The Release of Soluble Constituents from Washed Cells of *Pseudomonas aeruginosa* by the action of Polymyxin

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SUMMARY: The addition of polymyxin E to washed cell suspensions of a strain of *Pseudomonas aeruginosa* caused a release of materials which had absorption maxima at 260 mμ, pentose and phosphate from the cells. This release did not occur at 2° and in this respect differed from the leakage resulting when cells were suspended in distilled water or were treated with cetyltrimethylammonium bromide (CTAB). There was an optimum concentration of polymyxin for maximum release of cell constituents, higher concentrations inhibiting the release. A similar release of cell constituents, which was prevented at low temperatures, occurred when cells were suspended in buffer at pH 5.3. Morphological changes resulting from polymyxin treatment have been studied using the electron microscope.

Polymyxin, a material with antibiotic activity produced by *Bacillus polymyxa*, was first reported in America in 1947 (Stansly, Shepherd & White, 1947; Benedict & Langlykke, 1947). In the same year workers in this country isolated an antibiotic material produced by *B. aerosporus* (Ainsworth, Brown & Brownlee, 1947). These two organisms were subsequently shown to be similar, and the antibiotic materials to be closely related. Five polymyxins produced by different strains of *B. polymyxa* have now been recognized (Brownlee & Jones, 1948); these are all basic peptides with molecular weights of about 1200. It has been suggested that they may be cyclic peptides, resembling to some extent gramicidin S (Bell *et al.* 1949). In addition to a number of amino-acids all these compounds contain a C₉ saturated fatty acid which has been identified as 6-methyl-octan-1-oic acid (Wilkinson, 1949). Polymyxin E, which was used in all the work to be described, contains the amino-acids D-leucine, L-threonine and αβ-diaminobutyric acid.

There has been little work reported in the literature on the mode of action of polymyxin. Bliss, Chandler & Schoenbach (1949) observed that the antibiotic was rapidly bactericidal in low concentration against many Gram-negative bacteria, and that it was more effective at 37° than at 10°. The antibacterial activity was found to decrease with increasing size of inoculum. Some organisms, including strains of *Proteus* spp. and *Neisseria* spp., were naturally resistant, but repeated exposure to the antibiotic did not result in the development of resistance in sensitive strains. The activity of polymyxin was decreased by the presence of substances such as soap and lipositol which are known to antagonize cationic detergents. Latterade & Macheboeuf (1950) reported that polymyxin forms water-insoluble complexes with ribonucleic acids, mononucleotides, certain phospholipids and related compounds, and suggested that this may cause the agglutination of certain bacteria by poly-
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myxin. The agglutination is inhibited by neutral salts at low concentration. Szybalski & Bryson (1952) found that certain strains of Bacterium coli can be made resistant to polymyxin B. Cohen, Purdy & Kushnic (1952) reported that polymyxin inhibits esterase activity in a number of mycobacteria. 

Aqueous solutions of polymyxin are markedly surface active. Many surface-active agents are bactericidal, and various explanations have been put forward to account for this action. Baker, Harrison & Miller (1941) suggested that detergents disorganize the cell membrane and denature certain proteins. Hotchkiss (1946) reported the release of nitrogenous and phosphorus-containing compounds from bacteria which had been treated with detergents in bactericidal concentrations. In the case of Gram-positive organisms Gale & Taylor (1947) showed that the nitrogenous substances released included free amino-acids. Salton (1951) found purines, pyrimidines, pentose and inorganic phosphate to be released from Gram-positive and Gram-negative organisms when these were treated with cetyltrimethylammonium bromide (CTAB). Electron microscopy of cells treated with CTAB (Salton, Horne & Coslett, 1951) showed that this detergent caused marked morphological changes. A change in surface charge of such cells was shown by McQuillen (1950), who used a micro-electrophoresis technique.

Knox, Auerbach, Zarudnaya & Spirtes (1949) showed that the enzymic activity of certain cell-free preparations of Bact. coli was inhibited at detergent/protein ratios which were bactericidal for the intact cell. They suggested that the specific inhibition of such detergent-sensitive enzymes could account for the metabolic inhibition, cell death and increased permeability observed in bacteria treated with bactericidal amounts of cationic detergent. However, Salton (1951) suggested that it is unnecessary to invoke the inhibition of a detergent-sensitive enzyme as being the primary cause of cell death, and concluded that there is little evidence which disagrees with the original hypothesis of Baker et al. (1941) that detergents act by disorganization of a cell membrane.

