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STUDIES ON THE MECHANISM OF ACTION OF COLISTIN II. ALTERATION OF PERMEABILITY OF *ESCHERICHIA COLI* BY COLISTIN

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SUMMARY An alteration in the permeability of *E. coli* cells caused by colistin was detected by observation of the release of materials absorbing at 260 mμ from *E. coli* cells. The release took place at 37°C but not at 2°C. The release was prevented by divalent cations. Most of the material released was found to be low molecular ribocompounds. Deacylcolistin did not cause release of this material.

INTRODUCTION

In the preceding paper (NAKAJIMA and KAWAMATA, 1965 a) the authors reported that *E. coli* was rendered sensitive to actinomycin D by treatment with a subinhibitory dose of colistin. It was suggested that this sensitivity to actinomycin D was produced by an alteration in the permeability of the cell surface of *E. coli* caused by colistin.

Chemical studies on the structure of colistin showed that it consists of at least two components, polymyxin E_1 and polymyxin E_2 (SUZUKI *et al.*, 1963; 1965). Thus, the mechanism of action of colistin seems to resemble that of polymyxin E. It has been suggested by NEWTON (1953) 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 the cell membrane or osmotic barrier. The addition of polymyxin to a washed cell suspension of a strain of *Pseudomonas aeruginosa* resulted in the release from the cells of pentose, phosphate and materials with an absorption maximum at 260 mμ. A similar result was reported by KISHIDA *et al.* (1965) with colistin.

The primary site of action of polymyxin is claimed to be polyphosphates of the bacterial cell surface. An alteration in the bacterial cell surface was demonstrated with N-tolyl-α-naphthylamine-8-sulfonic acid, which penetrated the bacterial cell surface only when *Ps. aeruginosa* had been treated with polymyxin (NEWTON, 1955). An alteration was also demonstrated in the release of materials absorbing at 260 mμ after treatment of *Ps. aeruginosa* with polymyxin (NEWTON, 1953).

Morphological observation by electron microscopy of polymyxin treated *Ps. aeruginosa* (FEW, 1954) and of colistin treated *E. coli* (SUGANUMA *et al.*, 1965) also suggested an alteration of the bacterial cell surface.

It is of interest to compare the mode of action of colistin, which is now used clinically, with the actions of other polymyxins and also to elucidate the actual mechanism of action of this antibiotic from the stand point of the alteration of the bacterial cell surface. This paper reports detailed studies on the release of cellular materials absorbing at 260 mμ from *E. coli* on treatment with colistin.

MATERIALS AND METHODS

1. *Organism*

A colistin sensitive *E. coli*, strain B, was used.

2. *Medium and conditions of culture*

The organism was grown at 37°C in Simmons' medium (containing 2.5 g $(NH_4)_2HPO_4$, 1.5 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 5 g NaCl and 3 g Sodium glutamate per liter of H_2O) containing 0.3 per cent glucose. Shaking flasks, each containing 100 ml of medium were inoculated with 10 ml of an overnight culture in the same medium and shaken by rotary shaker at 130 rpm until they reached the logarithmic phase of growth.

3. *Antibiotics*

The colistin sulfate used was a product of Kayaku Antibiotics Research Co., Ltd., Tokyo and deacylcolistin was kindly given by Prof. T. SUZUKI (Institute for Protein Research, Osaka University, Osaka).

4. *Measurement of release of material with an absorption maximum at 260 mμ*

To a 9 ml volume of washed cell suspension of *E. coli* in Simmons' medium was added 1 ml of antibiotic solution in distilled water thus giving a volume of 10 ml, this was rocked in an L-shaped culture tube at 37°C or 2°C for the desired period. Then the suspension was chilled quickly in an ice water bath to stop the reaction and centrifuged at 2°C for 15 minutes at 5,000 rpm. The supernatant was again centrifuged for 10 minutes at same speed. The absorption spectrum of the resulting supernatant was recorded in a Shimazu automatic recording spectrophotometer. When studying the effect of cations on the release of material, Simmons' medium was replaced by 0.9 per cent saline solution.

