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STUDIES ON THE MECHANISM OF ACTION OF COLISTIN
I. FORMATION OF AN INSOLUBLE COMPLEX WITH NUCLEIC ACIDS

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SUMMARY Colistin, a peptide antibiotic of the polymyxin group, forms insoluble complexes with deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA is precipitated with a much smaller amount of colistin than RNA. The complex formation occurs between deacylcolistin and nucleic acids. The formation of these complexes is antagonized by divalent cations. The optimal pH for formation of the insoluble complex was found to be 6.4.

INTRODUCTION

Colistin is one of a group of basic peptide antibiotics, named polymyxins, which was first reported by KOYAMA *et al.* (1950). Chemical studies have shown that it is composed of at least colistin A, B and C (SUZUKI *et al.* 1963 a). SUZUKI *et al.* (1965) determined its chemical structure and found that colistin A and B correspond to polymyxin E_1 and E_2, respectively. The proposed chemical structures of colistin A and B (polymyxin E_1 and E_2) are illustrated in Fig. 1 (SUZUKI *et al.* 1963 b ; 1964).

Polymyxins, including colistin, are known to inhibit the growth of Gram-negative bacteria greatly and that of Gram-positive ones only slightly. Its mode of action has been studied by NEWTON (1954 a, b; 1956) and FEW and SCHULMAN (1953). According to NEWTON (1956), the site of action of polymyxin in bacterial cells is polyphosphate. However, as it has been reported by LATTERADE and MACHEBOEUF (1950) that polymyxin B forms an insoluble complex with RNA, further studies on the mode of action of polimyxin are necessary.

In the course of investigations on the mechanism of action of colistin, we observed the formation of an insoluble complex between colistin and DNA. Thus studies were made on the formation of the complexes using puri-

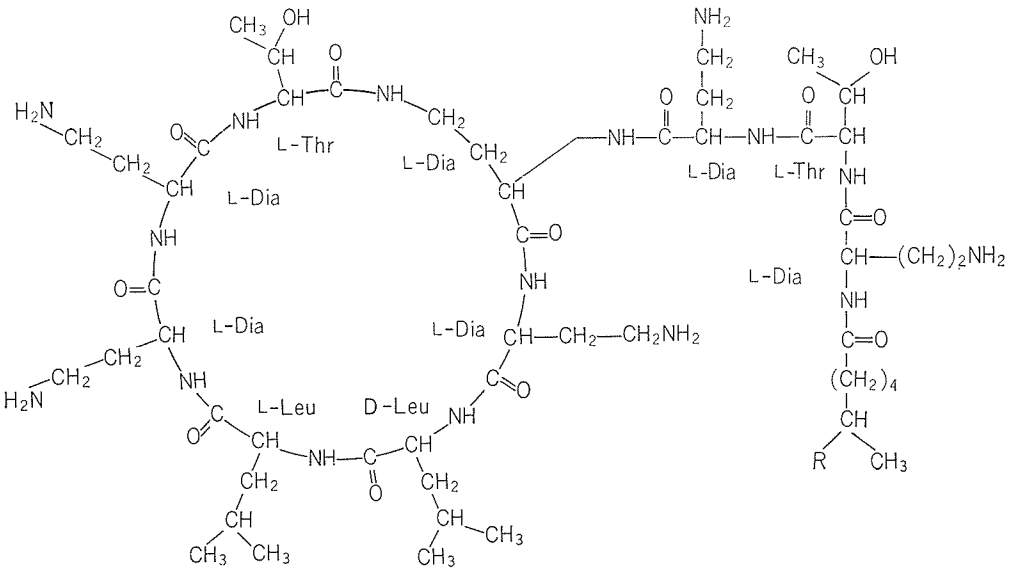

FIGURE 1 Chemical structure of colistin A and B.
Dia., Thr. and Leu. in the Figure are abbreviations for α, γ-diaminobutyric acid, Threonine and Leucine, respectively.
Colistin A: $R=C_2H_5$ Colistin B: $R=CH_3$

fied samples of colistin A and B and of polymyxins to clarify the relationship between complex formation and the action mechanism.

MATERIALS AND METHODS

1. *Antibiotics*

Colistin sulfate was a product of Kayaku antibiotics Research Co., Ltd., Tokyo. Polymyxin B_1, B_2, D_1, deacylcolistin, colistin A and B were kindly given by Prof. T. SUZUKI (Institute for Protein Research, Osaka University). Polymyxin B sulfate was a product of Taito-Pfizer Co., Ltd., Tokyo.