Since polymyxin is surface active it was decided to compare the action of this antibiotic with the cationic detergent CTAB. The organism chosen for these studies was Pseudomonas aeruginosa since polymyxin has been used with some success in the treatment of wounds infected with this organism (Jackson, Lowbury & Topley, 1951).

METHODS

Organism. A strain of Ps. aeruginosa isolated from a patient at the Birmingham Accident Hospital by Dr E. J. L. Lowbury was used in all experiments. The growth of this organism was inhibited by 5 μg. polymyxin E/ml., when an inoculum of c. 10^6 cells/10 ml. 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 8 % (w/v) casein, at an initial pH value of 7.4-7.6. Roux bottles containing 150 ml. medium were inoculated with 2 ml. of an overnight culture in the same medium. Cells were harvested by centrifugation, washed three times in 1 % (w/v)
sodium chloride, and finally suspended in saline of the same strength to give a suspension containing between 12 and 15 mg. dry weight cells/ml. Dry weights were determined by drying 1·0 ml. samples of the suspension to constant weight in an air oven at 105°.

Treatment of cells. The release of cell constituents was examined after treatment of the cells in the following ways: (1) suspension in 1 % (w/v) sodium chloride; (2) suspension in distilled water; (3) suspension in 1 % (w/v) sodium chloride solutions containing polymyxin or CTAB; (4) suspensions in McIlvaine's (1921) citrate-phosphate buffers at various pH values; (5) after being pipetted into distilled water at 100° and held at that temperature for 15 min.; (6) extraction with 5 % trichloroacetic acid (TCA) at 5° for 1 hr.

In all cases the final suspension density was 1·2–1·5 mg. dry weight cells/ml.

Estimation of cell constituents released into supernatant fluid. The majority of the cells were removed from samples by centrifugation at 2000 g for 5 min.; the supernatant fluids were removed and clarified by centrifugation at 2000 g for 20 min. The ultraviolet absorption spectra of these cell-free supernatant fluids was studied by means of a Beckman spectrophotometer, corrections being made for the absorption of the suspending liquids. Total phosphate content of the supernatant fluid was determined by the method of Fiske & Subbarow (1925); pentose was estimated by the procedure of Mejbaum (1939) calibrated against D-ribose.

Electron micrographs. Washed cells suspended in 1 % (w/v) saline containing 0·08 m-phosphate adjusted to pH 7·2 were treated with various concentrations of polymyxin for 1 hr. at 80°. Micro-drops of these suspensions, suitably diluted with 1 % (w/v) saline, were placed on specimen grids covered with nitrocellulose film, fixed for 2 min. in osmic acid vapour, dried in a desiccator and then washed free of salts with distilled water. The specimens were shadowed with gold-palladium alloy (60:40%). The shadowing was at an angle of 80° from the plane of the supporting film. Observations were made in the Siemens electron microscope at a direct magnification of ×10,000–14,000.

RESULTS

The leakage from washed cells of material absorbing at 260 m\(\mu\).

Examination of the ultraviolet absorption spectra of the supernatant solutions from suspensions of cells in 1·0 % (w/v) sodium chloride or distilled water showed a maximum at 260 m\(\mu\). in each case (Newton, 1958). After 3 hr. incubation at 30° the absorption of the supernatant solution from cells in distilled water was about ten times that of the supernatant solution from suspensions in saline. Addition of polymyxin or CTAB (final concentration 100 \(\mu\)g./ml. in each case) to the suspensions in distilled water did not result in any increase in the leakage of 260 m\(\mu\). absorbing material. These results are recorded in Table 1.

The leakage from cells in distilled water was decreased by the presence of sodium chloride. The effect of salt concentration on the leakage is shown in Fig. 1: concentrations up to 1 % (w/v) sodium chloride caused a marked
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Table 1. Release of 260 mµ absorbing material from washed cells of Pseudomonas aeruginosa

Suspensions of cells in 1% (w/v) saline, distilled water and distilled water + 100 µg polymyxin or CTAB/ml. The amount of 260 mµ absorbing material released into the suspending fluid was estimated at various times during a 3 hr. incubation at 30°.

