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# STUDIES ON THE MECHANISM OF ACTION OF COLISTIN
# V. ACTION ON THE RIBOSOMES

KUNIHIRO NAKAJIMA and JUNICHI KAWAMATA

Department of Experimental Chemotherapy, Research Institute for Microbial Diseases, Osaka University, Osaka

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SUMMARY Colistin caused the degradation of purified 30 *S* ribosomes of *E. coli* resulting in the breakdown of 30 *S* ribosomal RNA but not that of 50 *S* ribosomes. Incubation of externally added $^{3}$H-RNA with 30 *S* ribosomes and colistin caused the degradation of $^{3}$H-RNA to acid soluble compounds, indicating the activation of " latent " ribosomal ribonuclease (RNase I) associated with the 30 *S* ribosomes.

Most of the radioactivity of $^{3}$H-colistin A taken up by *E. coli* cells was distributed in the ribosomal fractions.

$^{3}$H-colistin A combined with the 30 *S* and 50 *S* ribosomes but not the 70 *S* ribosomes.

## INTRODUCTION

During an investigation on the mechanism of action of colistin (polymyxin E), evidence was obtained that acid soluble, low-molecular weight ribocompounds were released from *E. coli* cells on treatment with colistin at 37°C (NAKAJIMA and KAWAMATA, 1965b) and that colistin formed an insoluble precipitate *in vitro* with a ribosomal preparation of *E. coli* (NAKAJIMA and KAWAMATA, 1966a). In the preceding paper we reported that colistin caused the breakdown of ribosomal RNA of *E. coli* to acid soluble compounds, and that externally added $^{3}$H-RNA was degraded by incubating the ribosome preparation with the antibiotic at 37°C (NAKAJIMA and KAWAMATA, 1966b). Evidence was also reported indicating the activation of " latent " ribonuclease and the depolymerization of cellular RNA *in vivo* (NAKAJIMA and KAWAMATA, 1966b).

The abundant evidence now available indicates that three enzymes with RNase activity are associated with the ribosomal fraction of *E. coli*. These enzymes are so-called " latent " ribonuclease (RNase I), which is an endonuclease (ELSON, 1959; SPAHR and HOLLINGWORTH, 1961; GESTELAND, 1965, 1966), potassium-activated phosphodiesterase (RNase II) (SPAHR and SCHLESSINGER, 1963; SPAHR, 1964; Singer and Tolbert, 1965) and polynucleotide phosphorylase (LITTAUER and KORNBERG, 1957; SEKIGUCHI and COHEN, 1963) respectively.

This paper is chiefly on the mechanism of activation of " latent " RNase (RNase I) by colistin associated with the 30 *S* ribosomes in connection with the mode of action of colistin.

The mode of binding of colistin to the ribosomes and its intracellular distribution in *E. coli* using a purified sample of $^{3}$H-colistin A are also presented.

## MATERIALS AND METHODS

1. *Organism*

A colistin sensitive *Escherichia coli* B was used.

2. *Medium and conditions of culture*

The organism was grown as described previously (NAKAJIMA and KAWAMATA, 1965c) at 37°C in Simmons' medium (pH 7.2) containing 0.3 per cent glucose and shaken by a rotary shaker at 130 rpm.

3. *Antibiotics*

The colistin sulfate used was a product of Kayaku Antibiotic Research Co., Ltd., Tokyo and streptomycin and dihydrostreptomycin sulfate were products of Riken Chemical Co., Ltd., Tokyo and Kyowa Hakko Co., Ltd., Tokyo, respectively. Gramicidin S hydrochloride was supplied by Prof. S. Otani (Medical School of Osaka City University). Colistin A hydrochloride was kindly provided by Prof. T. Suzuki (Institute for Protein Research, Osaka University).

