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Site of action of Polymyxin on *Pseudomonas aeruginosa*: Antagonism by Cations

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SUMMARY: *N*-tolyl- α -naphthylamine-8-sulphonic acid forms conjugates with protein which fluoresce when excited by ultraviolet light. When washed cell suspensions of *Pseudomonas aeruginosa* are treated with *N*-tolyl- α -naphthylamine-8-sulphonic acid no fluorescence develops, but when polymyxin is also added, fluorescence develops, thus demonstrating that the dye can penetrate to protein-containing portions of the cells. This technique has been used to study the competition between polymyxin and certain cations for sites on the cells. From a comparison of the affinities of these cations for the polymyxin-combining groups of the cells with their ability to reverse the charge on certain colloids it is suggested that the polymyxin-combining loci of the cells may be polyphosphates.

The addition of polymyxin to a washed cell suspension of a strain of *Pseudomonas aeruginosa* results in a leakage from the cells of pentose, phosphate and materials which have an absorption maximum at 260 m μ . (Newton, 1953*a*). It has been suggested that the bactericidal activity of polymyxin may be due to its ability to combine with certain chemical groups on or just below the cell surface, with a resultant disorganization of a cell membrane or osmotic barrier. Similar findings have been described for other organisms (Few & Schulman, 1953). In a preliminary communication Newton (1953*b*) showed that cells could be protected against the bactericidal action of polymyxin by cations whose presence prevented the absorption of the antibiotic by the cells. There was no evidence for the formation of a polymyxin-metal ion complex, and it was suggested (Newton, 1953*c*) that there is a competition between polymyxin and cations for sites on or in the cells. The present paper gives a more detailed account of this competition from which some indication of the nature of the 'polymyxin-combining' groups of the cells can be obtained.

METHODS

Organism. The strain of *Ps. aeruginosa* previously described (Newton, 1953*a*) was used.

Medium, conditions of culture and harvesting. The organism was grown for 15 hr. at 30° in tryptic digest of casein which contained the equivalent of 3% (w/v) casein, and had an initial pH value of 7.4-7.6. Roux bottles, each containing 150 ml. medium, were inoculated with 2 ml. of an overnight culture in the same medium. Cells were harvested by centrifugation, washed twice in 1% (w/v) sodium chloride and finally suspended in saline of the same strength to give a suspension of *c.* 10 mg. dry-wt. cells/ml. (Newton, 1953*a*). Dry

weights were determined turbidimetrically on a Hilger absorptiometer previously calibrated in terms of the organism used.

The use of N-tolyl- α -naphthylamine-8-sulphonic acid to detect a change in permeability of washed cells. Aqueous solutions of N-tolyl- α -naphthylamine-8-sulphonic acid (TNS) do not fluoresce when excited by ultraviolet light; in the presence of protein the dye combines with negatively charged groups, and the conjugate fluoresces strongly when excited by light of wavelength 436 m μ . (Weber & Laurence, 1954). When washed cells of *Ps. aeruginosa* were suspended in dilute solutions of this dye no fluorescence was observed, indicating that there were no groups on the surface of the cells with which the dye could combine. The addition of polymyxin to such cell suspensions resulted in an immediate fluorescence; presumably the antibiotic caused an alteration of cell permeability and allowed the dye to penetrate into the cell where it combined with cell protein. The measurement of the intensity of fluorescence of polymyxin-treated cell suspensions in the presence of TNS has provided a means of determining the rate of combination of polymyxin with cells in the presence of certain cations.

Measurement of intensity of fluorescence. Intensities of fluorescence of treated cell suspensions were measured by comparison with a standard fluorescent solution with a modified Pulfrich photometer. The apparatus described by Weber (1952) was modified so that measurements could be made under conditions of constant temperature. The exciting light from a mercury arc was filtered through a 5850 Corning glass filter and the fluorescence observed through a 335 Corning glass filter.

Standard solution of N-tolyl- α -naphthylamine-8-sulphonic acid (TNS). A 10^{-3} M solution of TNS in 1% (w/v) sodium chloride was used in all experiments. A trace of sodium bicarbonate was added to give a final pH value of 7.

Standard fluorescent solution. A fluorescent solution for use as a standard in the Pulfrich photometer was prepared by adding 2 ml. standard TNS solution ($\equiv 2$ μ mole) to 2.5 ml. of a 0.1% (w/v) solution of crystalline bovine serum albumin (Armour Laboratories, batch no. 17150) in 0.01M-phosphate buffer, (pH 6.3). This solution was made up to 10 ml. with 1% (w/v) sodium chloride.

