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Morphological and Physiological Investigations on the Action of Polymyxin B on *Escherichia coli*

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Summary. Cultures of *Escherichia coli* (exponential phase of growth) were exposed to various concentrations of polymyxin and studied with different methods. Viable counts showed that the effect of polymyxin depends on the antibiotic concentration, on the density of the bacterial suspension and the duration of treatment. Measurements of the oxygen consumption have shown that the polymyxin effect begins immediately after addition of the substance and reaches its highest intensity after about 10 to 15 min. For electronmicroscopical studies controls and treated bacteria were fixed according to Ryter and Kellenberger (1958), and stained with uranyl acetate. Embedded in vestopal or methacrylate. The influence of polymyxin on the substructure of the coli cells at a suspension density of 1.2 mg wet weight of bacterial/ml can be devided into 2 phases: polymyxin concentrations up to 10 µg/ml produce protuberances at the cell surface the number of which increases with increasing concentration of the antibiotic, but they cause little intracellular changes. Polymyxin doses exceeding 10 µg/ml produce, besides the formation of protuberances, a rapidly proceeding cell autolysis which becomes manifest firstly by a brightening of the nuclear area and then by destruction of the whole cytoplasm. It is probable that the moment of formation of the first protuberances coincides with the cell death.

The antibacterial properties of surface-active polypeptide antibiotics have been described by different authors in a number of communications. An excellent review of the mode of action of polymyxin has been given by Newton (1956). Other relevant reviews have been prepared by Davis and Feingold (1962) and Gale (1963). Polymyxins predominantly influence gramnegative bacteria (Brownlee and Bushby, 1948; Brownlee *et al.*, 1952). These bactericidal antibiotics have a mechanism of action similar to that of cationic detergents (Sous *et al.*, 1961; Rhodes *et al.*, 1953; Wahn *et al.*, 1966; White *et al.*, 1949; Chaplin, 1952; Chapman, 1962; Hotchkiss, 1946; Knox *et al.*, 1949; Newton, 1958).

There are only a few reports on the effects of surface-active polypeptides on the ultrastructure of sensitive bacteria (Chapman, 1962; Few, 1954; Kaye and Chapman, 1963; Mitchell and Crowe, 1947).

Early observations by MITCHELL and CROWE (1947) and FEW (1954) on the effect of polymyxin are primarily concerned with postmortal phenomena.

In recent years CHAPMAN (1962) and KAYE and CHAPMAN (1963) reported on the effect of colistin sulphate on the structure of gram-negative and gram-positive bacteria. These authors have shown that relatively high concentrations of colistin destroy the nuclear structure and then produce a general destruction of the cytoplasm. Protuberances at the cellular walls of treated *Escherichia coli* cells were interpreted by CHAPMAN (1962) as extracellular deposits, probably of the antibiotic itself. In an earlier communication, however, we have pointed out that this finding was closely connected with the action of polymyxin (WAHN and ROCKSTROH, 1967). The aim of the present work was to obtain an insight into the dynamics of cell damage by a comprehensive study including cytological investigations, comparative manometric measurements and viable counts.

Material und Methods

Escherichia coli B used in this study were cultivated in nutrient broth in 100 ml Erlenmeyer-flasks shaken by a vibrator, at 37° C. The process of growth was followed turbidimetrically. The employed Polymyxin B supplied the Pfizer GmbH Karlsruhe. Viable counts were made on fresh blood agar plates. Cell density was adjusted by turbidity measurements (Spekol, VEB Carl Zeiss, Jena). Manometric measurements were done by a Warburg apparatus. For the electronmicroscopic investigation a Porter-Blum ultramicrotome and a Siemens-Elmiscope I were employed.

For the viable counts bacteria cultivated in nutrient broth were adjusted photometrically to 1.2 or 0.6 mg wet weight/ml at the end of the logarithmic growth phase (after about 5 hours). Then 50 ml of either suspension were transferred into 2 sterile Erlenmeyer flasks each and exposed to polymyxin concentration of 5 μg/ml and 10 μg/ml for 10 and 60 min, respectively. After the desired time had elapsed 10 ml of the suspension were taken from each flask and diluted with sterile physiological NaCl solution to a bacterial wet weight of 10^{-5} mg/ml. Of this solution 10 μl were inoculated with a celibrated loop on fresh blood agar plates. The readings of the formed bacterial colonies were taken after 40 hours.

