A Fluorescent Derivative of Polymyxin: its Preparation and Use in Studying the Site of Action of the Antibiotic

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SUMMARY: A fluorescent derivative of polymyxin (DANSP) was prepared by coupling 1-dimethylaminonaphthalene-5-sulphonyl chloride with the γ amino group of $\alpha\gamma$ diaminobutyric acid radicals in the polymyxin molecule. As in the case of polymyxin, DANSP is readily absorbed by polymyxin-sensitive organisms and is rapidly bactericidal; cells can be protected against this bactericidal activity by pretreatment with certain cations. Mechanical disintegration of DANSP-treated cells showed that the fluorescent compound was associated with two fractions: (a) cell walls and (b) small particles. Fractionation of three strains of *Pseudomonas aeruginosa* showed that the fluorescent conjugate was equally distributed between the two fractions, but in the case of three Gram-positive, lysozyme-sensitive, organisms 90 % of the fluorescent conjugate was found in the small particle fraction; controlled lysozyme treatment of these cells showed that the DANSP was associated with a membrane underlying the cell wall from which the small particle fraction is formed on mechanical disintegration.

Previous studies (Few & Schulman, 1953; Newton, 1953*a*) have shown that the addition of polymyxin to washed cell suspensions of sensitive organisms causes a release of soluble constituents from the cells; it was suggested by these workers that the bactericidal activity of polymyxin might be due to its ability to combine with certain groups near the cell surface and, in so doing, cause a disorganization of a cell membrane or osmotic barrier. Sensitive organisms can be protected against the bactericidal activity of polymyxin by certain cations (Newton, 1953*b*). This protection is due to a competition between polymyxin and cations for sites on the cells, pretreatment of the cells with cations preventing absorption of the antibiotic. A comparison of the affinities of a number of bi- and tervalent cations for the polymyxin-combining groups of a strain of *Pseudomonas aeruginosa* with the ability of these cations to reverse the charge on certain types of colloids suggested that the polymyxin-combining loci of these cells may be polyphosphates (Newton, 1954*a*).

As yet there has been no account of the localization of polymyxin within the bacterial cell; in a preliminary communication (Newton, 1954*b*) it was reported that cell walls prepared from a polymyxin-sensitive strain of *Pseudomonas aeruginosa* by mechanical disintegration rapidly absorbed as much as 300 $\mu\text{g.}$ polymyxin/mg. dry wt. cell walls, whereas cell walls prepared from a polymyxin-resistant strain of the same organism absorbed less than 70 $\mu\text{g.}/\text{mg.}$; electron microscopy showed that cell walls of sensitive strains were more electron-dense after polymyxin treatment. Similar results have been obtained with cell wall preparations from a number of other organisms

(Few, 1954), and Few & Schulman (1953) state that 'the isotherms for the absorption of polymyxin by both intact bacteria and bacterial cell wall preparations favour absorption at sites within the bacterial cell wall with but little penetration inside the cell'. However, experiments with isolated cell walls are difficult to interpret and give no indication of the amount of antibiotic which would be absorbed by the cell wall when other cellular components are present. A more direct approach to this problem has been made possible by the preparation of a fluorescent derivative of polymyxin.

Weber (1952) described the preparation of fluorescent conjugates of ovalbumin and bovine serum albumin with 1-dimethylaminonaphthalene-5-sulphonyl chloride. He found that coupling with the dye did not induce any observable denaturation of ovalbumin as judged by solubility at the isoelectric point; also that the enzymic properties of fumarase and ribonuclease were unaffected by coupling 1 to 3 molecules of the naphthalene derivative per protein molecule. There is a large difference in affinity of sulphonyl chloride for —OH and =NH compared with —NH_2 groups and it seems likely that only $\text{—SO}_2\text{NH—}$ bonds are formed, although this has not yet been demonstrated. Weber found no evidence for spontaneous breakdown of the conjugates, as shown by the appearance of free naphthalene sulphonic acid, while the fluorescence of the compounds was unaffected by changes of pH between 1.6 and 14.