4. *Isolation and purification of $^{14}$C-uracil labelled ribosomes from E. coli*

Preparation of $^{14}$C-uracil labelled ribosomes was carried out by labelling 200 ml of exponentially growing cells of *E. coli* in glucose-Simmons' medium ($E_{660}=0.5$) at 37°C for 45 minutes with 0.05 $\mu$c/ml of 2-$^{14}$C-uracil (specific activity=4.2 mc/mM, Daiichi Chemical Co., Ltd., Tokyo). At the end of the labelling period, the culture was chilled in an ice-water bath and centrifuged. Isolation and purification of the ribosomal fraction were carried out as described previously (NAKAJIMA and KAWAMATA, 1966a).

5. *Assays of "self-degradation" and "latent" RNase of the E. coli ribosomal fraction*

All procedures were carried out as described in the preceding paper (NAKAJIMA and KAWAMATA, 1966b). For assay of "self-degradation" of ribosomal fraction, the reaction mixture consisted of 1 ml of a suspension of purified *E. coli* ribosome preparation ($E_{250}=5.0$) in $10^{-2}$ M tris (pH 7.4)-$10^{-4}$ MMg acetate, 0.8 ml of the same buffer and 0.2 ml of the drug solution in distilled water. After incubation at 37°C for 30 minutes, the reaction mixture (2 ml) was chilled in an ice-water bath and acidified by the addition of 0.1 ml of cold 10 N perchloric acid (PCA) at a concentration of 0.5 N. It was then stood in the cold for 30 minutes and the resulting precipitate was immediately removed by filtration through a Millipore filter (HA 0.45 $\mu$). The absorbancy of this acid soluble filtrate was read at 260 m$\mu$ with a Hitachi spectrophotometer (EPU-2) employing 0.5 N PCA as a blank.

For assay of "latent" RNase, the incubation mixture (2 ml) consisted of 0.1 ml of $^{3}$H-labelled RNA ($E_{260}=13.0$, 24,000 cpm/ml) from *E. coli* and 1 ml of ribosome suspension ($E_{260}=5.0$) in $10^{-2}$M tris (pH 7.4) containing $10^{-4}$M Mg acetate, 0.7 ml of the same buffer and 0.2 ml of an aqeous solution of colistin of the concentration shown for each experiment. After incubation at 37°C for 30 minutes, the reaction mixture (2 ml) was chilled in an ice-water bath and mixed with 0.1 ml of 10 N PCA at a concentration of 0.5 N. It was stood in the cold for 30 minutes and the resulting precipitate was immediately removed by filtration through a Millipore filter (HA 0.45 $\mu$). The acid insoluble precipitate on the Millipore filter was layered on an aluminum planchet, dried and assayed for radioactivity of acid insoluble $^{3}$H-RNA with a Nuclear-Chicago gas flow counter.

6. *Separation and purification of 30S and 50S ribosomes from E. coli*

Preparation of 30*S* and 50*S* ribosomes was carried out by sedimentation in a sucrose density gradient (5–20%). An aliquot of 3 ml of this ribosome suspension ($E_{260}=100$) was layered on top of 26 ml of sucrose gradient (exponentially 5–20%) containing $10^{-2}$M tris (pH 7.4) and $10^{-4}$M Mg acetate. The gradient was centrifuged for 5 hours at 25,000 rpm at 4°C in a Hitachi RPS-25 swinging bucket rotor. The bottom of the tube was then punctured with a hypodermic needle. Specimens of 50 drops each were taken to measure the absorbancy at 260 m$\mu$ (0.1 ml in 3 ml $H_2O$) with a Hitachi spectrophotometer (EPU-2). The peak fractions corresponding to 30 *S* and 50 *S* ribosomes were collected, and then dialyzed against buffer containing $10^{-4}$M $Mg^{2+}$ at 4°C for 18 hours.

7. *Preparation and purification of $^{3}$H-colistin A*

$^{3}$H-colistin A (polymyxin $E_1$) was prepared by the

gas exchange method with tritium by Dr. T. Komai at the National Institute of Health, Tokyo. This preparation of randomly labelled $^3$H-colistin A was purified by silicic acid-celite (2 : 1=w/w) column chromatography and dried *in vacuo*.