For the measurement of oxygen consumption of resting bacteria the bouillon cultures were centrifuged, resuspended in phosphate buffer according to Sörensen (pH 7) plus 0.5% glycerol and adjusted photometrically to a density of 1.5 mg wet weight/ml. Then, 2 ml of this suspension were transferred into the main chamber of each Warburg vessel. For the elimination of CO_2, 0.2 ml of 5% KOH were given into the central cylinder of the Warburg vessel. Then starting solutions of polymyxin (0.4 ml) were placed into the lateral vessels. The experimental temperature was 37° C, the gaseous phase in the vessel consisted of air.

For the determination of oxygen consumption of proliferating bacteria the original bacterial cultures were diluted by sterile bouillon to give 0.1 mg wet weight/ml. The Warburg vessels were filled as described above. Polymyxin was added as the cultures had reached a suspension density of 0.3 mg bacterial wet weight/ml (photometrical check). The readings were done at intervals of 20 min.

Each polymyxin concentration was represented by 3 comparative vessels in one experiment. Each experiment was repeated several times.

For the electronmicroscopical study bouillon cultures of a suspension density of 1.2 mg bacterial wet weight/ml were exposed to polymyxin concentrations graded from 1 to 100 μg/ml for 1 hour. Then the bacteria were fixed according to the method of RYTER and KELLENBERGER (1958), however, without addition of tryptone. Staining was carried out by 1% uranyl acetate (1 hour), embedding in vestopal W and in methacrylate. The preparations were prepolymerized overnight at room temperature and subsequently hardened at 60° C for 2 days.

Results

The influence of bactericidal surface-active compounds on bacteria is largely dependent on the ratio between the quantity of cells and the concentration of the antibacterial substance (BLISS *et al.*, 1949; MITCHELL and CROWE, 1947). Also, in sensitive strains their influence varies from strain to strain. For this reason, in the first part of this work correlations existing between suspension density of tested strains, the polymyxin concentration and the effective period of the antibiotic were investigated more closely by viable counts. The results of the colony counts after a 40 hours period of incubation are given in the table. The bacterial counts are calculated from 4 successive experiments each including 10 comparative plates. An evaluation of the table with regard to the viable counts shows 1. a concentration-dependent effect, 2. a clear correlation between the amount of affected bacteria and polymyxin concentration, and 3. a dependence upon the time of exposure to polymyxin. This result largely agrees with findings obtained with a similar experimental arrangement with the quarternary ammonium salt, dimethylamino acetic acid dodecylamide chlorobenzylate (WAHN and ZAPF, 1966).

Table. *The effect of polymyxin in relation to the density of the bacterial suspension and exposure. Inoculum 10 μl of a suspension of 10^{-5} mg bacterial wet weight/ml*

Polymyxin-concentration	bacterial wet weight/ml	exposure 10 min	60 min
		number of colonies	
5 μg/ml	1.2	129	75
	0.6	52	5
10 μg/ml	1.2	53	25
	0.6	23	1
control	1.2	190	184

As to the electronmicroscopical investigations described below, we were especially interested in the influence of different polymyxin concentrations on bacteria at a suspension density of 1.2 mg wet weight/ml after 60 min exposure. It has been found that at a concentration of 5 μg polymyxin/ml about 60% of the affected bacteria had lost their ability of cell division.

Warburg manometry permits detection of earlier metabolic changes than do viable counts. Fig. 1 demonstrates the influence of different polymyxin concentrations on the oxygen consumption of resting *Escherichia coli B* cells. One can see that the action of polymyxin starts immediately after it has been added. There is an obvious relation between polymyxin concentration and decrease of respiration. In our experiments using 1.5 mg bacterial wet weight/ml, oxygen consumption was significantly inhibited by 5 μg polymyxin/ml. Higher polymyxin dosages, according to concentration, lead to a more drastic decrease of respiration. A comparison of the graph with the table shows that a great deal of the decrease of respiration must be due to the irreversibly injured cells. By the term "irreversibly injured" we mean here all bacterial cells which have lost their ability of reproduction, i.e. both dead and subletally injured cells.