Polymyxin B is a basic peptide of molecular weight *c.* 1200; it contains the amino acids phenylalanine, D-leucine, L-threonine and L- $\alpha\gamma$ -diaminobutyric acid in addition to a C_9 saturated fatty acid identified as methyl octan-1-oic acid (Wilkinson, 1949). A cyclic structure has been postulated for the molecule by Bell *et al.* (1949) who identified the free amino groups of intact polymyxin with the γ -amino groups of $\alpha\gamma$ -diaminobutyric acid and found five molecules of this amino acid per polymyxin molecule. A fluorescent derivative of polymyxin has been prepared by coupling 1-dimethylaminonaphthalene-5-sulphonyl chloride with the free amino groups of the antibiotic; limiting amounts of the sulphonyl compound being used so that each polymyxin molecule carried on the average one naphthalene group. The derivative (DANSP in the sequel) had approximately the same bactericidal activity as untreated polymyxin. The present paper describes the preparation of the compound and its localization in polymyxin sensitive organisms.

METHODS

Organisms. Organisms used in this work were three strains of *Pseudomonas aeruginosa* isolated by Dr E. J. L. Lowbury at the Birmingham Accident Hospital; *Bacillus megaterium* strain KM; a laboratory strain of *Sarcina lutea* and one of *Micrococcus lysodeikticus* (NCTC no. 2665).

Media, conditions of growth and harvesting. Strains of *Pseudomonas aeruginosa* were grown in tryptic digest of casein containing the equivalent of 3% (w/v) casein at an initial pH value of 7.4–7.6, the conditions of culture and harvesting being the same as previously described (Newton, 1953*a*).

Bacillus megaterium, *Sarcina lutea* and *Micrococcus lysodeikticus* were grown in 2% Difco peptone contained in Pyrex tubes (4×20 cm.) with a maximal depth of liquid of 8 cm. Tubes were inoculated with 2 ml. of an overnight culture in the same medium and the organisms grown for 15 hr. at 30° with aeration. Cells were harvested by centrifugation, washed once in 0.03 M-phosphate buffer (pH 7.0) and resuspended in the same buffer to give a suspension of *c.* 10 mg. dry wt. cells/ml. Dry weights were determined on a Hilger absorptiometer previously calibrated against the organisms used.

Preparation of 1-dimethylaminonaphthalene-5-sulphonamidopolymyxin (DANSP). Polymyxin B sulphate (250 mg.) and sodium bicarbonate (50 mg.) were dissolved in water, cooled to 2° and mixed with a solution of 1-dimethylaminonaphthalene-5-sulphonyl chloride (60 mg.) in 0.5 ml. acetone; the mixture was shaken for 3 hr. and the resulting conjugate separated from any free sulphonate by repeated precipitation with ethanol followed by slow filtration through a column of Dowex 2 (mesh 200, alkaline form). This treatment removed all traces of the free acid, small amounts of which were readily detected by paper chromatography as below.

Chromatographic separation of DANSP and 1-dimethylaminonaphthalene-5-sulphonic acid. The materials dissolved in 60% ethanol were spotted on Whatman no. 1 filter-paper, molar phosphate buffer, pH 7.0 was used as solvent and ascending chromatograms were run for 45 min. Papers were photographed in ultraviolet light using a light filter of saturated sodium nitrite solution 1 cm. thick (Weber, 1953). The R_F value of the sulphonamide derivative was effectively 0 and that of the sulphonate was 0.6; Pl. 1, fig. 1, is a photograph of a chromatogram showing the removal of free sulphonate by filtration through Dowex 2.

Absorption spectra of 1-dimethylaminonaphthalene-5-sulphonic acid, polymyxin B and DANSP. The absorption spectra of 1-dimethylaminonaphthalene-5-sulphonic acid, polymyxin B and DANSP are shown in Fig. 1. Polymyxin B shows an absorption maximum at $256 m\mu$. due to its phenylalanine content. The sulphonic acid and its sulphonamide derivatives show an absorption band in the $300\text{--}400 m\mu$. region; the position of the maximum depends upon the ionic character of the $=SO_2$ group (Weber, 1952); coupling with polymyxin resulted in a significant displacement towards the red (from 315 to $333 m\mu$.).

Determination of limiting inhibitory concentrations of polymyxin B and DANSP for Pseudomonas aeruginosa and Bacillus megaterium. Serial dilutions of polymyxin B sulphate or DANSP were made into sterile tryptic digest of casein contained in test tubes (2.5×15 cm.) which were closed with 'Oxoid' metal caps. Tubes, containing 5 ml., were inoculated with *c.* 10^5 viable cells/ml. medium and incubated at 30° for 24 hr. The concentration of antibiotic in the first tube which showed no growth after 24 hr. was taken as the limiting inhibitory concentration; incubation for 48 hr. did not alter the end point.