Treatment of cell suspensions. Washed cell suspension (0.1 ml.; 10 mg. dry-wt. cells/ml.) was added to (7.9- x) ml. 1% (w/v) sodium chloride to which 2 ml. standard TNS solution had been added; x ml. polymyxin (200 μ g./ml.) were then added and the intensity of fluorescence measured after 1 min. When the effect of cations on the development of fluorescence was studied the cations were added to the cell suspension 5 min. before the addition of polymyxin, and the intensity of fluorescence was measured at intervals, commencing 1 min. after the addition of polymyxin. In some experiments cells were pretreated by suspending in uranyl chloride solutions, followed after 5 min. by washing with 1% (w/v) NaCl before treatment with polymyxin in the presence of TNS as described above.

RESULTS

Relationship between polymyxin concentration and intensity of fluorescence

The intensity of fluorescence of cell suspensions in the presence of 2 μ mole TNS increased linearly with increasing polymyxin concentration (Fig. 1). Maximum fluorescence was obtained with 120 μ g. polymyxin/mg. dry-wt.

Fig. 1. Relationship between intensity of fluorescence and polymyxin concentration. Washed cells (1 mg. dry wt.) suspended in 1% (w/v) NaCl + 2 μ mole *N*-tolyl- α -naphthylamine-8-sulphonic acid + polymyxin; final volume 10 ml. Intensity of fluorescence measured 1 min. after the addition of polymyxin.

cells; increasing the concentration of TNS did not increase the maximum intensity of fluorescence. When cell suspensions were heated to 90° for 10 min. and then cooled, addition of TNS resulted in an immediate fluorescence which was of the same intensity as that obtained with unheated suspensions treated with 120 μ g. polymyxin/mg. dry-wt. cells.

Competition between polymyxin and cations

When excess polymyxin (150 $\mu\text{g./mg. dry-wt. cells}$) was added to cells suspended in 1% (w/v) saline + 2 $\mu\text{mole TNS}$, fluorescence developed at a rate too rapid to be measured so that for experimental purposes maximum fluorescence resulted immediately. When bivalent cations were added to the cell suspension before the polymyxin the fluorescence of the suspension increased gradually, at a rate dependent on the cation concentration. Fig. 2*a* shows the effect of magnesium concentration on the rate of increase of fluorescence; there is a linear relationship between rate and concentration over the range 5–20 $\mu\text{mole Mg/mg. dry-wt. cells}$ (Fig. 2*b*).

Fig. 2*a, b*. Effect of magnesium concentration on the rate of increase in intensity of fluorescence of cell suspensions in presence of *N*-tolyl- α -naphthylamine-8-sulphonic acid and polymyxin. Washed cells (1 mg. dry wt.) suspended in 1% (w/v) NaCl + 2 $\mu\text{mole TNS}$ + magnesium chloride; 150 $\mu\text{g. polymyxin}$ added 5 min. after the addition of magnesium and intensity of fluorescence measured after 1 min. and at intervals thereafter. *a*: intensity of fluorescence plotted against time, figures on graph indicate magnesium concentration (μmole) added to cell suspensions. *b*: rate of increase in fluorescence (V) plotted against magnesium concentration.

A number of uni-, bi- and trivalent cations were tested (Fig. 3*a, b*). Univalent ions (Na^+ , K^+ , Li^+ and NH_4^+) did not compete with polymyxin for sites on the cells, with the result that addition of polymyxin to cells suspended in the presence of these cations and TNS produced 'immediate' maximum fluorescence. Four bivalent cations were tested at a concentration of 10 $\mu\text{mole/mg. dry-wt. cells}$; it was found that the rate of increase in fluorescence varied with the different cations in the order $\text{Mg}^{++} > \text{Sr}^{++} > \text{Ca}^{++} > \text{Ba}^{++}$. The rate in each case has been taken as a measure of the affinity of a particular cation for the polymyxin-combining groups of the cells, i.e. the greater the degree of dissociation of the cation-cell complex the greater the rate of increase

in fluorescence on addition of polymyxin. Tervalent cations were effective at lower concentrations ($0.2 \mu\text{mole/mg. dry-wt. cells}$) than bivalent cations; cerium was found to have a greater affinity than lanthanum for the polymyxin-combining groups of the cells. In all experiments chlorides of metals were used (except for cerium, which was used as sulphate), but it was found that the nature of the anion did not affect the result.

