of *Streptococcus faecalis*

BY E. F. GALE AND E. SHIRLEY TAYLOR

*Medical Research Council Unit for Chemical Microbiology,
Biochemistry Department, Cambridge*

With a Note on Electron Micrographs of Normal and Tyrocidin-lysed Streptococci

BY P. D. MITCHELL, *Biochemistry Department, Cambridge*

AND G. R. CROWE, *Cavendish Laboratory, Cambridge*

SUMMARY: Treatment of *Streptococcus faecalis* cells containing high internal concentrations of lysine and glutamic acid with tyrocidin, Aerosol O.T., cetyltrimethylammonium bromide or phenol resulted in loss of the internal amino-acids. The substances used affected the cell wall so that the internal amino-acids leaked into the external environment; the rate of leakage was followed by a manometric method. The lytic action of tyrocidin, cetyltrimethylammonium bromide, Aerosol O.T., and phenol is sufficient to explain the disinfecting action of these substances.

It was shown (Gale, 1947) that lysine and glutamic acid passed into the internal environment of certain streptococci (Lancefield Group D) and that the internal concentration was markedly greater than that in the external environment at equilibrium. When the cells were removed from an amino-acid-rich medium and resuspended in distilled water or a suitable salt solution during 24–48 hr. at 4°, no loss of internal amino-acid occurred by diffusion. Hotchkiss (1944) stated that when bacterial cells were treated with tyrocidin or certain detergent substances a leakage of nitrogenous and phosphorus-containing substances occurred into the suspending fluid. It seemed that this might indicate that the action of tyrocidin, etc. was such that the amino-acids concentrated within the internal environment were released into the external environment and that the nitrogenous material estimated by Hotchkiss might consist partly of such amino-acids. The results reported in this paper show that this is the case (cf. Gale & Taylor, 1946).

METHODS

Organism and method of culture

The organism used was the same as that described previously (Gale, 1947). It was desirable to prepare cells which would contain a high internal concentration of lysine and glutamic acid; this can be done by growing the organism for approx. 12 hr. at 37° in medium A consisting of: casein digest; 0.1 % Marmite; 1.0 % glucose. The cells were washed once after harvesting.

Estimation of amino-acids. *l*-(+)-Lysine and *l*-(+)-glutamic acid were estimated manometrically by the use of specific amino-acid decarboxylase preparations (Gale, 1945).

Preparation of tyrocidin. Tyrothricin was obtained from the Wallerstein Co. through the courtesy of Dr R. Dubos and tyrocidin hydrochloride prepared therefrom by the method of Hotchkiss & Dubos (1941). The material was recrystallized once from methanol. We are indebted to Dr A. R. Trim of this department for samples of Aerosol O.T. and cetyltrimethylammonium bromide.

Antibiotic activities of tyrocidin, etc.

Table 1 shows the concentrations of the various antibiotics tested found necessary to prevent the growth of or to sterilize a culture of the group D *Streptococcus* used as test organism.

Table 1. *Inhibition of Strep. faecalis by antibiotic substances*

Antibiotic substance	Concentrations (mg./ml.)		
	Inhibition titre 10 ⁶ cells/ml.	Sterilization of 10 ⁸ cells/ml.	Cytolysis of 10 ⁸ cells/ml.
Tyrocidin	0.001	0.1	1.0
Cetyltrimethyl- ammonium bromide	0.001	0.2	1.0
Aerosol O.T.	0.1	0.1	1.0
Phenol	2.5	10.0	30.0
Gramicidin	0.001	0.1	No effect
Patulin	0.1	20.0	No effect
Gentian violet	0.01	> 2.0	No effect
Acriflavin	0.01	> 2.0	No effect
Sulphathiazole	1.0	—	No effect
Penicillin	8 Oxford units	—	No effect

The *inhibition titre* in each case was determined by taking a series of tubes of medium A, adding the antibiotic in serial dilution at intervals such that each tube contained one-fifth the concentration of antibiotic of the previous one in the series, inoculating with a standard inoculum of 10⁶ cells/ml. medium, and determining the limiting inhibitory concentration after 48 hr. incubation at 37°. In order to determine whether the action was bactericidal or bacteriostatic and also whether the inhibitory concentration varies with the number of cells present, a further test was carried out as a modification of the Rideal-Walker test. A 24 hr. culture of the organism in medium A containing approx. 10⁸ viable cells/ml. was taken and serial dilutions of the antibiotics added as before to the complete culture, after 30 min. a loopful of culture was taken from each tube and streaked on to nutrient agar. The plate cultures were examined after 24 hr. incubation at 37° and the concentration of antibiotic necessary to sterilize the culture under these conditions noted; this value is given in Table 1 as the 'Sterilization of 10⁸ cells/ml.' figure.