Plate-count estimation of surviving cells. Bacterial suspensions were treated with different concentrations of the antibiotic for 5 min. at 30° , 1 ml. samples

were then withdrawn and transferred to 99 ml. sterile saline (1 %, w/v), suitable dilutions in saline were then plated out in casein digest growth medium containing 2 % agar and the colonies counted after 48 hr. incubation at 30°.

Mechanical disintegration of bacteria and isolation of cell walls. The procedure used was essentially the same as that described by Salton (1953). Ten ml. of a washed cell suspension containing c. 10 mg. dry wt. cells/ml. were shaken with 10 ml. ballotini glass beads (grade 12), in the Mickle disintegrator (Mickle, 1948) for 15 min. The ballotini were removed by filtration through

Fig. 1.

Fig. 1. *Absorption spectra.* ●—●, 1-dimethylaminonaphthalene-5-sulphonic acid (100 μ g./ml. in 60 % ethanol); ×—×, polymyxin B sulphate (500 μ g./ml. in 60 % ethanol); ○—○, 1-dimethylaminonaphthalene-5-sulphonamido-polymyxin (DANSP; 100 μ g./ml. in 60 % ethanol).

Fig. 2.

Fig. 2. *Distribution of DANSP between the cell wall and small particle fractions of disrupted *Bacillus megaterium*.* Washed cells of *B. megaterium* treated with various concentrations of DANSP for 20 min. at 30° and washed repeatedly in distilled water until no fluorescence remained in washings. Intensity of fluorescence of intact cells and of cell fractions obtained by mechanical disintegration and differential centrifugation. Fluorescence of cell fractions expressed as percentage of fluorescence of intact cells. Number of cells surviving DANSP treatment estimated by plate counts.

a no. 1 sintered glass funnel. Under these conditions of disintegration more than 90 % of the cells were broken; any intact cells were removed from suspension by centrifugation for 10 min. at 1000 *g*. The cell walls were then separated from the 'cytoplasmic fraction' by centrifugation for 20 min. at 10,000 *g*; washed once in 1 *M*-sodium chloride and then repeatedly with distilled water until the supernatant fluids no longer gave a precipitate with silver nitrate. Small particles were separated from the 'cytoplasmic fraction' by centrifugation for 15 min. at 100,000 *g* in a Spinco Model L centrifuge.

Lysozyme treatment of Bacillus megaterium. Protoplasts were prepared from *B. megaterium* by treatment with lysozyme under the conditions described by Weibull (1953).

Fluorescence microscopy. Fluorescence photomicrographs were taken by Mr J. Smiles and Mr M. R. Young of the National Institute for Medical Research, Mill Hill, London, using a high-pressure mercury vapour lamp (Type G.E.C. 'Osira' 80 W) as ultraviolet light source with a Chance 0 × 7 filter and 4 % nickel sulphate solution as primary filter. The secondary filter placed in the projection ocular was a Leitz 'blue-green' filter. Photographic plates were Ilford Selochrome; exposure time *c.* 10 min.

Measurement of intensity and polarization of fluorescence. Intensities of fluorescence of DANSP-treated cell suspensions and cell fractions were measured by comparison with a standard fluorescent solution using a modified Pulfrich photometer as previously described (Newton, 1954*a*). The polarization of fluorescence was determined by the method of Weber (1952).

Preparation of specimens for the electron microscope. Specimens were washed twice with distilled water and finally suspended in distilled water. Protoplasts and protoplast membranes were fixed with mercuric chloride-formalin solution before washing. Micro-drops of suspensions were placed on specimen grids covered with nitrocellulose film and shadowed with gold palladium alloy (60:40 %). The shadowing was at an angle of 30° from the plane of the supporting film. Observations were made in the Siemens electron microscope, generally at a direct magnification of × 10,000–14,000.

RESULTS

Limiting inhibitory concentrations of polymyxin B and DANSP

Growth tests showed that the antibiotic properties of polymyxin were not significantly affected by coupling with 1-dimethylaminonaphthalene-5-sulphonyl chloride. The limiting inhibitory concentration of polymyxin B for *Pseudomonas aeruginosa* was 1.5 µg./ml. in growth tests using an inoculum of *c.* 10⁶ viable cells/ml. In similar growth tests the limiting inhibitory concentration of DANSP was 3.0 µg./ml. Growth of *Bacillus megaterium* was inhibited by 0.75 µg. polymyxin/ml. and by 1.0 µg. DANSP/ml.