1) *Preparation of the developing solvent*

The solvent system employed was composed of 5 ml pyridine and 100 ml of the upper layer of the mixture of acetic acid : n-butanol : water=1 : 4 : 5 (v/v).

2) *Preparation of the chromatographic column*

20 g of silicic acid (Nakarai Chemical Co., Ltd., Kyoto) and 10g of celite (Nakarai Chemical Co., Ltd., Kyoto, 45 gals/sq. ft/hour) were mixed in a mortar in the presence of 100 ml of the solvent. The resulting suspension was poured into the column (2×70 cm) and the column was washed with about 200 ml of the solvent.

3) *Operation of the column*

Two ml of $^3$H-colistin A solution (containing 50 mg) which had previously been dissolved in the solvent, were placed on the column. After the colistin solution had been adsorbed onto the column, about 300 ml of the same solvent were then placed on the column. Elution was performed at 20°C usually at the flow rate of 10 ml per hour and 2 ml fractions were collected.

Small portions of the individual fractions were taken up and N-contents and radioactivity of the individual fractions were measured with ninhydrin reagent and a Nuclear-Chicago gas flow counter, respectively. The ninhydrin positive fractions, which coincide with the radioactivity of $^3$H-colistin A, were collected and the organic solvent was eliminated in a rotary evaporator in a water bath at 30–35°C. The resulting residue was dissolved in a small volume of distilled water and the water was evaporated off *in vacuo* in the cold. This procedure was repeated six times and a white powder of $^3$H-colistin A was obtained (about 20 mg).

4) *Purity of the purified $^3$H-colistin A preparation*

The purity of the preparation was examined by paper chromatography (Toyo No. 50 filter paper, 2×40 cm) developing with the solvent system for 12 hours. The purified preparation of $^3$H-colistin A showed a single spot of the characteristic purple colour of peptide with ninhydrin reagent ($R_F \fallingdotseq 0.52$) and a specific activity of 75.2 mc/mM. Antibacterial activity and radioactivity on the paper were located only in this spot.

8. *Interaction of $^3$H-colistin A with E. coli ribosomes*

A ribosome suspension ($E_{260}=10.0$) was dialyzed overnight against $10^{-2}$M tris (pH 7.4)–$10^{-4}$M or $10^{-2}$ M $Mg^{2+}$ buffer at 4°C and 5 ml of the dialyzed ribosome suspension were dialyzed again for 18 hours at 4°C against 500 ml of buffer ($10^{-4}$M or $10^{-2}$M $Mg^{2+}$) containing tritiated colistin A at a final concentration of 3 μg/ml. An aliquot of 0.2 ml of the ribosome suspension was layered on 4.4 ml of sucrose gradient (5–20%) and the gradient was spun at 37,000 rpm at 4°C for 90 minutes ($10^{-2}$M $Mg^{2+}$) or 150 minutes ($10^{-4}$M $Mg^{2+}$). Fractions of 0.15 ml (about 8 drops) were collected and diluted with 3 ml of water. The absorbancy at 260 mμ was measured and the radioactivity of the samples was counted with a Nuclear-Chicago gas flow counter.