It can be seen from Table 1 that 1 µg. tyrocidin/ml. was sufficient to prevent the growth of the inoculum corresponding to 10⁶ cells/ml., but 0.1 mg./ml. was necessary to sterilize a culture of approx. 10⁸ cells/ml. It would appear that there is some quantitative relationship between the number of cells and the

amount of tyrocidin necessary for their sterilization. The same was true for cetyltrimethylammonium bromide. In the case of Aerosol O.T. a concentration of 0.1 mg./ml. was required to sterilize either 10^6 or 10^8 cells/ml. although 10^9 cells/ml. required 1 mg. of Aerosol/ml. (see later). It is probable that this is a special case in which the physical state of the detergent substance is important and that no antibacterial activity is shown in solutions of Aerosol O.T. which are too weak for micelle formation to occur (Alexander & Trim, 1946). It is clear from Table 1 that the action of these substances is bactericidal in the concentrations quoted.

Compared with tyrocidin, phenol is a weak disinfectant, 0.25 % being necessary to sterilize 10^6 cells/ml. and 1.0 % for 10^8 cells/ml. Substances representative of other groups of antibiotics were also tested; gramicidin (Hotchkiss, 1944) and patulin (Raistrick, 1943) were both bactericidal towards the organism while gentian violet, acriflavin, sulphathiazole and penicillin were not bactericidal in concentrations 500 times the inhibition coefficient.

Effect on maintenance of internal free amino-acid concentration

When *Strep. faecalis* cells are grown in medium A for 12–14 hr. the cells contain a high internal concentration of lysine and glutamic acid in a free state and this internal amino-acid does not diffuse out of the cells if the latter are suspended in distilled water or salt solution at 4° (48 hr.) or 37° (6 hr.) (Gale, 1947). It is possible that some antibiotic substances may affect the cell wall in such a way that this is no longer the case. To test this, the organism was grown in medium A under optimum conditions for the production of a high internal concentration of free lysine and glutamic acid, the cells centrifuged out of the medium, washed once and a portion assayed for these amino-acids as previously described (Gale, 1947). Further portions of the cells were then suspended in distilled water to a final cell-suspension strength of approx. 5 mg./ml. (approx. 10^9 cells/ml.) and suitable concentrations of various antibiotic substances added. The suspensions were then incubated for 3 hr. at 37° after which the cells were again centrifuged out of suspension and the internal amino-acid content assayed again.

Table 2 shows that there was no significant loss of internal lysine or glutamic acid when the cells were suspended in distilled water but that the presence in the suspending fluid of tyrocidin, cetyltrimethylammonium bromide, or Aerosol O.T. (1 mg./ml.) or of phenol (10 mg./ml.) resulted in a complete disappearance of these amino-acids from the internal environment. The other antibiotic substances tested had no significant effect on the internal environment in this respect.

Manometric demonstration of lysis by tyrocidin, etc.

The results shown in Table 2 suggest that the action of tyrocidin and the detergent substances is to alter the permeability of the cell wall so that the free amino-acids in the internal environment leak into the suspending medium. That this is the case was shown as follows:

A thick washed suspension of *Strep. faecalis* cells grown in medium A was prepared and the internal lysine and glutamic acid content determined as usual on intact and

boiled cells. One ml. of the suspension was then placed with 1 ml. buffer in the main compartment of a Warburg manometer cup fitted with two side-arms; into one side-arm 0.5 ml. of specific decarboxylase preparation in buffer was placed, and into the other 0.5 ml. of a suitable solution of tyrocidin or detergent substance. When the glutamic acid content was being studied M/5-acetate buffer pH 4.5 was used; with lysine, M/5-phosphate buffer at pH 6.0. After equilibration in a thermostat at 30°, the enzyme preparation was tipped into the main compartment and the 'external' amino-acid of the cell suspension assayed as usual (Fig. 1, curve 1). When the CO₂ evolution had ceased, after 10–15 min., the tyrocidin was added from the second side-arm.

Table 2. *Effect of antibiotic substances on maintenance of free amino-acid concentration in internal environment*

Cells suspended in distilled water containing substances as below for 3 hr. at 37° in suspension = approx. 5 mg. dry weight cells/ml. Dry weight of cells assayed = 52 mg.