5. *Turbidimetric determination of bacterial growth*

Unless otherwise stated, the growth of *E. coli* in L-shaped tubes was determined turbidimetrically at 660 mμ using a Coleman junior type spectrophotometer.

6. *Determination of nucleic acids and protein*

Bacteria were chilled to 2–0°C in an ice water bath and acidified by addition of cold 10 N perchloric acid to a final concentration of 0.5 N. The acid insoluble fraction was suspended in 0.5 N perchloric acid and heated at 90°C for 15 minutes. The DNA and RNA contents were determined by Burton's method (1956), RNA by Mejbaum's method (1939) and, after dissolving the residue in a small volume of 1 N NaOH, protein was determined by the method of LOWRY *et al.* (1951).

RESULTS

1. *Release of materials absorbing at 260 mμ from bacterial cells*

The effect of colistin on the release of UV-absorbing materials from *E. coli* B is shown in Fig. 1. In this experiment, cells at the logarithmic phase of growth were harvested and exposed to various concentrations of colistin. After incubation at 37°C, UV-absorbing material released from the cells was assayed. The release of material was detectable with 1 μg/ml of colistin and was marked with 10 μg/ml of colistin. When colistin treatment was carried out at 2°C, no release of material was detectable (Fig. 2).

The release of material increased with the incubation period. The profile illustrated in Fig. 3 shows that the increase was gradual and that even after 180 minutes the release still continued. This is in contrast to the case when *Staphylococcus aureus* is treated with gramicidin J (MIKI, 1960), in which the release reached a maximum after 10 minutes treatment and subsequently remained unchanged.

Since deacylcolistin is known to be less active in inhibiting bacterial growth than colistin, its effect on the release of materials absorbing at 260 mμ was investigated. As illustrated

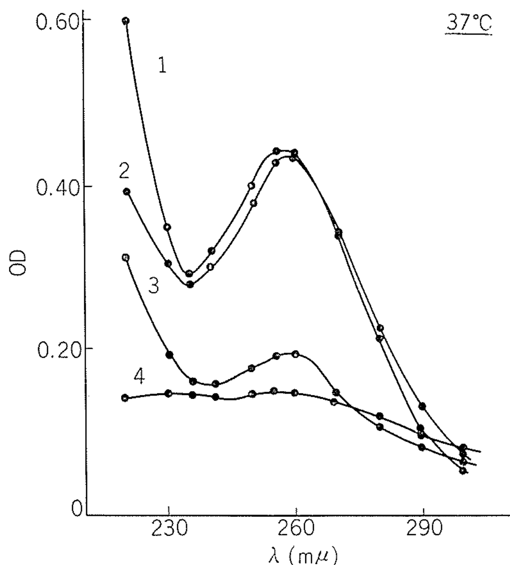

FIGURE 1 Release by colistin of material absorbing at 260 mμ from washed cells of *E. coli* B.
9 ml of washed cell suspension of *E. coli* B (optical density 0.3 at 660 mμ) were incubated with 1 ml of aqueous colistin solution for 60 minutes at 37°C. The mixture was centrifuged twice at 2°C for 15 and 10 minutes at 5,000 rpm and the absorption of the resulting supernatant was recorded with a Shimazu automatic recording spectrophotometer. Spectra 1, 2 and 3 were obtained with colistin at concentrations of 100, 10 and 1 μg per ml, respectively. Spectrum 4 was that of the control without colistin.

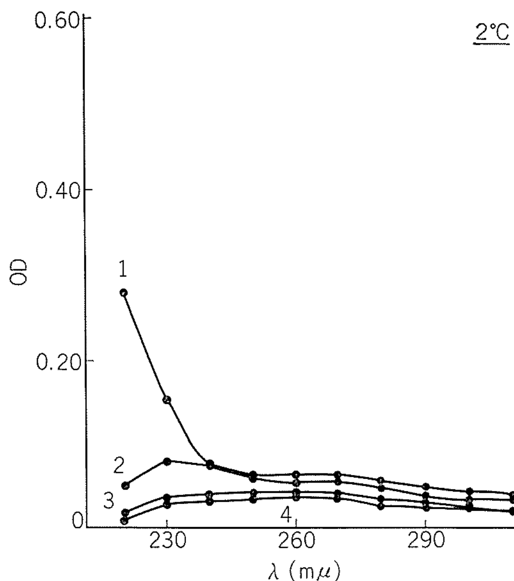

FIGURE 2 Release by colistin of material absorbing at 260 mμ from washed cells of *E. coli* B.
Curves 1, 2 and 3 obtained with 100, 10 and 1 μg of colistin per ml, respectively. The tubes were rocked at 2°C in an ice water bath for 60 minutes. The experimental procedures were as for Fig. 1. Curve 4 is that of the control.

in Fig. 4, it caused no release of this material.