2. *Nucleic acids*

DNA was prepared from *E. coli* strain B, and *B. subtilis*, strain SB-15, by Marmur's method (1961) and from calf thymus by the method of KAY *et al.* (1952). DNA of T2*r* phage was isolated and purified by Mandell and Hershey's method (1960). To obtain a heat denatured preparation of DNA, the solution of DNA was heated in a boiling water bath for 15 minutes and then chilled rapidly in an ice water. From *E. coli* strain B ribosomal RNA was prepared by Kurland's method (1960) and soluble RNA by Tissières' method (1959). The RNA preparations were analyzed by sucrose density gradient centrifugation to detect the 23S, 16S and 4S fractions.

3. *Protein*

Bovine serum albumin (Fraction V of Sigma Co., Ltd.) was used.

4. *Triphosphates*

Cytidine-5′-triphosphate and uridine-5′-triphosphate disodium salt (Calbiochem. Co., Ltd.), adenosine-5′-triphosphate and guanosine-5′-triphosphate disodium salt (Schwarz. Co., Ltd.) were used.

5. *Determination of the polymyxin-nucleic acid complex*

Nucleic acids were dissolved in distilled water at concentrations of 80 μg-P/ml and protein or nucleotide-triphosphates were dissolved in distilled water at concentrations of 200 μg/ml. To 1 ml of the

material to be tested, 1 ml of an aqueous solution of polymyxin was added and the volume was adjusted to 4 ml by adding 2 ml of CO_2-free distilled water. The mixture was stood for 30 minutes at 25°C and the resulting turbidity of the solution was estimated at 420 mμ in a Beckman type Hitachi spectrophotometer.

RESULTS

1. *Formation of insoluble complexes between colistin and nucleic acids*

As shown in Table 1, when 20 μg-P/ml of DNA of *E. coli* was mixed with 50 μg/ml of colistin or polymyxins, formation of insoluble complexes was detected by an increase in optical density at 420 mμ. It is noteworthy that deacylcolistin, that is colistin lacking a C_9-saturated fatty acid, forms almost as much insoluble complex as intact colistin. Bacitracin, one of the group of peptide antibiotics, failed to form a complex. The fact that deacylcolistin forms an insoluble complex suggests that the cyclic structure of the peptide moiety of polymyxin group antibiotics, including colistin, is responsible for the formation of the complex.

TABLE 1 *Formation of insoluble complexes between various kinds of polymyxins and E. coli DNA*

Polymyxins	Polymxin-DNA complex
Polymyxin B	0.252
Polymyxin B_1	0.250
Polymyxin B_2	0.232
Polymyxin D_1	0.240
Deacyl colistin	0.198
Colistin A (Polymyxin E_1)	0.250
Colistin B (Polymyxin E_2)	0.235
Colistin sulfate	0.275
Protamin sulfate	0.065
Bacitracin	0
Streptomycin sulfate	0.048

DNA extracted from *E. coli* strain B (20 μg-P/ml) was mixed with 50 μg/ml of colistin or polymyxins, and the mixture was stood for 30 minutes at 25°C. The resulting turbidities of these mixtures were measured in a Beckman type Hitachi spectrophotometer at 420 mμ. Polymyxin-DNA complex formation was expressed as the optical density at 420 mμ.

Table 2 shows the formation of insoluble complexes between colistin and nucleic acids obtained from various sources. All the DNA preparations tested, including heat denatured DNA, formed as much insoluble complex with colistin as *E. coli* DNA. RNAs formed complexes to a lesser extent than DNAs.

Neither protein nor the nucleotide preparations formed any complex with colistin. The quantitative relationships in the formation of complexes between colistin and nucleic acids are illustrated in Fig. 2.

With 20 μg-P/ml of *E. coli* DNA there was a visible precipitate with 5 μg/ml of colistin. As indicated in Table 3, DNA was recovered quantitatively from the precipitate when 80 μg-P/ml of DNA were incubated with 5 μg/ml of colistin. RNA, however, was only precipitated at a concentration of 500 μg/ml of colistin. This shows that DNA forms insoluble com-

TABLE 2 *Formation of an insoluble complex between colistin and various kinds of nucleic acids*

Sources of nucleic acids	Colistin-DNA complex
DNA	
Escherichia coli B	0.274
Calf thymus	0.264
Bacillus subtilis (SB-15)	0.282
T_2r bacteriophage	0.256
Heat-denatured (*E. coli* B)	0.280
RNA	
Ribosomal (*E. coli* B)	0.028
Soluble (*E. coli* B)	0.010

Colistin (50 μg/ml) was mixed with various kinds of DNA and RNA (20 μg-P/ml), and these mixtures were allowed to stand under the same conditions as described in Table 1.