Suspending medium	Amino-acid content of internal environment	
	Lysine (μ l.)	Glutamic acid (μ l.)
Initial content of cells	122	116
Distilled water alone	118	105
Tyrocidin 1 mg./ml.	0	0
Cetyltrimethylammonium bromide 1 mg./ml.	0	0
Aerosol O.T. 1 mg./ml.	0	0
Phenol 10 mg./ml.	0	0
Gramicidin 1 mg./ml.	120	111
Gentian violet 10 mg./ml.	110	56
Patulin 10 mg./ml.	No significant decrease over control in distilled water	
Acriflavin 10 mg./ml.		
Sulphathiazole 1 mg./ml.		
Penicillin, 8 Oxford units/ml.		

If the action of the tyrocidin is to liberate amino-acids from the internal environment of the streptococcal cells, this would be shown by evolution of CO₂, as the lysine or glutamic acid is attacked by the decarboxylase preparation. Fig. 1 shows the results obtained in such an experiment. The addition of 1.0 mg. tyrocidin at time 15 min. resulted in a rapid liberation of glutamic acid from the cells and the amount liberated was equal to that assayed in boiled cells (curve 2) and so corresponded to the total amount in the internal environment. The addition of smaller amounts of tyrocidin resulted in the liberation of correspondingly smaller amounts of glutamic acid: thus while 1.0 mg. tyrocidin gave rise to 100 μ l. glutamic CO₂ in the case quoted, 600 μ g. gave rise to 59 μ l. and 300 μ g. to 27 μ l., suggesting a close quantitative relation.

The rate of evolution of glutamic CO₂ was much the same however much tyrocidin was added, and it was the final quantity that varied with the amount of tyrocidin added. It appears that the smaller amounts of tyrocidin released the internal amino-acid from a proportion of the cells only. To test this in another experiment 0.5 mg. tyrocidin was added to the cell suspension and the glutamic CO₂ evolution followed to cessation, when a further 0.5 mg. tyrocidin was added. Fig. 3 shows that the second addition causes the release of more glutamic acid from the cells, the two separate additions of 0.5 mg. have a combined effect equal to that of a single addition of 1.0 mg. The addition of

further tyrocidin to the manometer in which 1.0 mg. had already been added had no further effect when the dry weight of cells present was of the order of 40–50 mg. Fig. 2 shows that similar results were obtained when the experiments were repeated using lysine decarboxylase instead of the glutamic enzyme.

Fig. 1. Effect of tyrocidin on *Strep. faecalis*: liberation of glutamic acid from internal environment. Curve 1: glutamic acid assay of intact cells. Curve 2: glutamic acid assay of boiled cells. Curve 3: 1.0 mg. tyrocidin added at arrow 15 min. after addition of enzyme. Curve 4: 0.6 mg. tyrocidin added at arrow 15 min. after addition of enzyme. Curve 5: 0.3 mg. tyrocidin added at arrow 15 min. after addition of enzyme. Dry weight of cells assayed = 47.0 mg. Temperature = 30°. Manometer vessels contain initially 1.0 ml. M/5-acetate pH 4.5; 1.0 ml. washed suspension of *Strep. faecalis* cells (main compartment); 0.5 ml. glutamic decarboxylase (side-bulb 1); 0.5 ml. tyrocidin (side-bulb 2).

The weight of tyrocidin per cell needed for complete lysis in these experiments was of the same order as that shown in Table 1 for the amounts necessary to sterilize cultures. It seems probable that the lytic effect is sufficient to explain the antibiotic action of tyrocidin. From Figs. 1–3 it appears that while 1.0 mg. tyrocidin will lyse and sterilize 50 mg. streptococcal cells, 0.5 mg. will lyse only half these cells. To confirm that this was also true of the disinfecting action of tyrocidin, the experiment was repeated and viable counts carried out on (1) the untreated washed suspension, (2) the suspension treated with 1.0 mg. tyrocidin/50 mg. cells, and (3) the suspension treated with 0.3 mg. tyrocidin/50 mg. cells. Table 3 shows that whereas the larger amount of tyrocidin decreased the number of viable cells from 38×10^8 to 2×10^8 , 0.3 mg. decreased

the viable count by approx. one-third of this amount. It would seem from this evidence that about 10^8 molecules of tyrocidin are required to lyse and kill one *Streptococcus* cell.