The experiments described above clearly show that the treatment of *E. coli* with colistin at a level of 10 or 100 μg/ml results in the release of UV-absorbing materials into the medium. To see the relationship between the release of UV-absorbing material and the viability of the cells, cells were exposed to colistin and viable cells were assayed by plating on nutrient agar. As seen in Table 1, 1 μg/ml of colistin had no inhibitory effect on the viability of *E. coli*, whereas treatment with 10 μg/ml of colistin resulted in a remakable decrease in viable cells. This coincides with the result shown in Fig. 1.

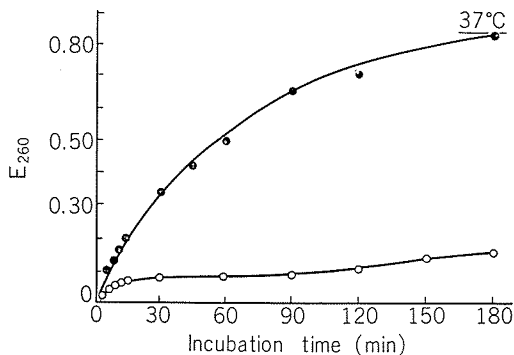

FIGURE 3 Rate of release by colistin of material absorbing at 260 mμ from washed cells of *E. coli* B.
E. coli B cells in the logarithmic growth phase were harvested and suspended in Simmons' medium. The cell suspension was incubated with (●——●) or without (○——○) colistin at a concentration of 10 μg per ml at 37°C. At intervals, aliquots of cell suspension were removed and UV-absorbing material released into the medium was measured.

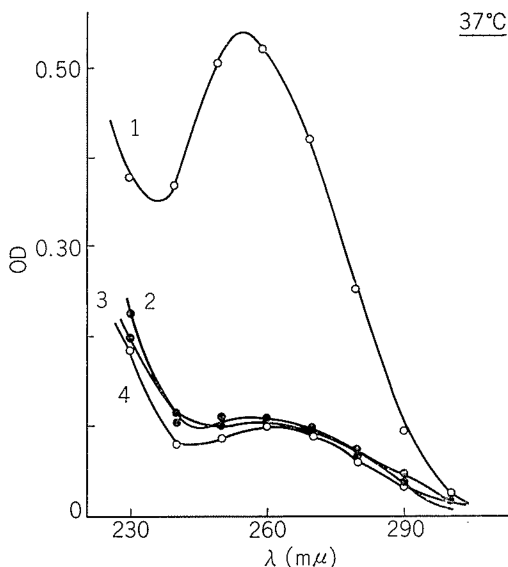

FIGURE 4 Effect of colistin and deacylcolistin on the release of material absorbing at 260 mμ from washed cells of *E. coli* B.
E. coli B cells in the logarithmic growth phase were collected and incubated with deacylcolistin at a concentration of 100 μg (curve 2) and 10 μg (curve 3) per ml for 60 minutes at 37°C. As controls, cells were incubated with colistin at a concentration of 10 μg per ml (curve 1) and without drug (curve 4).

TABLE 1 *Effect of colistin on the viability of E. coli B*

Time of incubation (min)	Concentration of colistin (μg/ml)	Viable cells (per ml)
0	0	2.3×10^{8}
60	0	7.7×10^{8}
60	0.1	7.2×10^{8}
60	0.5	7.8×10^{8}
60	1.0	7.6×10^{8}
60	5.0	4.7×10^{7}
60	10.0	2.1×10^{7}

E. coli B cells in the logarithmic phase of growth were harvested and iucubated in glucose-Simmons' medium containing various concentrations of colistin with shaking for 60 minutes at 37°C. Then the incubation mixtures were diluted with Simmons' medium and the viability of the bacteria was measured on nutrient agar plates by the conventional counting technique.