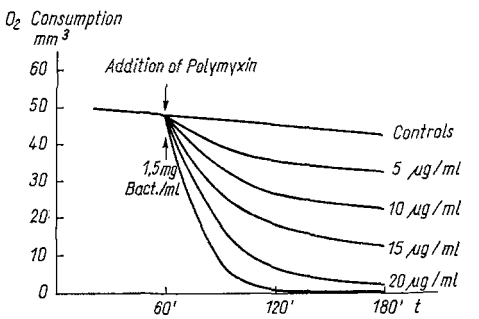

Fig. 1

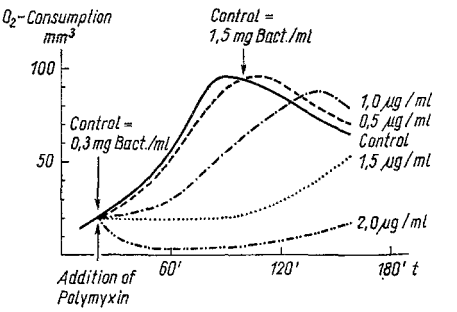

Fig. 2

Fig. 1. Oxygen consumption of resting *Escherichia coli B* cells under the influence of different polymyxin concentrations. Inoculum 1.5 mg bacterial wet weight/ml

Fig. 2. Oxygen consumption of *Escherichia coli B* cells under the influence of different polymyxin concentrations. 0.3 mg bacterial wet weight/ml, when polymyxin was added

The effect of polymyxin B on proliferating cells is represented by Fig. 2. One can also see that the fall of respiration begins immediately after addition of polymyxin. As little as 1 μg polymyxin/ml significantly reduces the oxygen consumption. The action of polymyxin on growing cells is essentially the same as that on resting cells, where at a 5-fold suspension density the first reduction of respiration occured with 5 μg polymyxin/ml. At higher polymyxin concentrations the curves demonstrate a correspondingly greater fall of the oxygen consumption.

The inclination of the curves can thus be explained that by addition of polymyxin, according to concentration certain amounts of the germs become irreversibly damaged and do no longer participate in further

divisions. On the other hand, the resistant bacteria undergo further divisions and consequently the oxygen consumption which is somewhat delayed with increasing polymyxin concentrations, ultimately approaches the maximum value of the controls.

This finding is also similar to results obtained with ion-active detergents (WAHN and ZAPF, 1963, 1965).

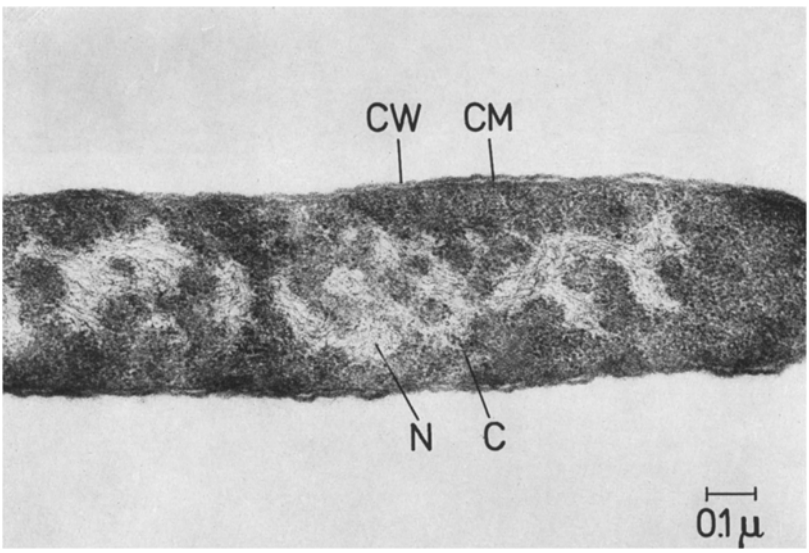

Fig. 3. Longitudinal section through a control cell. Cell wall (*cw*) and cytoplasm membrane (*cm*) (see arrow) the granular basic plasma (*c*) and the weak electron-dispersing nuclear areas (*n*) which are filled up with fine-stranded DNA material, can be seen. Vestopal embedding

Since the fine structure of *Escherichia coli* has already been described in full detail by different authors (BRIEGER, 1963; CONTI and GETTNER, 1962; RYTER and KELLENBERGER, 1958), we shall confine ourselves to a brief outline (Fig. 3). The envelope of the cell consists of a cell wall which mostly appears in 5 layers, and the double contoured membrane of the cytoplasm. In the central portion of the coli cell one can perceive at the medianal sections, as a rule, 1—2 extended nuclear areas, which due to lateral prominences of peripheral sections often appear more cleaved. They are completely filled with fine-stranded DNA material. The basic cytoplasma is uniformly intermingled with ribosomal particles. Intracellular membraneous structures so far have been described only in a few papers (COTA-ROBLES, 1966; FISCHMAN and WEINBAUM, 1967; RYTER and JACOB, 1966; SCHNAITMAN and GREENAWALT, 1966).

