JOLIPEPTIN, A NEW PEPTIDE ANTIBIOTIC
II. THE MODE OF ACTION OF JOLIPEPTIN

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Jolipeptin causes a release of 260 mμ absorbing materials from the jolipeptin-sensitive strains, *Escherichia coli* B and *Bacillus subtilis* PCI 219. Moreover, jolipeptin lyses not only protoplasts of *B. subtilis* PCI 219 and *Micrococcus lysodeikticus*, but also spheroplasts of *E. coli* B and *Pseudomonas aeruginosa* IFO 3901. In contrast, colistin, produced simultaneously with jolipeptin, lysed spheroplasts of *E. coli* B and *P. aeruginosa* IFO 3901, but not protoplasts of gram-positive bacteria, the latter being colistin-insensitive strains. On the basis of this study, it was concluded that jolipeptin acts primarily on the cellular membrane.

Polypeptide antibiotics, such as gramicidin[1], polymyxin[2], and colistin[3] damage primarily cell membranes of antibiotic-sensitive microorganisms, and alter cell permeability. Consequently intracellular materials are released into the medium. Moreover, it has been described that these antibiotics[4,5,6] and bacitracin[7] lyse protoplasts and spheroplasts of such microorganisms.

Jolipeptin produced by a colistin-producing strain, *Bacillus polymyxa* var. *colistinus* KOYAMA, is a polypeptide antibiotic, as described in the previous paper[8]. As the mode of action against jolipeptin-sensitive microorganisms, it is considered that jolipeptin acts on the cell membranes and consequently leakage of intracellular material occurs, as in the case of polypeptide antibiotics described above.

In this paper, the action of jolipeptin was investigated, particularly on protoplasts and spheroplasts of jolipeptin-sensitive gram-positive and gram-negative bacteria when compared with the action of colistin.

Materials and Methods

Organisms and culture conditions: *Bacillus subtilis* PCI 219, *Micrococcus lysodeikticus*, *Escherichia coli* B and *Pseudomonas aeruginosa* IFO 3901 were used. *B. subtilis* PCI 219 and *M. lysodeikticus* were grown in nutrient broth and harvested in the late phase of exponential growth. *E. coli* B and *P. aeruginosa* IFO 3901 were replaced in the fresh medium during the same growth phase, allowed to grow for 2 hours, and then harvested. The harvested cells were washed twice with physiological saline and used for the preparation of protoplasts or spheroplasts.

Preparation of protoplasts: Washed cells of *B. subtilis* PCI 219 and *M. lysodeikticus*, were each suspended in 0.05 M phosphate buffer (pH 7.2) containing 20 % sucrose and lysozyme with a final concentration of 100 μg/ml. The cell suspension was incubated for 60 minutes at 30°C.

Preparation of spheroplasts: The method described by REPASKE was used[9]. Washed cells of *E. coli* B and *P. aeruginosa* IFO 3901, respectively, were suspended in 0.05 M Tris-HCl buffer (pH 8.0) containing 500 μg/ml of EDTA, 100 μg/ml of lysozyme and 20 % sucrose. Incubation was carried out at 30°C for 60 minutes. The formation of protoplasts and spheroplasts was checked by microscopic observation.

Determination of the release of intracellular ultraviolet-absorbing materials: The cells of *B. subtilis* PCI 219 and *E. coli* B were each cultured in nutrient broth at 30°C. At the late portion of the exponential growth phase, the cells were harvested and washed twice with physiological saline, suspended in 0.05 M phosphate buffer (pH 7.2) containing 9 % NaCl and the suspension was incubated in this minimal medium at 30°C for 1 hour with shaking. The cells were then washed once with physiological saline and resuspended in the same fresh phosphate buffer. Jolipeptin or colistin was added to the washed cell suspension at the indicated concentration, and incubation was continued at 30°C. At intervals, a part of the cell suspension was withdrawn, immediately centrifuged at 15,000 $\times g$ for 10 minutes, and the absorbance of the supernatant at 260 mμ was measured with a Hitachi-Beckman spectrophotometer.