9. *Intracellular distribution of $^3$H-colistin A in E. coli*

Two hundred ml of exponentially growing cells of *E. coli* B in glucose-Simmons' medium ($E_{660}=0.5$) were grown at 37°C and 3 μg/ml of $^3$H-colistin A was added 30 minutes before harvesting the cells. Cells were washed repeatedly in Simmons' medium until no trace of radioactivity remained in the washings and were kept frozen at –20°C before use. Cellular fractionation by differential centrifugation was carried out according to a slight modification of the method of Connamacher and Mandel (1965). The bacteria were resuspended in 10 ml of $10^{-2}$M tris (pH 7.4) containing $10^{-4}$M Mg acetate and disintegrated mechanically in a B. Brown cell homogenizer (MSK) with 20 g of glass beads (0.1 mm in diameter) at 2°C at 2,000 rpm for 4 minutes. Then the broken cell suspension was centrifuged for 10 minutes at 3,000×$g$ to remove unbroken cells, cell debris and glass beads. On centrigugation of the supernatant fraction, three different fractions were obtained, namely the cell wall fraction which appeared cell free under the microscopic and may contain cytoplasmic membranes (precipitated by centrifugation at 10,000×$g$ for 10 minutes and at 20,000×$g$ for 10 minutes), the ribosomal fraction (precipitated at 105,000×$g$ for 120 minutes) and the supernatant fraction (supernatant after centrifugation at 105,000 ×$g$ for 120 minutes). All procedures were carried out in a cold room. The radioadtivity of $^3$H-colistin A in each fraction was determined in a Nuclear-Chicago gas flow counter.

RESULTS

1. *Degradation of $^{14}$C-uracil labelled ribosomal RNA by colistin*

Samples of $^{14}$C-uracil labelled ribosomes of *E. coli* were incubated with concentrations of colistin ranging from 1 to 1,000 $\mu$g/ml at 37°C for 30 minutes in $10^{-2}$M tris buffer (pH 7.4) containing $10^{-4}$M Mg acetate. As illustrated in Table 1, remarkable degradation of inherent, radioactive ribosomal RNA was observed with 100 and 1,000 $\mu$g/ml of colistin. Some antibiotics having a basic character, such as streptomycin and dihydrostreptomycin, were also found to cause the breakdown of the radioactive ribosomal RNA of *E. coli* to acid soluble ribocompounds, whereas on incubation with $10^{-3}$M or $10^{-4}$M gramicidin S no decrease of ribosomal RNA detected. A similar result was obtained with ribosomes of *E. coli* prepared in the logarithmic as well as the stationary phases of growth, although the degradation of the latter was greater.

TABLE 1 *Degradation of $^{14}$C-uracil labelled ribosomal RNA by colistin and basic compounds*

| Colistin conc. ($\mu$g/ml) | Acid insoluble $^{14}$C-uracil labelled ribosomal RNA |
|---|---|
| 0 (0 min.) | 11,528 |
| 0 (30 min.) | 12,200 |
| 1 | 11,468 |
| 10 | 9,998 |
| 100 | 1,935 |
| 1000 | 3,560 |
| 0.01 M EDTA (pH 7.4) | 780 |
| 4 M urea | 1,240 |
| streptomycin* | 10,998 |
| streptomycin** | 2,561 |
| dihydrostreptomycin* | 11,050 |
| dihydrostreptomycin** | 2,467 |
| gramicidin S* | 12,050 |
| gramicidin S** | 10,986 |

$^{14}$C-uracil labelled *E. coli* ribosomes ($E_{260}$=2.5, 13,500 cpm) were incubated with colistin at levels of 1, 10, 100 and 1,000 $\mu$g per ml in $10^{-2}$ M tris (pH 7.4) containing $10^{-4}$ M Mg acetate. After incubation at 37°C for 30 minutes, the reaction mixture (2 ml) was chilled in an ice-water bath and acidified by the addition of 0.1 ml of 10 N perchloric acid (PCA) at a final concentration of 0.5 N. The mixture was stood for 30 minutes in the cold and the resulting precipitate was filtered through a Millipore filter (HA 0.45$\mu$). The radioactivity of $^{14}$C on the Millipore filter was counted with a Nuclear-Chicago gas flow counter. Degradation of ribosomal RNA is indicated by the decrease of acid insoluble radioactive compounds.

As controls, ribosomes were incubated with 0.01 M EDTA (pH 7.4) or 4 M urea. (*)=$10^{-4}$ M, (**)=$10^{-3}$ M.