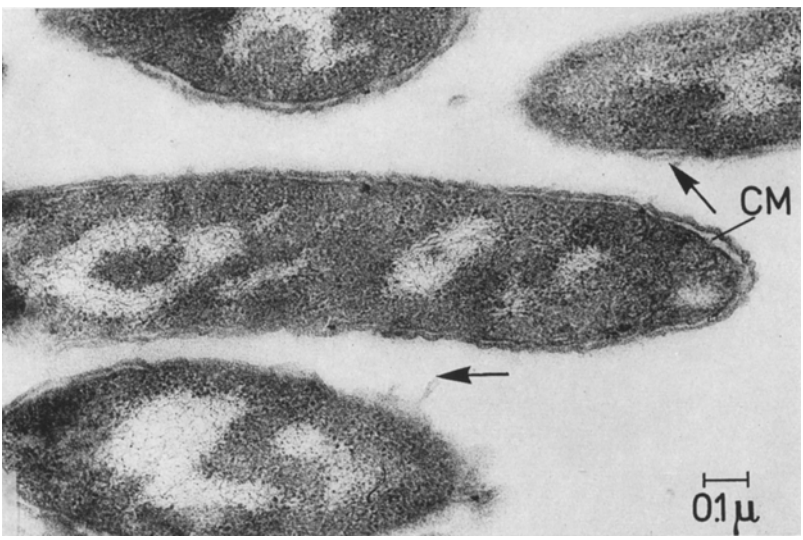

Fig. 4. Section through *Escherichia coli* treated with 2.5 µg polymyxin/ml for 1 hour. The intracellular structure of the sections does not differ from that of the controls. At the cell wall, however, protuberances appear sporadically (see arrow). The cytoplasmic membrane (*cm*) is clearly visible at some points. Vestopal embedding

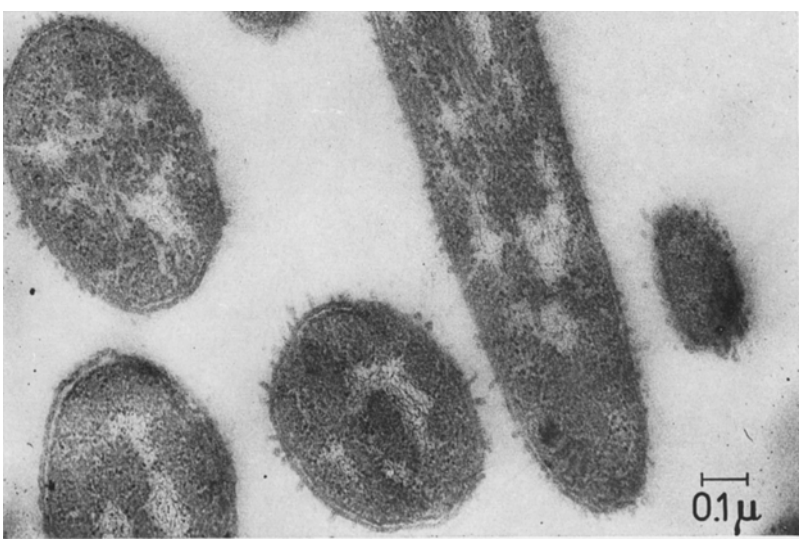

Fig. 5. Section through cells influenced by 5 µg polymyxin/ml for 1 hour. Most of the sections show at the cell surface more or less protuberances. Some nuclear regions appear to be slightly brightened. Vestopal embedding