Assay methods of protoplast- or spheroplast-lysing activities: The lysing activity on protoplasts or spheroplasts was determined as follows: to 2.7 ml of protoplast or spheroplast suspension, 0.3 ml of solution containing various concentrations of jolipeptin or colistin were added to 20 % sucrose solutions and mixed gently. The absorbance of the suspension at 660 mμ was measured with a Hitachi spectrophotometer at room temperature (about 28°C). The readings were made in 1-cm glass cuvettes.

Results

1. The Release of Intracellular Materials by Jolipeptin or Colistin

The cells of *E. coli* B and *B. subtilis* PCI 219 in the exponential growth phase were employed throughout these experiments after suspending in minimal media. These microorganisms were sensitive to jolipeptin. Fifty μg/ml of jolipeptin was added to each washed cell suspension and a sample from each tube was withdrawn at intervals of 0, 15, 30, 45 and 60 minutes. The sample withdrawn was divided into two parts and one was used to measure the turbidity of the cell suspension at 660 mμ. The other part was immediately centrifuged at 15,000$\times g$ for 10 minutes and the absorption of the supernatant liquid was measured at 260 mμ. Figs. 1 and 2 show the kinetics of the release of 260 mμ absorbing materials from the cells in the presence or absence of jolipeptin. In the case of the exposure of jolipeptin to *B. subtilis* PCI 219, the release of 260 mμ absorbing materials from cells occurred gradually. An immediate release of the 260 mμ absorbing materials was observed in *E. coli* B.

In the control suspension, without jolipeptin, the value of 260 mμ absorbance was very low during the whole period of the experiment. With a decrease of the turbidity of the cell suspension as measured by the optical density at 660 mμ. A remarkable decrease of turbidity was not observed and it was evident that under these conditions neither autolysis nor plasmolysis of the cells has occurred.

On the other hand, when the cells were incubated with colistin, release of 260 mμ absorbing materials was observed in the colistin-sensitive organism, *E. coli* B, but not in the colistin-insensitive organism, *B. subtilis* PCI 219 (Figs. 3 and 4).

The ultraviolet spectrum of the intracellular materials released in the supernatant fluid by jolipeptin or colistin was measured on the sample withdrawn at 60 minutes.

Fig. 1. Effects of jolipeptin on the release of intracellular materials and on the turbidity of *B. subtilis* PCI 219. The assay method is described in methods.

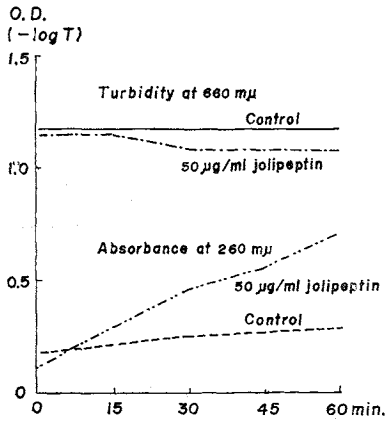


Fig. 2. Effects of jolipeptin on the release of intracellular materials and on the turbidity of *E. coli* B. The assay method is described in methods.

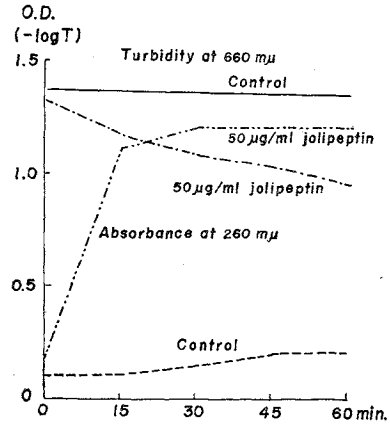


Fig. 3. Effects of colistin on the release of intracellular materials and on the turbidity of *B. subtilis* PCI 219. The assay method is described in methods.