For comparison, experiments with 0.01M EDTA (pH 7.4) and 4M urea were also carried out. Both agents caused remarkable degradation of $^{14}$C-uracil labelled ribosomal RNA of *E. coli.*

2. *Self-degradation of 30 S ribosomes by colistin*

It was shown in the preceding paper (NAKAJIMA and KAWAMATA, 1966b) that colistin caused the activation of "latent" RNase in the ribosome fraction, which was reported to be found associated with only 30 *S* ribosomes (Elson, 1958, 1959; Elson and Tal, 1959; Spahr and Hollingworth, 1961; Tal and Elson, 1963; Commack and Wade, 1965; Gesteland, 1965, 1966). Therefore, 30 *S* and 50 *S* ribosomal subunits were isolated from *E. coli* ribosomes by sucrose density gradient centrifugation and the mechanism of activation of RNase by colistin was investigated. A purified preparation of each ribosome subunit was incubated with various amounts of colistin at 37°C for 30 minutes in a buffer similar to that used in the above experiment.

As shown in Table 2, colistin only caused the breakdown of 30 *S* ribosomes to acid soluble compounds but not that of 50 *S* ribosomes.

3. *Demonstration of "latent" ribonuclease (RNase I) activity on 30 S ribosomes by colistin*

While investigating the self-degradation of 30 *S* ribosomes by colistin, it was found that incubation of externally added $^{3}$H-RNA with

TABLE 2 *Self-degradation of a purified sample of 30 S ribosomes by colistin*

| Colistin conc. ($\mu$g/ml) | Acid soluble materials ($E_{260}$) | |
|---|---|---|
| | 30 *S* ribosomes | 50 *S* ribosomes |
| 0 (0 min.) | 0.005 | 0.004 |
| 0 (30 min.) | 0.004 | 0.005 |
| 1 | 0.005 | 0.005 |
| 10 | 0.019 | 0.005 |
| 100 | 0.780 | 0.010 |
| 1000 | 0.550 | 0.018 |
| 0.01 M EDTA (pH 7.4) | 1.020 | 0.680 |
| 4 M urea | 0.987 | 0.650 |

Purified samples of 30 *S* and 50 *S* ribosomes ($E_{260}$=2.5) of *E. coli* B were incubated with colistin at concentrations of 1, 10, 100 and 1,000 $\mu$g per ml, respectively, in $10^{-2}$ M tris (pH 7.4) containing $10^{-4}$ M Mg acetate. After incubation at 37°C for 30 minutes, the reaction mixture was chilled in an ice-water bath and acidified by the addition of cold 10 N PCA at a final concentration of 0.5 N. The mixture was stood for 30 minutes in an ice-water bath and resulting precipitate was removed by filtration through a Millipore filter (HA 0.45$\mu$). The absorbancy of the acid soluble fraction was measured with a Hitachi spectrophotometer (EPU-2) at 260 m$\mu$. As controls, 50 *S* and 30 *S* ribosomes were incubated with 4 M urea or 0.01 M EDTA (pH 7.4).

colistin and 30 *S* ribosomes, but not with 50 *S* ribosomes, of *E. coli* resulted in the breakdown of $^3$H-RNA to acid soluble compounds. The effects of 4M urea and 0.01M EDTA (pH 7.4), which are thought to activate "latent" RNase, namely "RNase I" bound to 30 *S* ribosomal particles, were studied for comparison.

As illustrated in Table 3, 100 and 1,000 $\mu$g/ml of colistin resulted in a remarkable activation of the RNase on the 30 *S* ribosomes but not on the 50 *S* ribosomes within 30 minutes at 37°C in $10^{-2}$M tris buffer (pH 7.4) containing $10^{-4}$M Mg acetate. EDTA and urea caused the degradation of externally added radioactive RNA to acid soluble compounds when they were incubated not only

TABLE 3 *Domonstration of ribonuclease activity on the 30 S ribosomes of E. coli by colistin*

| Colistin conc. ($\mu$g/ml) | Acid insoluble $^3$H-RNA (cpm) | |
|---|---|---|
| | 30 *S* ribosomes | 50 *S* ribosomes |
| 0 (0 min.) | 2,210 | 2,246 |
| 0 (30 min.) | 2,246 | 2,180 |
| 1 | 2,246 | 2,350 |
| 10 | 2,198 | 2,200 |
| 100 | 359 | 1,987 |
| 1000 | 698 | 1,989 |
| 0.01 M EDTA (pH 7.4) | 139 | 479 |
| 4 M urea | 287 | 498 |