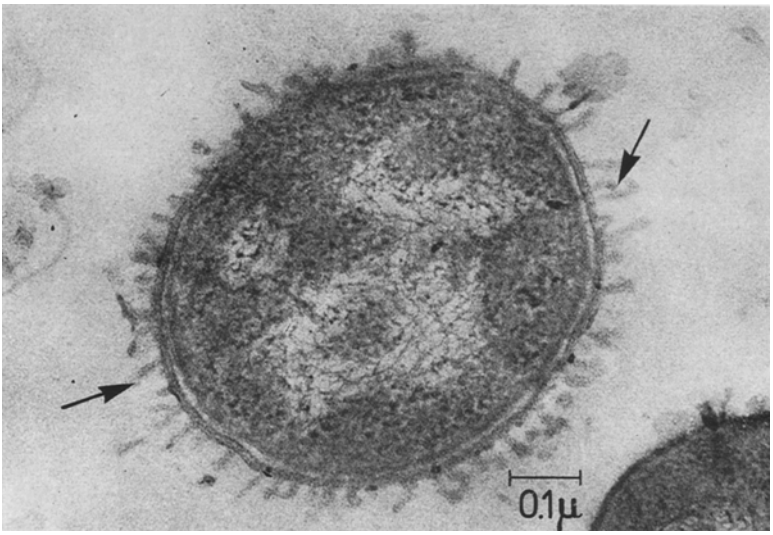

Fig. 6 Cell-cross-section, vestopal embedding. The cells were treated with 10 µg polymyxin for 1 hour. The five-layered cell wall shows numerous protuberances which appear to be connected with the cytoplasm of the cell at some points (see arrow). The intracellular structure does not reveal any changes

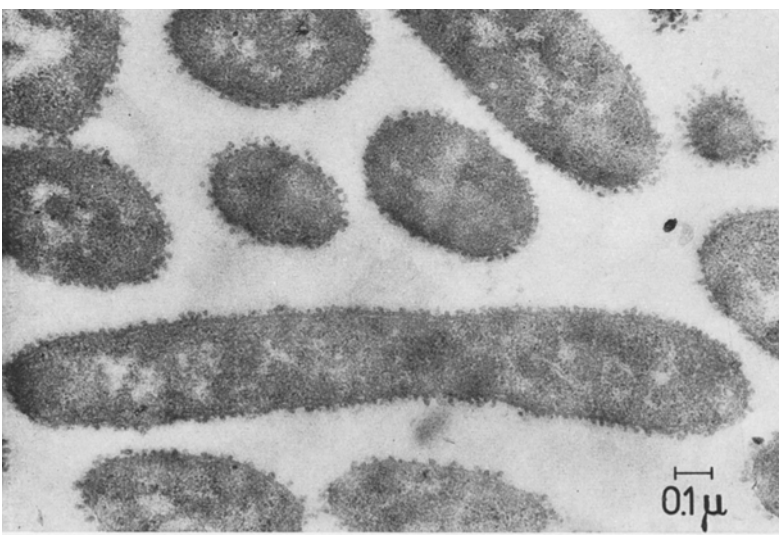

Fig. 7. Cells treated with 10 µg polymyxin/ml. Methacrylate embedding. It can be seen that treatment with 10 µg polymycin/ml crows each bacterium with a dense layer of tip-like processes. The cells seem to have more shrinks then with vestopal embedding

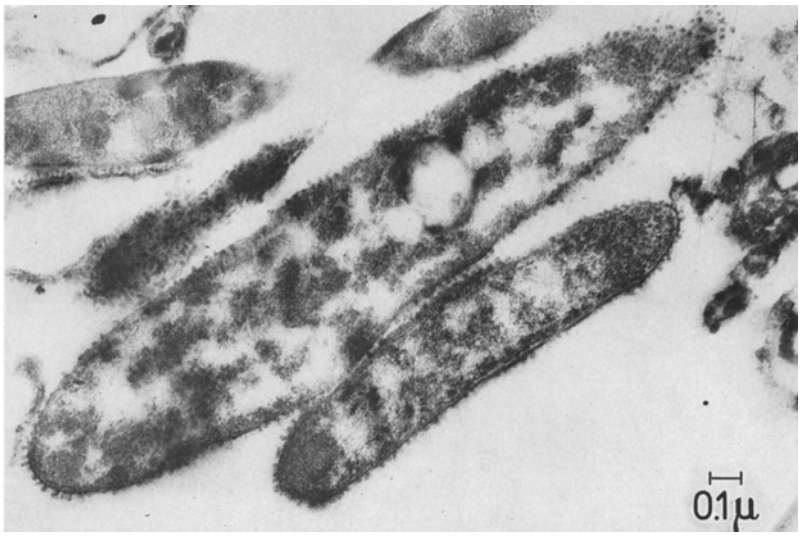

Fig. 8. Influence of 15 µg polymyxin/ml for 1 hour. Metacrylate embedding. The cells show besides the protuberances a partial decay of the cytoplasm with large transparent zones, especially in the areas of the nucleus