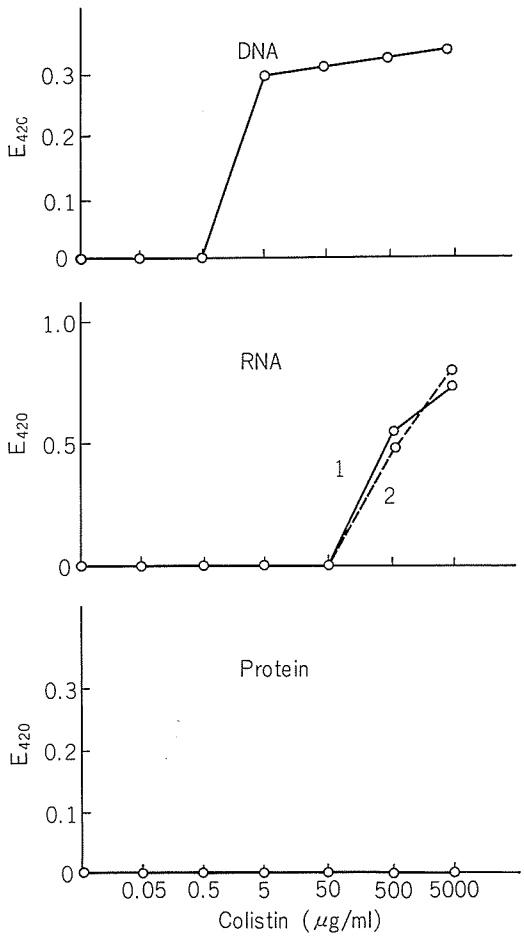

FIGURE 2 Formation of insoluble complexes between nucleic acids and colistin.
In this experiment, *E. coli* DNA (20 μg-P/ml), RNA (20 μg-P/ml) and Bovine serum albumin (50 μg/ml) were incubated with various concentrations of colistin, as described in Table 1. For complex formation with RNA, ribosomal RNA (1) and soluble RNA (2) from *E. coli* were used.

plexes with colistin much more readily than RNA.

2. *Effect of cations on the formation of insoluble complexes*

It has been reported that cells can be protected against the bactericidal action of polymyxin by divalent cations (NEWTON, 1953).

TABLE 3 *Recovery of nucleic acids from aqueous solution by colistin treatment*

Colistin (μg/ml)	Contents of nucleic acids in supernatant fraction (3,000 rpm 15 min) after colistin precipitation	
	DNA (μg-P/ml)	RNA (μg-P/ml)
0	19.6	20.5
0.5	18.8	20.1
5	0.7	19.8
50	0.3	17.6
500	<0.1	0.3
5000	<0.1	<0.1

DNA and RNA extracted from *E. coli* B (20μg-P/ml) were incubated with various concentration of colistin for 30 minutes at 25°C, and the precipitate obtained was centrifuged at 3,000 rpm for 15 minutes. The supernatant fraction was acidified to 0.5 N with cold 10 N perchloric acid, and kept in the cold for 30 minutes. After centrifugation, the acid insoluble fraction was assayed for DNA and RNA by methods of BURTON (1956) and MEJBAUM (1939).

Therefore, attempts were made to demonstrate the effects of neutral salts on the formation of a complex between colistin and DNA. DNA of *E. coli* corresponding to 20 μg-P/ml was incubated with 50 μg/ml of colistin and 0.01 M of various kinds of neutral salts for 30 minutes at 25°C and the resulting turbidity was measured. As shown in Table 4, only bi- and trivalent cations, particularly Mg^{+2} and Ca^{+2}, exhibited strong inhibitory effects on complex formation. Sodium citrate also antagonized complex formation.

3. *Effect of pH on formation of insoluble com plexes*

To investigate the effect of pH on complex formation, 20 μg-P/ml of *E. coli* DNA was mixed with 50 μg/ml of colistin in buffers of various pH values and the resulting turbidity was measured. As seen in Fig. 3, maximum turbidity was obtained at pH 6.4, the turbidity decreasing at higher and lower pH values.