<table>
<thead>
<tr>
<th>Suspending medium</th>
<th>1% (w/v) saline</th>
<th>Distilled water</th>
<th>Water + 100 µg. polymyxin/ml.</th>
<th>Water + 100 µg. CTAB/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min.)</td>
<td>Log I_o/I (at 260 mµ)/mg. dry-weight cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.028</td>
<td>0.80</td>
<td>0.78</td>
<td>0.92</td>
</tr>
<tr>
<td>30</td>
<td>0.043</td>
<td>1.80</td>
<td>1.64</td>
<td>1.91</td>
</tr>
<tr>
<td>60</td>
<td>0.085</td>
<td>2.17</td>
<td>2.06</td>
<td>2.16</td>
</tr>
<tr>
<td>120</td>
<td>0.190</td>
<td>2.40</td>
<td>2.23</td>
<td>2.42</td>
</tr>
<tr>
<td>180</td>
<td>0.260</td>
<td>2.61</td>
<td>2.41</td>
<td>2.67</td>
</tr>
</tbody>
</table>

decrease in the leakage; further increase in concentration had little additional effect. The addition of polymyxin or CTAB to cells suspended in 1.0% (w/v) saline caused a rapid release of 260 mµ absorbing material. The ultraviolet absorption spectra of the supernatant solutions after 3 hr. incubation at 30° is shown in Fig. 2.

Fig. 1. The effect of salt concentration on the leakage of 260 mµ absorbing material. Washed cells of *Ps. aeruginosa* suspended in sodium chloride of varying concentrations were incubated at 30°. The absorption of the supernatant fluids at 260 mµ. was measured after 10 min. (•—•), 30 min. (□—□) and 180 min. (○—○).

Fig. 2. Ultraviolet spectra of supernatant fluids from *Ps. aeruginosa* suspensions (1.5 mg. dry-weight cells/ml) in 1.0% (w/v) saline alone or +100 µg. polymyxin or CTAB/ml. Temperature, 30°. Curve 1, saline suspension, initial sample; curve 2, saline suspension incubated for 180 min.; curve 3, suspension in saline + CTAB incubated for 180 min.; curve 4, suspension in saline + polymyxin incubated for 180 min.
The rate of release of 260 mμ absorbing material from suspensions of cells in distilled water, 1-0% (w/v) saline or 1% (w/v) saline + 100 μg. polymyxin/ml. was followed for 22 hr. and compared with the amount of material released by treatment at 100°. Fig. 3 shows that the release from cells in distilled water was complete in about 8 hr. Cells standing in saline released 260 mμ absorbing material at a slow steady rate during the whole period of 22 hr. The addition of polymyxin to such suspensions resulted in an initial rapid release during the first 4–5 hr. followed by a slower release during the next 17 hr. The amount of 260 mμ absorbing material released from polymyxin-treated cells in 22 hr. exceeded the amount which was released from the cells by treatment at 100°. Similar results were obtained when CTAB was added to cells suspended in saline.

![Graph showing rate of release of 260 mμ absorbing material from Ps. aeruginosa suspended in various conditions.](image)

**Fig. 3.** Rate of release of 260 mμ absorbing material from *Ps. aeruginosa* suspended in 1-0% (w/v) saline (●—●); distilled water (○—○); 1-0% (w/v) saline + 100 μg. polymyxin/ml. (●—○). Temperature, 30°.

**The effect of polymyxin and CTAB concentration on the release of 260 mμ absorbing material**

Estimations were made of the amounts of 260 mμ absorbing material released from cells during treatment with a range of polymyxin or CTAB concentrations from 10 to 1000 μg./ml. Samples were taken at various times during a 6 hr. incubation at 30°. Figs. 4 and 5 show that the curves obtained for polymyxin and CTAB-treated cells were essentially the same. There was an optimum concentration for maximum release of 260 mμ absorbing material, and increase in concentration above this level caused less to be released. In the case of CTAB-treated cells, concentrations above 500 μg./ml. resulted in the immediate release of a small amount of 260 mμ absorbing material with
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no further release during the next 6 hr. The amount released by these high concentrations was found to be equal to the amount extractable from the cells with cold 5% TCA; this was only one-tenth of the amount which could be extracted from the cells by treatment at 100°. With polymyxin-treated cells similar results were obtained, but the agreement was not quite as close since the absorption of polymyxin at 260 m.µ. is appreciable in concentrations above 500 µg./ml. A satisfactory method for determining the amount of polymyxin remaining in the supernatant solutions has yet to be found, and the curves are not corrected for the absorption due to this.