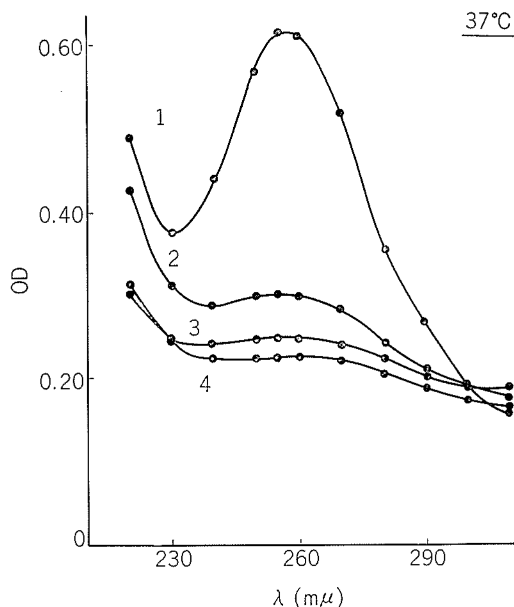

FIGURE 5 Effect of Mg^{+2} and Ca^{+2} ions on the release by colistin of material absorbing at 260 mμ from washed cells of *E. coli* B.
Experimental procedures were as for Fig. 1 except that incubation was carried out in 0.9 per cent saline solution.
1 : 10 μg/ml of colistin
2 : 10 μg/ml of colistin plus 0.01 M $MgCl_2$
3 : 10 μg/ml of colistin plus 0.01 M $CaCl_2$
4 : No colistin

2. *Effect of cations on the release of materials absorbing at 260 mμ*

In a preceding paper it was demonstrated that divalent cations such as Mg^{+2} and Ca^{+2} exhibited a strong inhibitory effect on the formation of an insoluble complex between DNA and colistin (NAKAJIMA and KAWAMATA, 1965 b). Therefore, the effect of cations on the release of materials absorbing at 260 mμ from cells treated with colistin was tested. As illustrated in Fig. 5, these cations inhibited the release of materials from the cell.

3. *The nature of the materials absorbing at 260 mμ*

To analyze the nature of the materials absorbing at 260 mμ which were released into the