TABLE 4 *Effect of neutral salts on the formation of the colistin-DNA complex*

Salt	Colistin-DNA complex
H_2O	0.270
$MgCl_2$	0.052
$MgSO_4$	0.049
$CaCl_2$	0.030
$MnCl_2$	0.052
$ZnCl_2$	0.056
$FeCl_3$	0.048
Na-citrate	0.025
Na-acetate	0.260
NaCl	0.258
LiCl	0.250
NH_4Cl	0.262

DNA extracted from *E. coli* strain B (20 μg-P/ml) was mixed with 50 μg/ml of colistin in the presence of 0.01 M solutions of various neutral salts. The experimental procedures were the same as shown in Table 1.

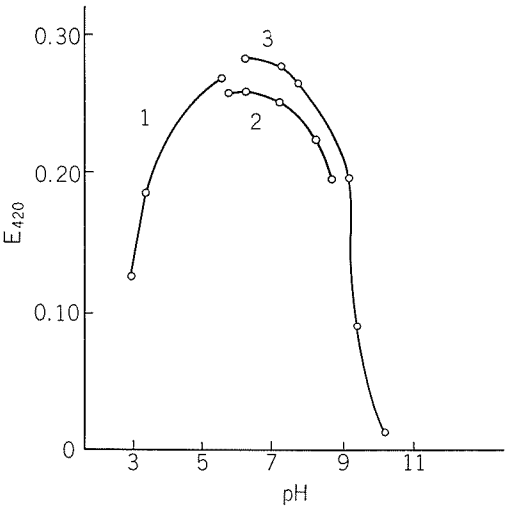

FIGURE 3 Influence of pH on the formation of an insoluble complex between DNA and colistin. Complex formation was carried out in (1) 0.01 M glycine buffer, (2) 0.01 M Sörensen phosphate buffer and (3) 0.01 M veronal buffer. The experimental procedures were the same as those described in Table 1.

DISCUSSION

The interaction of antibiotics and nucleic acids has been reported by many investigators. Actinomycin forms a complex preferentially with DNA (KAWAMATA and IMANISHI, 1960; KIRK, 1960; RAUEN *et al*, 1960). Streptomycin is known to precipitate DNA and RNA by forming complexes with these substances (COHEN, 1947; GROS, 1948). Mitomycin C was found to form cross-links with DNA (IYER and SZYBALSKI, 1963; 1964) and causes an overall inhibition of duplication of cellular DNA (SHIBA *et al.*, 1959). Complex formation of polymyxin B with RNA has also been reported (LATTERRADE and MACHEBOEUF, 1950).

The mechanism of action of polymyxin B and of its binding with RNA remains unknown. From a comparison of the affinities of various cations for the polymyxin-combining group of the cell with their ability to reverse the charge on certain colloids, NEWTON (1954) has suggested that polymyxin E binds polyphosphates in the bacterial cell surface leading to a change in the surface charge of the cells and an alteration in the permeability of the cell membrane which results in cell death.

In our experiments colistin combined firmly and predominantly with DNA and the degree of complex formation with RNA was found to be only 1/100 of that with DNA. A similar tendency was found with deacylcolistin. This means that the basic, cyclic peptide moiety of colistin is essential for complex formation. The reason why deacylcolistin is a weak inhibitor of the growth of bacteria is to be attributed to the absence of C9-fatty acid which is thought to facilitate the penetration of peptide moiety through the permeability barrier of the cell. The biological significance of this complex formation with DNA has not yet been

clarified. However, it is supposed that by formation of such complexes, the physiological function of nucleic acids might be inhibited. In another series of experiments the formation of complexes between colistin and phospholipids such as cephalin and lecithin was observed (FEW and SCHULMAN, 1955; NAKAJIMA and KAWAMATA, unpublished data). Further experiments are necessary to determine the primary site of action of colistin.

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REFERENCES

BURTON, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**, 315–323.

COHEN, S. S. (1947). Streptomycin and deoxyribonuclease in the study of variation in the properties of a bacterial virus. *J. Biol. Chem.* **168**, 511–526.

FEW, A. V. and SCHULMAN, T. H. (1953). The absorption of polymyxin E by bacteria and bacterial cell walls and its bactericidal action. *J. Gen. Microbiol.* **9**, 454–466.

FEW, A. V. (1955). The interaction of polymyxin E with bacterial and other lipids. *Biochim. Biophys. Acta* **16**, 137–145.