![Graph](image_url)

**Fig. 4.** The effect of polymyxin concentration on the release of 260 m.µ. absorbing material from *Ps. aeruginosa* at 30°. Cells were suspended in 1-0% (w/v) saline + polymyxin and the absorption of the supernatant solution measured at 260 m.µ. after the following periods of incubation: curve 1, 30 min.; curve 2, 2 hr.; curve 3, 4 hr.; curve 4, 6 hr.

**Fig. 5.** The effect of CTAB concentration on the release of 260 m.µ. absorbing material from *Ps. aeruginosa* at 30°. Cells were suspended in 1-0% (w/v) saline + CTAB, and the absorption of the supernatant solution measured at 260 m.µ. after the following periods of incubation: curve 1, 30 min.; curve 2, 1 hr.; curve 3, 2 hr.; curve 4, 5 hr.; curve 5, 8 hr.

**The release of pentose and phosphate from cells**

The ultraviolet absorption of supernatant fluids from cells suspended in saline or saline +100 µg./ml. polymyxin was measured at 260 m.µ., and samples were then analysed for pentose and total phosphate content. Fig. 6 shows that the release of pentose and phosphate occurred at the same rate as the release of 260 m.µ. absorbing material. Similar results were obtained with CTAB-treated cells and with cells suspended in distilled water.

**The effect of pH on the release of 260 m.µ. absorbing material**

Washed cells were suspended in McIlvaine's (1921) citrate phosphate buffer, in the presence or absence of polymyxin, at a range of pH values between 3 and 8. Samples were taken after incubating at 30° for 1 and 3 hr., and the absorption of the cell-free supernatant solutions measured at 260 m.µ. Fig. 7 shows that after 1 hr. incubation, while there was marked release at pH 5-8 there was little release from the cells suspended in buffer alone at pH values between 6-5 and 8. After 3 hr. incubation the leakage from cells in buffer alone at
pH 5.3 exceeded the leakage from polymyxin-treated cells and was also greater than the amount which could be extracted from the cells by treatment at 100°.

Fig. 6. Rate of release of cellular constituents from *Ps. aeruginosa* suspensions at 30°. (●—●), total phosphate; (○—○), 260 mµ. absorbing material; (■—■), pentose. (A) cells suspended in 1.0 % (w/v) saline; (B) cells suspended in 1.0 % (w/v) saline + 100 µg. polymyxin/ml.

The effect of temperature on the release of 260 mµ. absorbing material

It has been shown that the release of 260 mµ. absorbing materials was accelerated by suspension of cells in (1) distilled water, (2) saline + polymyxin, (3) saline + CTAB, and (4) buffer at pH 5.3. The rate of release was followed at 2 and 30° in each case, and it was found that the release was of two types: (a) that which occurred at both temperatures, and (b) that which did not
occur at $2^\circ$ (Figs. 8, 9). The leakage from suspensions of cells in distilled water occurred at both temperatures, whereas the leakage from cells suspended in buffer at pH 5-8 and in saline + polymyxin did not occur at $2^\circ$. In the case of CTAB-treated cells there was an initial release at $2^\circ$ which was complete after 30 min., no further release occurring during the next 5 hr. The amount of 260 m. $\mu$ absorbing material released from cells by CTAB at $2^\circ$ was approximately the same as that obtained by extraction of the cells with cold 5\% TCA.

![Figure 7](image-url)  
Fig. 7. Effect of pH on the release of 260 m. $\mu$ absorbing material. (●—●), cells suspended in McIlvaine's citrate phosphate buffers; (○—○), suspensions in buffer +100 $\mu$g. polymyxin/ml.

Electron microscopical observations on polymyxin-treated cells

Electron micrographs of polymyxin-treated cells showed that the release of soluble cell constituents was accompanied by marked morphological changes. Concentrations of polymyxin which caused maximum release of 260 m.$\mu$. absorbing material also caused a maximum loss of 'electron-dense' material from the cells. Cells treated with higher concentrations of polymyxin, which inhibited the release of 260 m.$\mu$. absorbing material, retained the majority of their 'electron-dense' material although they show marked surface damage (Pl. 1, figs. 1–5).