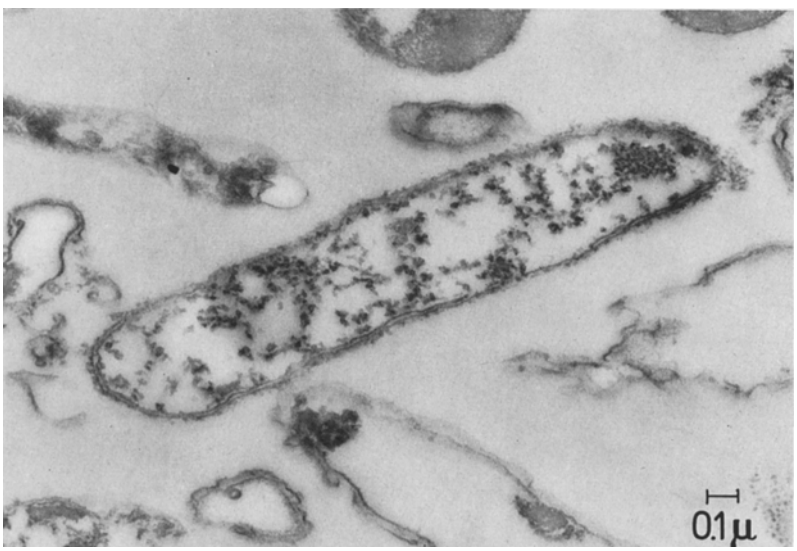

Fig. 9. Cells after action of 100 µg polymyxin/ml for 1 hour. The picture shows a state of nearly perfect autolysis. Vestopal embedding

After action of polymyxin, essentially two stages of changes can be observed in *Escherichia coli*: 1. Polymyxin concentrations which

correspond to the therapeutically available blood levels, cause at the cell surface more or less numerous protuberances which reach a maximal number at about 10 μg/ml. 2. Polymyxin concentrations exceeding 10 μg/ml lead in any case to pronounced destruction of the cell, in addition to the formation of protuberances.

Under the influence of 2.5 μg polymyxin B/ml in some cells (Fig. 4) tip-like processes had formed sporadically at the cell surface. At 5 μg/ml both the number of protuberances on the affected single cell and the number of cells changed in this manner increase considerable (Fig. 5). At this polymyxin concentration the oxygen consumption (Fig. 1) decreases by about 25 per cent and also the viable counts reflect a strong reduction of the colony number (Table).

No intracellular changes, however, have been observed at 5 μg polymyxin/ml.

Under the influence of 10 μg polymyxin/ml almost all bacteria appear to be entirely covered with protuberances (Figs. 6 and 7). The nuclear equivalent and the basic plasma mostly are unchanged by this concentration. At the cross-section of the cell (Fig. 6) it seems that the protuberances of the cell surface are directly connected with the cytoplasm in some places.

Addition of 15 μg of polymyxin produced, besides the processes at the surface, also pronounced intracellular destructions (Fig. 8). Obviously, an irregular decay of the cytoplasm is going on and the nuclear areas are no longer clearly distinct from the remaining plasm. Apparently, numerous bacteria exposed to 15 μg polymyxin/ml begin almost immediately to undergo progressive destruction. The oxygen consumption of bacteria pretreated in the same way declined to about 30 per cent of the control value at the same polymyxin dosage. Still higher polymyxin concentrations stimulate the lytic process. Besides numerous partially destroyed cells there are only cell walls and wall fragments after 20 μg polymyxin/ml.

Since several authors (SYKES, 1939; TAI and VAN HEYNINGEN, 1951) have reported a lysis-inhibiting action for high doses polymyxin and ionactive detergents, we have also used the relatively high concentration of 100 μg/ml in this study. The micrographs, however, reveal that nearly all bacteria undergo extensive autolysis when exposed to this concentration of antibiotic (Fig. 9).

Discussion

A comparison of the results obtained with different methods permits conclusions as to the biological condition of the microscopically investigated material. Thus, polymyxin doses exceeding 20 μg/ml after 1 hour's exposure, in all probability, will kill the whole bacterial population.

Therefore, it can be expected that such polymyxin concentrations induce structural changes in nearly all bacteria. On the other hand, at concentrations below 20 μg/ml we expect to see a heterogeneous picture since earlier investigations have shown that there is a mixture of extensively damaged and undamaged organisms.