Fig. 3a, b. Effect of bi- and trivalent cations on the rate of increase in intensity of fluorescence of cell suspensions in the presence of *N*-tolyl- α -naphthylamine-8-sulphonic acid and polymyxin. Washed cells (1 mg. dry wt.) suspended in 1% (w/v) NaCl + $2 \mu\text{moles TNS}$ + cations (bivalent; $10 \mu\text{mole/mg. dry-wt. cells}$; trivalent; $0.2 \mu\text{mole/mg. dry-wt. cells}$). Polymyxin ($150 \mu\text{g.}$) was added 5 min. after addition of cations and the intensity of fluorescence measured after 1 min. and at intervals thereafter.

Protection of cells against polymyxin by uranyl ions (UO_2^{++})

In contrast to the results obtained with other cations the uranyl complex with the cells appeared to be relatively undissociated so that polymyxin did not combine with uranyl-treated cells even after long exposures to the antibiotic. Fig. 4a shows the relationship between the amount of uranyl chloride used to treat a suspension containing 1 mg. dry-wt. cells and the percentage protection against polymyxin. Uranyl-treated cells were washed repeatedly with 1% (w/v) sodium chloride without any decrease in the percentage protection against polymyxin. Uranyl ions could be removed from the cells by washing with polyphosphates, sodium hexametaphosphate being the most effective. The protection resulting from treatment of cells with $0.1 \mu\text{mole uranyl chloride/mg. dry-wt. cells}$ was 50% annulled by $2 \mu\text{mole sodium hexametaphosphate}$ and 100% annulled by $10 \mu\text{mole}$ (Fig. 4b). Table 1 shows the relative powers of other polyphosphates to annul the protective action of uranyl ions. Hexose diphosphate, sodium dihydrogen phosphate and sodium citrate had no annulling action at the concentrations tested.

Table 1. *Annulment of uranyl chloride protection*

Washed cells (1 mg. dry wt.) suspended in 1% (w/v) NaCl + 0.1 μ mole UO_2Cl_2 for 5 min., centrifuged, washed with 1% NaCl and treated with the substances shown below for 5 min., then centrifuged, washed with 1% NaCl and resuspended in 1% NaCl + 2 μ moles *N*-tolyl- α -naphthylamine-8-sulphonic acid, and finally 150 μ g. polymyxin added. Intensity of fluorescence was measured after 1 min. Results expressed as % of maximum fluorescence in absence of cations (i.e. % annulment of uranyl chloride protection).

	Concentration (μ mole/mg. dry-wt. cells)	% annulment of UO_2Cl_2 protection
Sodium hexametaphosphate	5	75
Sodium pyrophosphate	10	74
Adenosine triphosphate	100	78
Hexose diphosphate	100	0
Sodium dihydrogen phosphate	1000	0
Sodium citrate	1000	0

a

b

Fig. 4a. Protection of cells against polymyxin by pretreatment with uranyl chloride. Washed cells (1 mg. dry wt.) suspended in 1% (w/v) NaCl, treated with UO_2Cl_2 at concentrations shown for 5 min., centrifuged, washed once with 1% NaCl, resuspended in 1% NaCl + 2 μ mole *N*-tolyl- α -naphthylamine-8-sulphonic acid and 150 μ g. polymyxin added. Intensity of fluorescence measured after 1 min.

Fig. 4b. Reversal of uranyl chloride protection by sodium hexametaphosphate. Washed cells (1 mg. dry wt.) suspended in 1% (w/v) NaCl + 0.1 μ mole UO_2Cl_2 for 5 min., centrifuged, washed once with 1% NaCl and suspended in sodium hexametaphosphate, at the concentrations shown, for 5 min., centrifuged, washed with 1% NaCl, resuspended in 1% NaCl + 2 μ mole *N*-tolyl- α -naphthylamine-8-sulphonic acid and 150 μ g. polymyxin added 1 min. before intensity of fluorescence was measured.