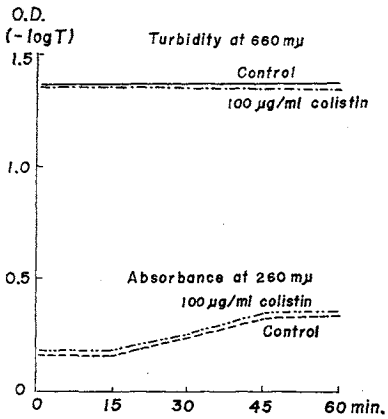


Fig. 4. Effects of colistin on the release of intracellular materials and on the turbidity of *E. coli* B. The assay methods are described in methods.

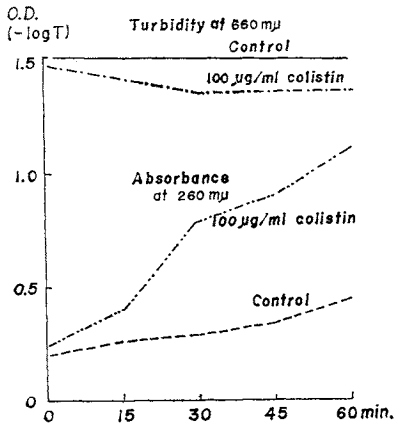
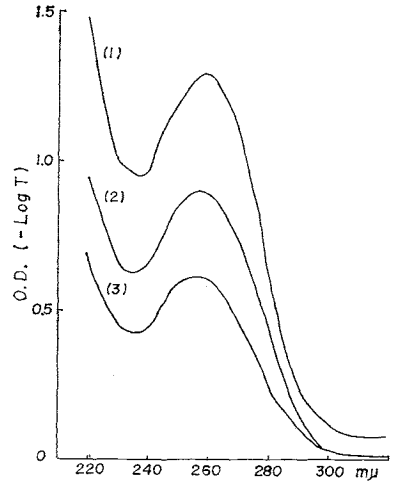


Fig. 5. Ultra-violet spectra of the intracellular materials released by jolipeptin or colistin from *B. subtilis* PCI 219 and *E. coli* B. Spectra 1 and 2 show absorbance of intracellular materials released by jolipeptin from *B. subtilis* PCI 219 and *E. coli* B, respectively. Spectrum 3 shows absorbance of material released by colistin from *E. coli* B.



As shown in Fig. 5, the ultraviolet spectrum in all cases resembled the spectrum of a nucleic acid with a maximum absorption band at 260 mμ.

2. Effects of Jolipeptin or Colistin on Protoplasts and Spheroplasts

Since release of 260 mμ absorbing materials by jolipeptin from *E. coli* B and *B. subtilis* PCI 219 was observed, it was

Fig. 6. Lysis of protoplasts of *B. subtilis* PCI 219 after treatment with jolipeptin (5 μg/ml) and colistin (100 μg/ml).

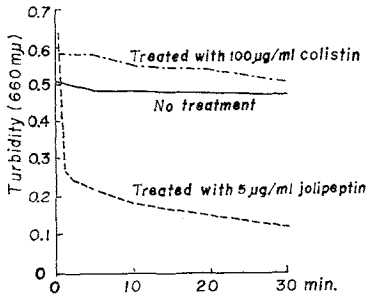


Fig. 7. Lysis of protoplasts of *M. lysodeikticus* after treatment with jolypeptin (5 μg/ml) colistin (100 μg/ml).

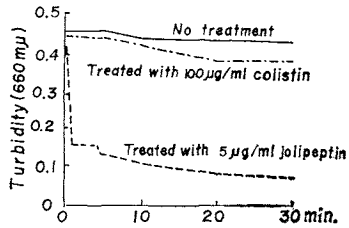


Fig. 8. Lysis of spheroplasts of *E. coli* B after treatment with jolipeptin (5 μg/ml) and colistin (100 μg/ml).