The protuberances at the cell surface can be explained in 2 ways: 1. The tip-like process on the cell wall of polymyxin-treated bacteria may be a form of the highly enriched antibiotic itself, and 2. they may be a protruding cell substance which solidifies at the outer surface of the bacteria due to excess of the surface-active antibiotic.

Support to the first possibility is given by the extremely high absorption of the antibiotic by sensitive-bacteria. Few and Schulman (1953) have calculated that the amount of polymyxin absorbed by a sensitive bacterium at the moment of saturation would suffice to cover a surface 12 times as large as the bacterial surface. This calculation, however, does not account for the possibility of penetration of polypeptide molecules into the cell. A similar enrichment has also described for cation-active detergents (Bliss *et al.*, 1949; McQuillen, 1950; Rockstroh, 1967; Salton, 1951).

The following objection to the assumption that the protuberances are adsorbed antibiotic particles may be raised: When using high doses of polymyxin B the resulting high number of protuberances (Figs. 6 and 7) suggests that the cell surface must obtain numerous points of affinity. For this reason it is hardly conceivable that the polypeptide, when given at low concentrations, should intensively add only at a few points (Fig. 4).

The second possibility enclosed a damage of the cellimiting membranes, particularly the cytoplasmic membrane which acts as an osmotic barrier. This is supported by the fact that the different effects reach their maximum very rapidly after addition of polymyxin. So, the substantial loss of bacteria described by several authors (Few and Schulman, 1953; Newton, 1953; Salton, 1951) occured immediately after addition of the antibiotic or the detergent. Newton (1954), moreover, has shown that addition of polymyxin causes an abrupt penetration of a fluorescent dyestuff which is nondiffusible for untreated cells, into the plasm of the bacterial cells. Tomcsik (1955) succeeded in showing lysozyme-sensitive bacteria which had been treated with polymyxin or a cation-active tenside prior to exposure to the enzyme that the protoplasts did no longer occupy the usual spheric form, but that the cytoplasmic membrane rather persisted in a given solidified form. Similar conclusions about synthetic wetting agents have been reached by Zapf (1960). In addition, Newton (1955) was able to show that the

proponderant portion of a fluorescenting polymyxin preparation is located in the cytoplasmic membrane. Therefore, these facts seem to indicate that the observed tips at the wall of *Escherichia coli* must be protruding cell substance.

Now the question is whether the primary action of surface-active compounds (polypeptide antibiotics, ion-active tensides) includes a physical deorganization of the cytoplasmic membrane, or whether the change of cell permeability is a secondary problem. Other effects of the polypeptide antibiotics on bacteria in addition to those described above have been observed, for instance inhibition of the enzymic activity (MERRICK, 1965; NEWTON, 1953b) and inhibition of the protein synthesis both on intact bacteria and in vitro (ENNIS, 1965; I. and II.).

The idea that the change of the membrane ist solely brought about by the surface activity is contradicted by 2 findings: SILAJEW (1952) has found that with polymyxin M by virtue of a migration of the — CO-binding of the α-group of diaminobutyric acid on the γ-amino group of this acid, none of the properties, not even the surface activity of the molecule, become affected, whereas the bactericidal action is abolished by transacylation. SOUS *et al.* (1961) combining 2 of the polypeptide antibiotics found an extraordinarily high increase of efficiency, so that e.g. 2 antibiotic doses which lie below the treshold of action (colistin sulphate + tyrothrycin) given in combination, arrest growth and kill the bacteria. The additive action of surface activity of the two components can by no means explain this synergism. Special information about the origin and the chemical quality of the membrane's protuberances could be expected from exposure to enzymes. Experiments are in progress investigating whether during the action of polymyxin the tip-like structures become detached from the surface of the bacteria into suspension medium, which would allow chemical analysis of the protuberances.

The results of this work show that the protuberances at the cell surface of *Escherichia coli* become visible at the threshold of letal dose of the antibiotic. Since a damage of the membrane occures immediately after addition of polymyxin, irrespective of whether it is a primary or a secondary phenomenon, the formation of protuberances is understood as a process which starts immediately after the membrane effect, probably at the moment of cell death. Polymyxin doses exceeding 10 μg/ml, as has been briefly mentioned in this work, at a germ-density of 1.2 mg wet weight/ml lead to a more or less strong destruction of the bacterial cells. Investigations on whether these intracellular changes are polymyxin-specific or whether such phenomena a merely polymyxin-induced unspecific autolytic process will be published by us in another paper.

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