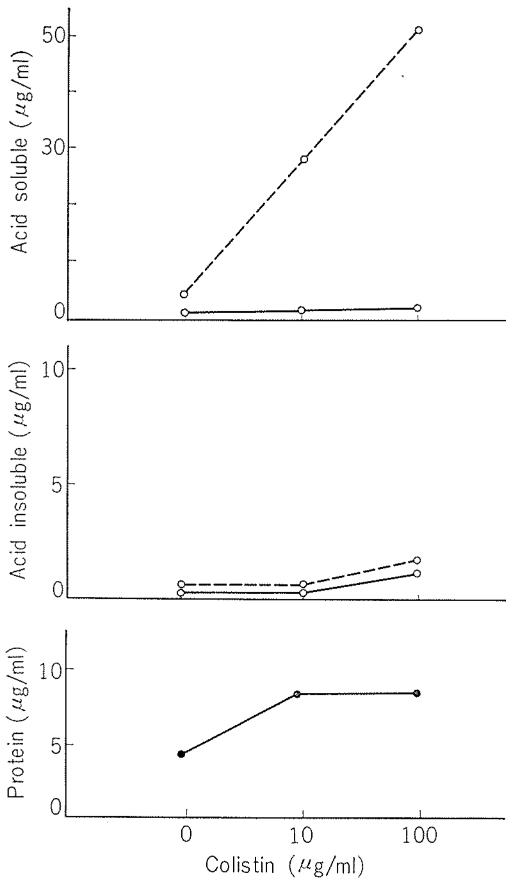

FIGURE 6 Analysis of UV-absorbing materials released from colistin treated *E. coli* B.
After incubation of *E. coli* B in Simmons' medium containing colistin (10 μg and 100 μg/ml) for 60 minutes at 37°C, $MgCl_2$ at a final concentration of 0.01 M was added to cell suspensions to prevent the formation of complexes of nucleic acids and colistin. An aliquot (10 ml) of cell suspension was centrifuged and the supernatant fraction was acidified by addition of perchloric acid to final concentration of 0.5 N. The acid insoluble fraction was suspended in 0.5 N perchloric acid and heated at 90°C for 15 minutes. Aliquots from the cold acid soluble and insoluble fractions were analyzed for deoxyribo-, and ribocompounds by the diphenylamine and orcinol methods, respectively. The hot perchloric acid insoluble fraction was dissolved in a small volume of 1 N NaOH and its protein was determined by the method of Lowry *et al.* (1951)
○——○ Deoxyribocompounds
○-----○ Ribocompounds
●——● Protein

medium, cell suspensions were centrifuged after incubation with colistin, and the resulting supernatant fraction was analyzed. As shown in Fig. 6, the release of a high molecular, acid insoluble fraction was negligible, whereas there was a large quantity of acid soluble fraction which reacted with orcinol.

DISCUSSION

In the preceding paper (NAKAJIMA and KAWAMATA, 1965 b) the formation of an insoluble complex between colistin and DNA was reported. Although the formation of a similar complex between DNA and deacylcolistin was observed, deacylcolistin was less effective in inhibiting the growth of bacteria. The release of materials absorbing at 260 mμ was observed when *E. coli* cells were treated with colistin but not with deacylcolistin.

The fact that the formation of an insoluble complex between colistin and DNA and also the release from cells of materials absorbing at 260 mμ are inhibited by divalent cations suggests that the two phenomena are closely related in connection with the antibacterial activity of colistin. The reason why deacylcolistin is inactive as a bactericidal agent, even though it forms a complex with DNA, may be that it has no C_9-fatty acid moiety and hence is unable to penetrate into the cell. Thus, the complete molecule of colistin may be necessary for it to exert activity on intact or whole cells.

With detergent or gramicidin J, the release of materials absorbing at 260 mμ from the cells takes place even at lower temperature (SALTON, 1951; MIKI, 1960), but colistin and other ploymyxins cause release only at a higher temperature, such as 37°C. These results indicate that some biological process rather than a mere physico-chemical one is necessary for polymyxin to function in the bacterial cell.

It was shown in a preceding paper, that colistin forms a stable complex with nucleic acids. It was also observed that complexes

were formed between polymyxin E and phospholipids isolated from certain bacterial membranes (FEW, 1955) and between the antibiotic and phospholipids, such as lecithin and cephalin, which are known to be components of the bacterial membrane (FEW, 1955; NAKAJIMA and KAWAMATA, unpublished data). These results, together with those described in the present paper, suggest that the action of colistin on the bacterial cell may be due to the formation of complexes with cellular components such as nucleic acids or phospholipid which are localized on, or within the bacterial membrane. The formation of these complexes would cause a modification of the membrane structure, resulting in an alteration of the permeability towards various cellular components and also extracellular materials. In support of this suggestion, it was shown in previous paper (NAKAJIMA and KAWAMATA, 1965 a) that treatment of *E. coli* with a subinhibitory dose of colistin renders the cell susceptible to the action of actinomycin. At the moment, the actual mechanism by which the binding of colistin with cellular components leads to an alteration of permeability is unknown.

At any rate, the alteration in the permeability of the bacterial cell surface allows the released of cellular components which are essential for the viability of the bacteria. Further studies must be made on the nature of the acid soluble fraction in these released materials. In unpublished experiments we have detected remarkable decreases in the amounts of both intracellular acid soluble, and acid insoluble RNA. Thus, the increase in the amount of the extracellular acid soluble fraction seems to be derived from both acid soluble, and insoluble RNA in the cells.

As NEWTON (1954) suggested, phospholipids may be an important site of action of polymyxin. However, the formation of complexes between colistin and DNA and probably also RNA, in the cells may also bring about not only structural disorganization but also alterations in the physiological functions of both types of nucleic acid.

Further studies on this point are now in progress.

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