Absorption of DANSP by washed cells of Pseudomonas aeruginosa and Bacillus megaterium

Suspensions of washed cells in 1 % (w/v) saline (containing *c.* 1 mg. dry wt. cells/ml.) were treated with 25 µg. DANSP/ml. for 5 min. at 30°. After centrifugation the cell pellet and supernatant fluid were examined in ultraviolet light. It was found that the cells had absorbed the DANSP and fluoresced strongly, and that the fluorescence was unaffected by repeated washing with 1 % saline. Pl. 1, figs. 2, 3, are fluorescence photomicrographs of *P. aeruginosa* and *B. megaterium* treated with DANSP as described above.

It has previously been shown (Newton, 1953*a, b*) that washed cells of *Pseudomonas aeruginosa* can be protected against the bactericidal activity

of polymyxin by pretreatment with certain cations, the cations preventing the absorption of antibiotic by the cells. Cations also prevent the absorption of DANSP by bacteria. Washed cells of *P. aeruginosa* suspended in 1% (w/v) saline or 1% saline + 0.05 M-calcium chloride were treated with DANSP (25 µg./ml.) for 5 min., the suspensions centrifuged and the cell pellets examined under ultraviolet light. Pl. 1, fig. 4 shows that in the presence of calcium ions little DANSP was absorbed by the cells.

Fractionation of DANSP-treated cells

Experiments with Pseudomonas aeruginosa. Washed cells of *P. aeruginosa* suspended in 1% (w/v) saline were treated with DANSP (25 µg./mg. dry wt. cells) for 20 min. at 30°, the suspension centrifuged, and the cells washed repeatedly with 1% saline until no trace of fluorescence remained in the washings when these were examined under ultraviolet light. The cells were resuspended in 0.01 M-phosphate buffer (pH 6.3) to give a suspension of c. 10 mg. dry wt. cells/ml. and shaken with ballotini in the Mickle disintegrator for 20 min. The cell wall fraction was then isolated as described above. It was found that after removal of the cell walls the supernatant fluid still fluoresced strongly when examined under ultraviolet light; measurement of the intensity of fluorescence of unbroken cells, the cell wall fraction and the 'cytoplasmic fraction' in the Pulfrich photometer showed that the fluorescence was equally distributed between the cell-wall fraction and the 'cytoplasmic fraction'. When the cell walls were shaken with ballotini in the Mickle disintegrator for a further 30 min. centrifugation showed that no fluorescent material had been removed from the walls by this treatment.

The fluorescence of the 'cytoplasmic fraction' was found to be strongly polarized; the degree of polarization did not vary over the temperature range 2–30°, indicating that DANSP was absorbed on to a macromolecule of molecular weight > 300,000 (Weber, 1952). Centrifugation of the cytoplasmic fraction at 10,000 *g* for 1 hr. sedimented part of the fluorescent material, the remainder being sedimented after centrifugation for 15 min. at 100,000 *g*. The 'pellet' obtained by this high-speed centrifugation was semi-transparent and dark red in colour; examination of this material in the electron microscope showed that it was mainly composed of small spherical particles 10–30 mµ. in diameter. Examination of a suspension of this small particle fraction with a Zeiss spectroscop showed general absorption below 450 mµ. but no marked bands at longer wavelengths. Addition of sodium dithionite resulted in the immediate appearance of an intense band at 550 mµ. and a weaker band at 559 mµ., suggesting that cytochromes *b* and *c* were associated with the particles. In this respect these particles resemble those isolated from sonic disintegrates of *Pseudomonas fluorescens* by Stanier, Gunsalus & Gunsalus (1953). A similar distribution of DANSP was found when two other strains of *P. aeruginosa* were studied.

Mechanical disintegration of Bacillus megaterium. Washed cells of *B. megaterium* were treated with a range of concentrations of DANSP (10–100 µg.

DANSP/mg. dry wt. cells) for 20 min. at 30°, washed repeatedly with distilled water until no trace of fluorescence remained in the washings, and finally resuspended in distilled water to give a suspension of *c.* 10 mg. dry wt. cells/ml. A sample of this suspension was taken for measurement of fluorescence intensity, the remainder shaken with ballotini in the Mickle disintegrator, and the cell wall and small particle fractions of the disintegrated cells separated by differential centrifugation. After sedimentation of the small particles no fluorescent material remained in the supernatant fluids. The intensity of fluorescence of the cell wall and small particle fractions was measured and expressed as a percentage of the fluorescence of the whole cells. Fig. 2 shows that for a range of four concentrations of DANSP between 10 and 100 $\mu\text{g./mg.}$ dry wt. cells, 90 % of the fluorescence was associated with the small particle fraction and only 10 % with the cell walls. Fig. 2 also shows the relationship between the percentage cells killed and DANSP concentration; *c.* 25 $\mu\text{g.}$ DANSP/mg. dry wt. cells was required for 99 % killing.