Manometric experiments of this type were carried out with cetyltrimethylammonium bromide and Aerosol O.T. Very similar results were obtained with

Fig. 2. Effect of tyrocidin on *Strep. faecalis*: liberation of lysine from internal environment. Curve 1: lysine assay of intact cells. Curve 2: lysine assay of boiled cells. Curve 3: 1.0 mg. tyrocidin added at arrow 15 min. after addition of enzyme. Curve 4: 0.5 mg. tyrocidin added at arrow 15 min. after addition of enzyme. Curve 5: 0.3 mg. tyrocidin added at arrow 15 min. after addition of enzyme. Dry weight of cells assayed = 40 mg. Temperature = 30°. Manometer vessels contain initially 1.0 ml. M/5-phosphate pH 6.0; 1.0 ml. washed suspension of *Strep. faecalis* cells (main compartment); 0.5 ml. lysine decarboxylase (side-bulb 1); 0.5 ml. tyrocidin (side-bulb 2).

similar quantities, i.e. approx. 1 mg. of detergent substance lyses approx. 50 mg. cells. The rate of liberation of glutamic acid or lysine appeared somewhat slower with Aerosol O.T. than with the other substances, 100 μ l. glutamic CO_2 being evolved after 20 min. after the addition of 1.0 mg. Aerosol O.T. compared with 10 min. for tyrocidin.

The experiments quoted in Table 2 suggest that phenol in 1.0 % concentration has an effect on the cells similar to that described for the detergents mentioned. An attempt was made to test this by the manometric test as above. Fig. 4 shows the results obtained with glutamic decarboxylase on the

addition of phenol to 66 mg. cells. The addition of 30 mg. phenol resulted in a slow evolution of glutamic CO_2 , while 10 mg. phenol had a similar effect,

Fig. 3. Relation between quantity of tyrocidin added and amount of glutamic acid released from internal environment. Dry weight of *Strep. faecalis* cells assayed = 50.0 mg. Temperature = 30°. Manometer vessels made up with 1.0 ml. $\text{m}/5$ -phosphate pH 6.0; 1.0 ml. washed suspension of *Strep. faecalis* cells, and 0.5 ml. glutamic decarboxylase (main compartment) with tyrocidin in side-bulbs. Manometers allowed to equilibrate and liberation of external glutamic CO_2 to cease before time = 0. Curve 1: 0.5 mg. tyrocidin tipped at time = 0. Curve 2: 0.5 mg. tyrocidin tipped at time = 0; further 0.5 mg. tyrocidin tipped at time = 20 min. (arrow). Curve 3: 1.0 mg. tyrocidin tipped at time = 0.

Table 3. Effect of tyrocidin on viability of *Strep. faecalis*

Strep. faecalis cells grown in medium A and made up into thick washed suspension (50 mg. dry weight/ml.). Tyrocidin added and left at room temperature for 30 min. before serial dilutions made for viable count.

Cell preparation	Viable count	
	Dilution 10^{-7}	Dilution 10^{-8}
Washed suspension 50 mg./ml.	425	38
Washed suspension 50 mg./ml. treated with 1.0 mg. tyrocidin/ml.	24	2
Washed suspension 50 mg./ml. treated with 0.3 mg. tyrocidin/ml.	220	26

giving a final evolution of CO_2 about one-fourth that produced by 30 mg. The slow rate of evolution is probably due to inhibition of the decarboxylase, as the enzyme is acting in the presence of 1.0 % phenol. It was not possible to carry out this type of experiment with lysine decarboxylase as this enzyme is more

sensitive to phenol than the glutamic decarboxylase; the addition of the phenol is followed by a small evolution of CO_2 which may amount to 20–30 $\mu\text{l.}$, after which the enzyme is inactivated. The results given in Table 2, however, leave no doubt that internal lysine is released from the cells. Phenol thus owes its disinfectant action to an effect upon the bacterial cell wall whereby essential constituents of the internal environment are released.

Fig. 4. Effect of phenol on *Strep. faecalis* cells: liberation of glutamic acid from internal environment. Curve 1: glutamic acid assay of intact cells. Curve 2: glutamic acid assay of boiled cells. Curve 3: 60 mg. phenol added at arrow 20 min. after addition of enzyme. Curve 4: 30 mg. phenol added at arrow 20 min. after addition of enzyme. Curve 5: 10 mg. phenol added at arrow 20 min. after addition of enzyme. Dry weight of cells assayed = 66.2 mg. Temperature = 30°. Manometer vessels contain initially 1.0 ml. $\text{N}/5$ -acetate pH 4.5; 1.0 ml. washed suspension of *Strep. faecalis* cells (main compartment); 0.5 ml. glutamic decarboxylase (side-bulb 1); 0.5 ml. phenol (side-bulb 2).

Action on other cells. Experiments similar to those described above were carried out with washed suspensions of *Staph. aureus* and *Saccharomyces carlsbergensis*; the internal amino-acids of these cells were released by the action of tyrocidin in a manner essentially similar to that described above for streptococci.

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