Purified preparation of 30 *S* and 50 *S* ribosomes of *E. coli* ($E_{260}$=2.5) were incubated with *E. coli* $^3$H-RNA ($E_{260}$=0.75, 2,460 cpm) and colistin at levels of 1, 10, 100 and 1,000 $\mu$g per ml, respectively at 37°C for 30 minutes in $10^{-2}$M tris (pH 7.4) containig $10^{-4}$M Mg acetate and the assay of latent RNase was carried out as described in the text. The $^3$H-radioactivity of the acid insoluble fraction was counted with a Nuclear-Chicago gas flow counter. As controls, 50 *S* and 30 *S* ribosomes were incubated with 4 M urea or 0.01 M EDTA (pH 7.4) respectively.

with 30 *S* ribosomes but also with 50 *S* ribosomes of *E. coli* although in the former case this was much greater. This indicates that colistin preferentially activates "latent" RNase, viz., "RNase I" associated with 30 *S* ribosome particles. Externally added $^3$H-RNA was degraded by incubating the 50 *S* ribosome preparation with 0.01M EDTA (pH 7.4) or 4M urea, which suggests that the RNase might also be located on the 50 *S* ribosome particles. It is, however, uncertain whether the RNase I is merely adsorbed onto the 50 *S* ribosomes during the course of preparation of cell extract or whether the enzyme is actually associated with 50 *S* ribosomes as well as 30 *S* ribosomes.

It is concluded from these results that the sensitivity of ribosomes to colistin is associated exclusively with the 30 *S* subunit of ribosomes by analogy with the action of strepto-

mycin (Davies *et al.*, 1964; Davies, 1964; Cox *et al.*, 1964).

The mechanism of activation of the enzyme was examined in the following way. Non-radioactive ribosomes were treated with colistin to provide a latent period before self-degradation took place. Self-degradation was measured by the appearance of acid soluble UV-absorbing materials. At zero time $^3$H-labelled RNA of high specific radioactivity was added. The degradation of the added substrate was determined by the appearance of $^3$H-RNA degradation compounds in the acid soluble fraction. The result is illustrated in Fig. 1. It is clear that the added RNA was largely degraded before the inherent RNA is attacked. Ribosomes that have not been treated with EDTA or 100 $\mu$g/ml of colistin did not attack either substrate. Thus, it is concluded that the RNase from the ribosomes is released by the action of colistin.

4. *Intracellular distribution of $^3$H-colistin A in the cells*

From the above observations, it was concluded that the ribosomes are a site of colistin action resulting from the formation of a complex between colistin and ribosomes and the degradation of ribosomal RNA as a result of the activation of " RNase I ".

The demonstration of the actual localization of colistin in the cells, therefore, seems to be a good approach to the elucidation of its action. Differential centrifugation of cells treated with a purified sample of $^3$H-colistin A was considered to be a useful method for this purpose.

*E. coli* cells were treated with 3 $\mu$g/ml of $^3$H-colistin A (75.2 mc/mM) at 37°C for 30