GROS, F. and RYBAK, B. (1948). Action de la pénicilline et de la stréptomycine sur le catabolisme de l'acide ribonucléique. *Helv. Chim. Acta* **31**, 1855–1883.

IYER, V. N. and SZYBALSKI, W. (1963). A molecular mechanism of mitomycin action: liking of complementary DNA strands. *Proc. Nat. Acad. Sci.* **50**, 355–362.

IYER, V. N. and SZYBALSKI, W. (1964). Mitomycins and porfiromycin: chemical mechanism of activation and cross-linking of DNA. *Science* **145**, 55–58.

KAWAMATA, J. and IMANISHI, M. (1960). Interaction of actinomycin with deoxyribonucleic acid. *Nature* **187**, 1112–1113.

KAY, E. R. M., SIMMONS, M. S. and DOUNCE, A. L. (1952). An improved preparation of sodium deoxyribonucleate. *J. Am. Chem. Soc.* **74**, 1724–1726.

KIRK, T. M. (1960). The mode of action of actinomycin D. *Biochim. Biophys. Acta* **42**, 167–169.

KOYAMA, Y., KUROSAWA, A., TSUCHIYA, A. and TAKAKURA, K. (1950). A new antibiotic, colistin, produced by spore-forming soil bacteria. *J. Antibiotics* Tokyo **3**, 457–458.

KURLAND, C. G. (1960). Molecular characterization of ribonucleic acid from *Escherichia coli* ribosomes. I. Isolation and molecular weight. *J. Mol. Biol.* **2**, 83–91.

LATTERRADE, C. and MACHEBOEUF, M. (1950). Recherches biochimiques sur le mode d'action de la polymyxine. *Ann. Inst. Pasteur* **78**, 753–458.

MANDELL, J. D. and HERSHEY, A. D. (1960). A fractionating column for analysis of nucleic acids. *Anal. Biochem.* **1**, 66–77.

MARMUR, T. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* **3**, 208–218.

MEJBAUM, W. (1939). Über die Bestimung kleinen Pentosemengen, insbesondere in Derivatien der Adenylsäure. *Hoppe Seyler's Z. Physiol. Chem.* **258**, 117–120.

NEWTON, B. A. (1953). Reversal of the antibacterial activity of polymyxin by divalent cations. *Nature* **172**, 160–161.

NEWTON, A. A. (1954 a). Site of action of polymyxin on *Pseudomonas aeruginosa:* Antagonism by cations. *J. Gen. Microbiol.* **10**, 491–499.

NEWTON, B. A. (1954 b). The absorption of polymyxin by a cell wall preparation from *Pseudomonas aeruginosa*. *J. Gen. Microbiol.* **10**, iii–iv.

NEWTON, B. A. (1956). The properties and mode of action of polymyxins. *Bacteriol. Rev.* **20**, 14–27.

RAUEN, H. M., KERSTEN, H. and KERSTEN, W. (1960). Zur Wirkungsweise von Actinomycinen. Hoppe-Seyler's *Z. Physiol. Chem.* **321**, 139-147.

SHIBA, S., TERAWAKI, A., TAGUCHI, T. and KAWAMATA, J. (1959). Selective inhibition of formation of deoxyribonucleic acid in *Escherichia coli.* by mitomycin C. *Nature* **183**, 1056–1057.

SUZUKI, T., INOUYE, H., FUJIKAWA, K. and SUKETA,

S. (1963 a). Studies on the chemical structure of colistin I. Fractionation, molecular weight determination, amino acids and fatty acid composition. *J. Biochem.* Tokyo **54**, 25–33.

SUZUKI, T., INOUYE, H., FUJIKAWA, K. and NAGASAWA, S. (1963 b). Studies on the chemical structure of colistin II. Amino acid sequence of colistin A. *J. Biochem.* Tokyo **54**, 173–180.

SUZUKI, T. and FUJIKAWA, K. (1964). Studies on the chemical structure of colistin IV. Chemical structure of colistin B. *J. Biochem.* Tokyo **56**, 182–189.

SUZUKI, T., HAYASHI, K., FUJIKAWA, K. and TSUKAMOTO, K. (1965). The chemical structure of polymyxin E: The identities of polymyxin E_1 with colistin A and of polymyxin E_2 with colistin B. *J. Biochem.* Tokyo **57**, 226–227.

TISSIÈRES, A. (1959). Some properties of soluble ribonucleic acid from *Escherichia coli*. *J. Mol. Biol.* **1**, 365–374.