DISCUSSION

The leakage of 260 m.$\mu$. absorbing material, pentose and phosphate which occurred when washed cells of *Ps. aeruginosa* were treated with CTAB was very different from the leakage from streptococci and staphylococci observed
by Salton (1951). In the case of these Gram-positive organisms the amount of material released by the detergent equalled the amount which could be extracted from the cells by treatment at 100°, and this release was complete in about 5 min. in the case of streptococci and 80 min. in the case of staphylococci. Increase in the concentration of detergent did not result in any

Fig. 8. Effect of temperature on release of 260 mJ. absorbing material. Cell suspensions in 1-0 % (w/v) saline (•—•); distilled water (○—○); saline + 100 µg. polymyxin/ml. (□—□); suspensions incubated at 30 and 2°, the amount of 260 mJ. absorbing material in the supernatant fluids is plotted against time.

Fig. 9. Effect of temperature on release of 260 mJ. absorbing material. Cell suspensions in citrate-phosphate buffer, pH 7-3 (●—●); pH 5-3 (x—x); pH 7-8 + 100 µg. CTAB/ml. (○—○); pH 7-8 + 100 µg. polymyxin/ml. (□—□). Suspensions incubated at 30 and 2°, the amount of 260 mJ. absorbing material in the supernatant fluids is plotted against time.
marked decrease in the amount of materials released. CTAB treatment of P. aeruginosa resulted in a slow leakage which continued for many hours, and eventually exceeded the amount which could be extracted from the cells at 100°C. This slow leakage did not occur at low temperatures or in high concentrations of the detergent. In each of these cases there was an initial rapid leakage of cell constituents, the amount of which corresponded very closely to the amount which could be extracted from the cells by cold 5% TCA.

These facts could be explained by assuming that the cells contain a small amount of nucleotide, or other purine or pyrimidine-containing material which was immediately released on the addition of the detergent; this leakage could be the result of a physical disorganization of the osmotic barrier of the cells as proposed by Baker et al. (1941). Such an effect would not be markedly affected by temperature. The further release of cell constituents may be due to an enzymic breakdown of larger non-diffusible cell components, this breakdown being inhibited by high concentrations of CTAB or by low temperatures.

Polymyxin, in causing the leakage of 260 mč. absorbing material, pentose and phosphate from washed cells, appeared to be acting in a manner similar to CTAB. As aqueous solutions of polymyxin are markedly surface active and the molecule contains a hydrophobic chain and a polar group, and as the bactericidal activity of the antibiotic is decreased by the presence of substances known to antagonize cationic detergents (Bliss et al. 1949), this conclusion seems reasonable. However, the fact that the leakage produced by polymyxin did not occur at 2°C suggests that it may have been entirely due to enzymic breakdown of cellular constituents, and that the antibiotic did not act primarily by altering the permeability of these cells. Alternatively, polymyxin may cause a disorganization of the osmotic barrier of the cell by chemical combination with some of its components; this combination may not occur to any significant extent at 2°C. CTAB may differ from polymyxin in its action, by causing a physical disorganization which cannot be prevented by a lowering of temperature.

The author is indebted to Dr E. F. Gale for encouragement and help. He is also grateful to Dr M. R. J. Salton for samples of CTAB; to Mr H. Pearson, Mr K. Harvey and Miss E. Green, of the Cavendish Laboratory, for technical assistance in taking electron-micrographs, and to Burroughs Wellcome and Co. for a supply of polymyxin E.

REFERENCES


EXPLANATION OF PLATE

Fig. 1. *Pseudomonas aeruginosa*. Washed suspension of cells from a 15 hr. culture.

Fig. 2. Washed cells treated for 1 hr. with 25 µg. polymyxin/ml. About 50% of the cells appear to have lost the majority of their ‘electron dense’ material.

Fig. 3. Washed cells treated for 1 hr. with 50 µg. polymyxin/ml. 98% of the cells have lost ‘electron dense’ material. This concentration of polymyxin causes maximum release of 260 mµ. absorbing materials.

Fig. 4. Washed cells treated for 1 hr. with 500 µg. polymyxin/ml. All the cells have retained their ‘electron dense’ material but show some surface damage. This concentration of polymyxin inhibited the leakage of 260 mµ. absorbing materials.

Fig. 5. Washed cells treated for 1 hr. with 1000 µg. polymyxin/ml. Cells still retain their ‘electron dense’ material but show marked surface damage.

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B. A. Newton—Polymyxin and *Pseudomonas aeruginosa*. Plate 1