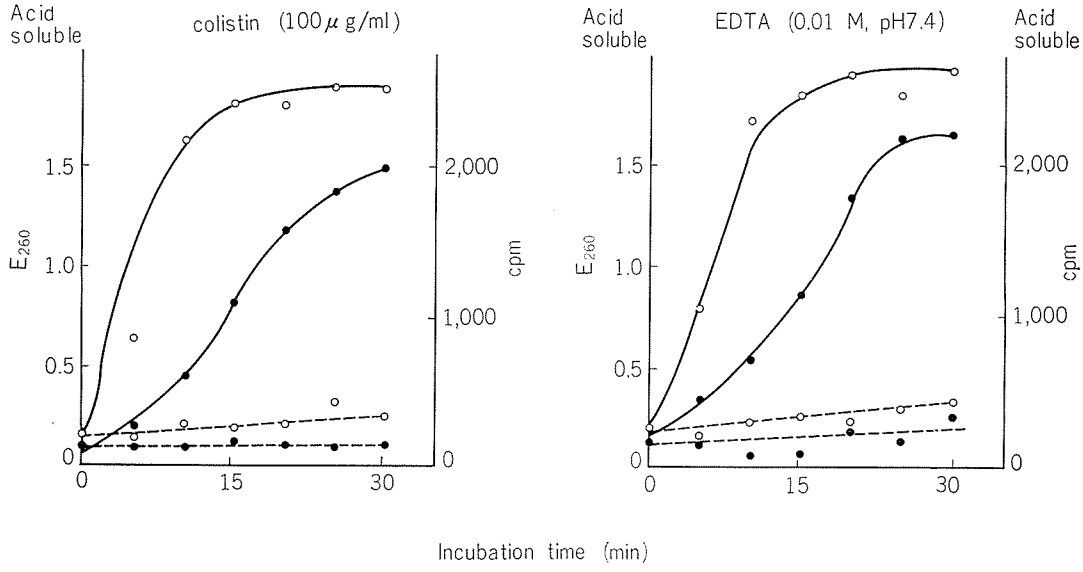


FIGURE 1 Degradation of externally added $^3$H-RNA and of nonradioactive inherent ribosomal RNA by colistin.
*E. coli* ribosomes ($E_{260}$=2.5) were incubated with purified *E. coli* $^3$H-RNA ($E_{260}$=0.75, 2,160 cpm) and colistin at a level of 100 $\mu$g per ml at 37°C in $10^{-2}$M tris (pH 7.4)–$10^{-4}$M Mg acetate. At intervals, the UV-absorbing materials and $^3$H-radioactivity in the acid soluble fraction were measured as described in the text. Degradation of externally added $^3$H-RNA is shown by the appearance of acid soluble $^3$H and that of inherent ribosomal RNA by acid soluble ultraviolet-absorbing compounds.
●——●: degradation of inherent ribosomal RNA of *E. coli*
○——○: degradation of externally added $^3$H-RNA of *E. coli*
In this figure, the dotted line shows the values of the untreated controls.

TABLE 4 *Intracellular distribution of $^3$H-colistin A in E. coli B*

| Cellular fractions | $^3$H-colistin A (cpm) | (%) |
|---|---|---|
| Intact cells | 81,030 | 100.0 |
| Cell wall fraction (containing membranes) 10,000×*g*, 10 min. | 22,875 | 28.2 |
| 20,000×*g*, 10 min. | 7,600 | 9.4 |
| Ribosome fraction (105,000×*g*, 120 min.) | 50,120 | 61.8 |
| Supernatant fraction (105,000×*g*, 120 min.) | 2,528 | 3.1 |

Two hundred m*l* of the cell suspension of *E. coli* ($E_{660}$=0.5) was shaken with 3 μg/m*l* of $^3$H-colistin A at 37°C in glucose-Simmons' medium for 30 minutes and the cells were disrupted by mechanically disintegration and fractioned by differential centrifugation. The radioactivity of each fraction was measured as described in the text.

minutes in glucose-Simmons' medium and then fractionated by centrifugation.

As shown in Table 4, the radioactivity of colistin was mainly distributed in either the "ribosomal fraction" (precipitated at 105,000 ×*g* in 120 minutes) or the "cell wall fraction" (precipitated at 10,000×*g* in 10 minutes and then at 20,000×*g* in 10 minutes). It is clear that the affinity of the ribosomal fraction of *E. coli* is much greater than that of the cell wall fraction. The accumulation of radioactivity particularly in the ribosome fraction was demonstrated after treatment of *E. coli* cells with $^3$H-colistin A.