DISCUSSION

The leakage of soluble constituents from washed cells of *Ps. aeruginosa* which results from the addition of polymyxin suggested that the bactericidal activity of this antibiotic might be due to its ability to disorganize the osmotic barrier of a cell by chemical combination with some of its components (Newton, 1953a). The penetration of *N*-tolyl- α -naphthylamine-8-sulphonic acid into polymyxin-treated cells has provided a more direct demonstration

of the immediate permeability change which occurs in the presence of this antibiotic.

Certain antibiotics appear to have an avidity for cations (Albert, 1953), the annulment of aureomycin inhibition of a cell-free nitro-reductase system by manganese being attributed to the formation of a chelate (Saz & Slie, 1953). There is no evidence for chelation between polymyxin and cations (Newton, 1953*b*); the results presented in the present paper indicate that the antagonism of this antibiotic by cations is due to a competition between the cations and polymyxin for sites on the cells. In this respect polymyxin appears to resemble cationic detergents; a number of workers have described competition between non-toxic cations and toxic surface active cations (Valko & DuBois, 1944; Ridenour & Armbrustin, 1948; Massart, 1952).

It is of interest to compare the affinities of bi- and trivalent cations for the polymyxin-combining groups of the cells with the ability of these ions to reverse the charge on certain types of colloids. Bungenburg de Jong (1949) studied the reversal of charge on certain types of colloids by series of cations. He found that with 'phosphate colloids' (i.e. 'colloids' with ester-phosphate as ionogenic groups, e.g. egg lecithin, soya-bean phosphatides, thymus and yeast nucleates) increasing the ion radius of trivalent cations ($\text{Ce}^{+++} \rightarrow \text{La}^{+++}$) increased the reversal of charge concentration. This did not apply to bivalent cations in which the order of efficacy of a number of cations differed irregularly with the particular 'phosphate colloid' studied. However, the UO_2^{++} ion though bivalent showed reversal of charge at extraordinarily low concentration, less than that of trivalent ions. In the case of 'carboxyl colloids' (i.e. 'colloids' with carboxyl groups as ionogenic groups, e.g. Na arabinatate, Na pectinate and Na pectate) increasing the ion radius of bivalent cations decreased the reversal of charge concentration ($\text{UO}_2^{++} < \text{Ba}^{++} < \text{Sr}^{++} < \text{Ca}^{++} < \text{Mg}^{++}$), UO_2^{++} occupying no exceptional position: only in the case of trivalent ions was the sequence still the same as for 'phosphate colloids' ($\text{Ce}^{+++} < \text{La}^{+++}$). For 'sulphate colloids' (i.e. 'colloids' with ester-sulphate as ionogenic groups, e.g. Na-agar and K-chondroitin sulphate), increasing the ion radius of bi- and trivalent ions decreased the reversal of charge concentration, Ce^{+++} , La^{+++} and UO_2^{++} being effective in nearly the same concentration as the bivalent ions. Table 2 sets out the various relationships diagrammatically and shows that there is a close relationship between the cation sequence for the reversal of charge on 'phosphate colloid' and the affinities of these ions for the polymyxin-combining groups of washed cells of *Ps. aeruginosa*. It would thus appear that polymyxin may combine with phosphate groups near the cell surface.

Rothstein & Larrabee (1948) found that yeast cells could bind UO_2^{++} ions and presented evidence for the formation of a highly undissociated complex containing uranium at the cell surface. In a later paper (Rothstein & Meier, 1951) it was suggested that the uranium complexing groups of the cell surface were polymers of phosphate. The fact that UO_2^{++} protection of washed cells of *Ps. aeruginosa* could be annulled by polymerized phosphates but not by inorganic phosphate suggests that the polymyxin-combining loci of the cell surface may also be polyphosphates.

Table 2. *Comparison of specific cation sequences for reversal of charge on certain types of colloids (Bungenburg de Jong, 1949) with the affinities of these cations for the polymyxin combining groups on washed cells of Ps. aeruginosa*

Cation sequences 1, 2 and 3 summarize the results of Bungenburg de Jong (1949) and show the concentrations at which cations reverse the charge on certain types of colloids (1, 'phosphate colloids'; 2, 'carboxyl colloids' and 3, 'sulphate colloids'). Cation sequence 4 summarizes the results recorded in the present paper and shows the concentrations at which cations protect washed cells of *Ps. aeruginosa* against the bactericidal activity of polymyxin. There is a close relationship between the cation sequence of the reversal of charge on 'phosphate colloids', and the affinity of these ions for the 'polymyxin-combining groups' of washed cells of *Ps. aeruginosa*.

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