When cell walls and small particles were prepared from washed cells of *Bacillus megaterium* it was found that the cell walls alone, on incubation with DANSP, absorbed up to 270 $\mu\text{g.}$ DANSP/mg. dry wt. cells, a figure in close agreement with the values already recorded for the absorption of polymyxin by cell walls of other sensitive organisms (Few & Schulman, 1953; Newton, 1954*b*). The DANSP absorbed by these cell walls was not removed by repeated washing with distilled water. When, however, the DANSP-treated cell walls were incubated with the small particle fraction for 10 min. at 30° and again separated by centrifugation, it was found that more than 90 % of the DANSP had been removed from the cell walls and had been taken up by this small particle fraction.

Lysozyme treatment of Bacillus megaterium. Weibull (1953) showed that when washed cells of *B. megaterium* suspended in dilute phosphate buffer were treated with lysozyme a rapid lysis occurred which left only spherical empty ghosts and small granules. When the cells are suspended in 0.2 M-sucrose during the lysozyme treatment, the bacterial cell wall becomes depolymerized but the rest of the cell remains as an intact structural unit—a spherical 'protoplast' (Pl. 2, fig. 7). Such protoplasts can be lysed by dilution of the sucrose; they then give rise to the ghosts and granules obtained by lysozyme treatment in phosphate buffer. Centrifugation of the lysed protoplasts at 10,000 *g* for 15 min. sediments a dark yellow layer consisting mainly of ghosts, which in the electron microscope appear to be flat membrane-like bodies (Pl. 2, fig. 8). When this fraction is treated for a short period in a sonic oscillator the membrane-like structure is destroyed, but the yellow material can be re-sedimented as a small particle fraction by centrifugation at 100,000 *g*. Weibull found that the cytochromes of the cell are associated with this yellow fraction.

Fluorescence photomicrographs of DANSP-treated *Bacillus megaterium* (Pl. 1, fig. 3) showed that the fluorescent compound was associated with the boundaries of the cell and the cross-walls. When such fluorescent cells were suspended in sucrose solution (0.2 M) and treated with lysozyme, the cell wall

depolymerized, leaving protoplasts which fluoresced strongly in ultraviolet light (Pl. 1, fig. 5). No detectable fluorescent material remained in the supernatant fluids after centrifugation of the protoplast suspension.

When a suspension of fluorescent protoplasts was subjected to supersonic vibration of 25 kc./sec. (generated by a 500 W. Mullard magnetostrictor oscillator) the protoplast structure was completely destroyed. The fluorescent material in the supersonic disintegrate was then found to be associated with a small particle fraction sedimented by centrifugation at 100,000 *g* for 15 min. Similar results were obtained with *Sarcina lutea* and *Micrococcus lysodeikticus*.

*Properties of protoplasts prepared from untreated and
polymyxin treated Bacillus megaterium*

Protoplasts prepared from washed cells of *B. megaterium* which had been treated with a bactericidal concentration of polymyxin or DANSP differed from protoplasts obtained from untreated cells of this organism in that they were not lysed by suspension in distilled water. Further, they consisted of cubical rather than spherical subcellular units, this is clearly shown in the electron micrographs (Pl. 2, figs. 6-9) and was confirmed by phase-contrast microscopy.

DISCUSSION

There is now considerable indirect evidence (Few & Schulman, 1953; Newton, 1953*a*, 1954*a*) that polymyxin combines with and disorganizes structures within the bacterial cell wall which are responsible for the maintenance of the osmotic equilibrium of the cell. A more direct approach to the problem of the site of action of polymyxin has been made possible by studies on protoplasts by the use of a fluorescent derivative of the antibiotic (DANSP). In the experiments described here it has been possible to demonstrate the combination of DANSP with cell walls and small particles from disrupted bacteria, while in the case of certain lysozyme-sensitive organisms it has been found that DANSP combines with a membrane underlying the cell wall. The association of DANSP with the small particles obtained by fractionation of disrupted bacteria is interesting in view of the observations of Burdon (1946) and of Mitchell & Moyle (1951). Burdon described a lipid layer in a number of bacteria which was readily stained by Sudan Black; this lipid layer, which lies inside the cell wall, is usually thought to represent the osmotic barrier of the cell. Mitchell & Moyle (1951) obtained a small particle fraction from *Staphylococcus aureus*; the particles had diameters ranging from 10 to 50 *mμ*. and contained a high proportion of phospholipid. Mitchell & Moyle suggested that the small particles may be associated with the protein envelope in the intact cell as an underlying membrane, and that the lipid of the particles may be the lipid observed by Burdon in bacteria stained with Sudan Black. The more recent work of Weibull (1953) showed that similar particles are formed by sonic disintegration of protoplast membranes which in the intact cell appear to be immediately underlying the cell wall. Thus it seems possible that the small particles which have been shown in the present work to bind the