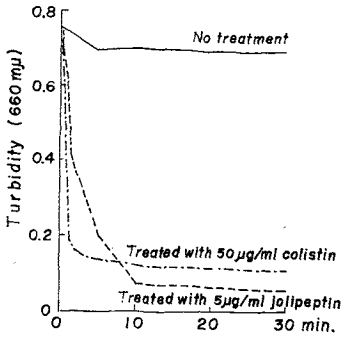
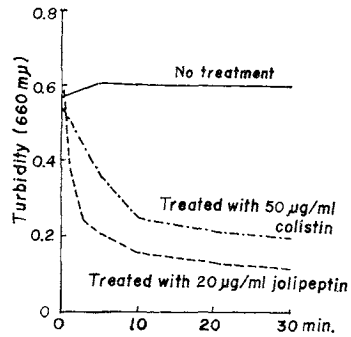


Fig. 9. Lysis of spheroplasts of *P. aeruginosa* IFO 3901 after treatment with jolipetin (20 μg/ml) and colistin (50 μg/ml).



considered that jolipeptin acted primarily on the cellular membrane of the test organisms and consequently the intracellular materials were released into the medium, as seen with other polypeptide antibiotics.

The action of jolipeptin on the cellular membrane of a typical gram-positive bacteria, *B. subtilis* PCI 219 and *M. lysodeikticus*, and of typical gram-negative bacteria, *E. coli* B and *P. aeruginosa* IFO 3901, was examined using protoplasts and spheroplasts of these microorganisms.

Protoplasts of *B. subtilis* PCI 219 and *M. lysodeikticus* were employed as representing gram-positive bacteria. These strains were sensitive to jolipeptin but insensitive to colistin. When 5 μg/ml of jolipeptin at the final concentration was added to these protoplast suspensions, a decrease of absorbance at 660 mμ was observed. As shown in Figs. 6 and 7, the lysis of protoplasts was immediately initiated by the addition of jolipeptin. However, in neither case were these protoplasts lysed by colistin even at a concentration of 100 μg/ml of colistin, despite the concentration, and the ability of colistin to lyse spheroplasts of *E. coli* B completely.

The spheroplasts were prepared from the gram-negative bacteria, *E. coli* B and *P. aeruginosa* IFO 3901. These microorganisms were sensitive to both jolipeptin and colistin as described previously[8]. The optical density of spheroplasts in suspension decreased immediately for both strains in the presence of jolipeptin or colistin (Figs.

8 and 9). The spheroplasts of *P. aeruginosa* IFO 3901 were lysed by 20 μg/ml of jolipeptin but not by 5 μg/ml of jolipeptin, which could lyse spheroplasts of *E. coli* B. This fact might be related to the minimal inhibitory concentration of jolipeptin against this strain.

The findings that protoplasts and spheroplasts were lysed by jolipeptin, but that protoplasts were not lysed by colistin suggested that the lytic process might be the basis for antibiotic activity of jolipeptin and colistin. In any event, it was concluded that jolipeptin acted primarily on cellular membrane of jolipeptin-sensitive strains.

Discussion

The studies described above confirmed that the primary action of jolipeptin is on cellular membrane.

As described in the introduction, it has been confirmed that the primary action of bacitracin and gramicidin is on cellular membranes and that these antibiotics cause the alteration of cell permeability. In addition to these actions of bacitracin and gramicidin, there is evidence that bacitracin inhibits dephosphorylation of lipid-PPi to the active phosphomonoester form, as described by SIEWERT and STROMINGER[10]. CHAPPELL and CROFTS have reported that gramicidin acted as uncoupler of oxidative phosphorylation in mitochondria[11]. On the basis of this evidence, it may be considered that there are other actions of jolipeptin on microbial physiology, and these are under study.

On the other hand, colistin lysed spheroplasts of gram-negative bacteria, but did not lyse protoplasts of gram-positive bacteria. AIDA and ITO have reported that a protoplast-dissolving factor isolated from *B. subtilis* 427-4 did not show specificity against bacterial protoplasts and spheroplasts, although it is not antibiotic[12]. YAMAGUCHI *et al.*[13] have also reported on the specificity of action on bacterial cellular membranes. For example, a protoplast-bursting factor isolated from pig pancrease, lysed the protoplasts of gram-positive bacteria, but not spheroplasts of gram-negative bacteria. It is interesting that the action of colistin shows specificity on protoplasts and spheroplasts. We think that the specificities of these antibiotics against microbial cellular membranes may be applied widely in the future in studies of cellular membranes.

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