fluorescent derivative of polymyxin may originate from a membrane which underlies the cell wall.

The chemical nature of the polymyxin-binding component of bacterial cells is as yet unknown, but there is some evidence to suggest that it may be a phospholipid. Bliss, Chandler & Schoenbach (1949) showed that phosphatides of the lecithin type interfered with the antibacterial activity of polymyxin; Latterade & Macheboeuf (1950) described the formation of polymyxin phospholipid complexes; and Newton (1954*a*) showed that the polymyxin-combining groups of *Pseudomonas aeruginosa* had properties of polyphosphates. With this possibility in mind the difference in distribution of DANSP between cell walls and small particles in the case of the two groups of organisms studied might perhaps be explained in terms of the difference in phospholipid content of Gram-positive and Gram-negative cells and cell walls. Salton (1952, 1953) found that the walls of Gram-negative organisms contain considerably more lipid than the walls of Gram-positive organisms, while Mitchell & Moyle (1954) found that Gram-positive organisms possess only half the amount of lipid phosphorus found in Gram-negative organisms.

From the results described in this paper it is clear that the affinity of the small-particle fraction of *Bacillus megaterium* for DANSP is much greater than that of the cell walls. The redistribution observed on the addition of small particles to DANSP-treated cell walls demonstrates that studies of the absorption of polymyxin by isolated cell-wall preparations (Few & Schulman, 1953) give no indication of the distribution of polymyxin in the intact cell. Such studies have, however, given some indication of the nature of the resistance to polymyxin shown by many Gram-positive organisms (Few & Schulman, 1953) and by one strain of *Pseudomonas aeruginosa* (Newton, 1954*b*). Cell walls of resistant organisms absorb only about one-fifth the amount of polymyxin absorbed by the walls of sensitive organisms, and it seems possible that in the case of resistant organisms there is little penetration of polymyxin through the cell wall to the underlying membrane or osmotic barrier.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Chromatographic separation of DANSP and 1-dimethylaminonaphthalene-5-sulphonic acid. Whatman no. 1 paper; solvent, molar phosphate buffer pH 7.0; fluorescence excited by mercury arc with Wood's filter. (1) DANSP after filtering through Dowex 2; (2) DANSP before filtering through Dowex 2; (3) 1-dimethylaminonaphthalene-5-sulphonic acid.
- Fig. 2. Fluorescence photomicrograph of *Pseudomonas aeruginosa* treated with 25 µg. DANSP/mg. dry wt. cells. × 1050.
- Fig. 3. Fluorescence photomicrograph of *Bacillus megaterium* treated with 25 µg. DANSP/mg. dry wt. cells. × 700.
- Fig. 4. Protection of cells by cations. (1) untreated cells; (2) cells treated with 25 µg. DANSP/ml. for 5 min.; (3) cells suspended in 0.05 M-calcium chloride before treatment with DANSP (25 µg./ml.) for 5 min. Fluorescence excited by mercury arc with Wood's filter.
- Fig. 5. Fluorescence photomicrograph of DANSP treated (25 µg./mg. dry wt. cells) *Bacillus megaterium* after depolymerization of the cell wall by treatment with lysozyme. × 700.

PLATE 2

Fig. 6. Washed cells of *Bacillus megaterium*. $\times 18,000$.

Fig. 7. Protoplast prepared from *Bacillus megaterium* by controlled lysozyme treatment; preparation fixed with mercuric chloride and formalin. $\times 26,950$.

Fig. 8. Protoplast membrane (*Bacillus megaterium*) resulting from lysis of protoplasts by suspension in distilled water; preparation fixed in mercuric chloride and formalin. $\times 26,950$.

Fig. 9. Sub-cellular unit obtained by lysozyme treatment of *Bacillus megaterium* which had been pretreated with a bactericidal concentration of polymyxin; preparation not fixed. No lysis resulted on repeated washing with distilled water. $\times 26,950$.

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