5. *Binding of $^3$H-colistin A with E. coli ribosomes*

To identify the mode of binding of $^3$H-colistin A with ribosomes, a sample of purified *E. coli* ribosomes was dialyzed in $10^{-2}$M tris buffer containing $10^{-4}$M or $10^{-2}$M Mg acetate at 4°C for 18 hours in the presence of $^3$H-colistin A and the ribosome-colistin complex was analyzed by sucrose density gradient centrifugation (5–20% sucrose).

It is clear from Fig. 2 that colistin was bound to both 50 *S* and 30 *S* ribosomes of *E. coli*. In a sucrose gradient containing $10^{-2}$M Mg acetate, colistin was not found to be bound to the 70 *S* ribosomes. Binding to the 50 *S* or 30 *S* ribosomes also took place when colistin was added directly to ribosomes at 2°C, indicating that the substance with which it com-

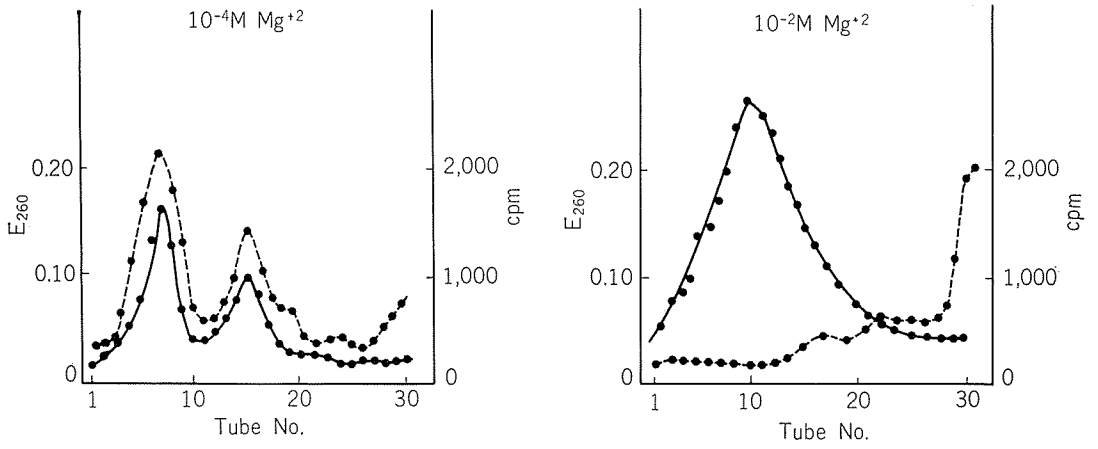


FIGURE 2 Binding of $^3$H-colistin A (polymyxin $E_1$) with ribosomes *of E. coli*
A purified sample of ribosome preparation ($E_{260}$=10.0) was dialyzed for 18 hours at 4°C against 500 ml of the buffer ($10^{-4}$M $Mg^{2+}$ or $10^{-2}$M $Mg^{2+}$) containing tritiated colistin A at a final concentration of 3 μg/ml and the analysis of $^3$H-colistin-ribosome complexes was carried out by sucrose density gradient centrifugation as described in the text. ●——● : O.D.$_{260}$ of each fraction; ●-----● : radioactivity of $^3$H-colistin A.

bined was not a metabolic product of the antibiotic.

To ascertain whether or not the particles of the complex of colistin with 50 *S* or 30 *S* ribosomes were sensitive to the action of the RNase released from 30 *S* ribosomes, attempts were made to depolymerize the ribosomal RNA of these particles with pancreatic RNase (Worthigton Biochem. Co., New Jersey). Ribosomes were dialyzed at 4°C for 18 hours against $10^{-2}$M tris buffer (pH 7.4) containing $10^{-4}$M Mg acetate and 3 $\mu$g/ml of $^{3}$H-colistin A. Then the dialyzed preparation of ribosomes was treated with 25 $\mu$g/ml of pancreatic RNase at 37°C for 15 minutes and then analyzed by sucrose density gradient centrifugation (5–20% sucrose).

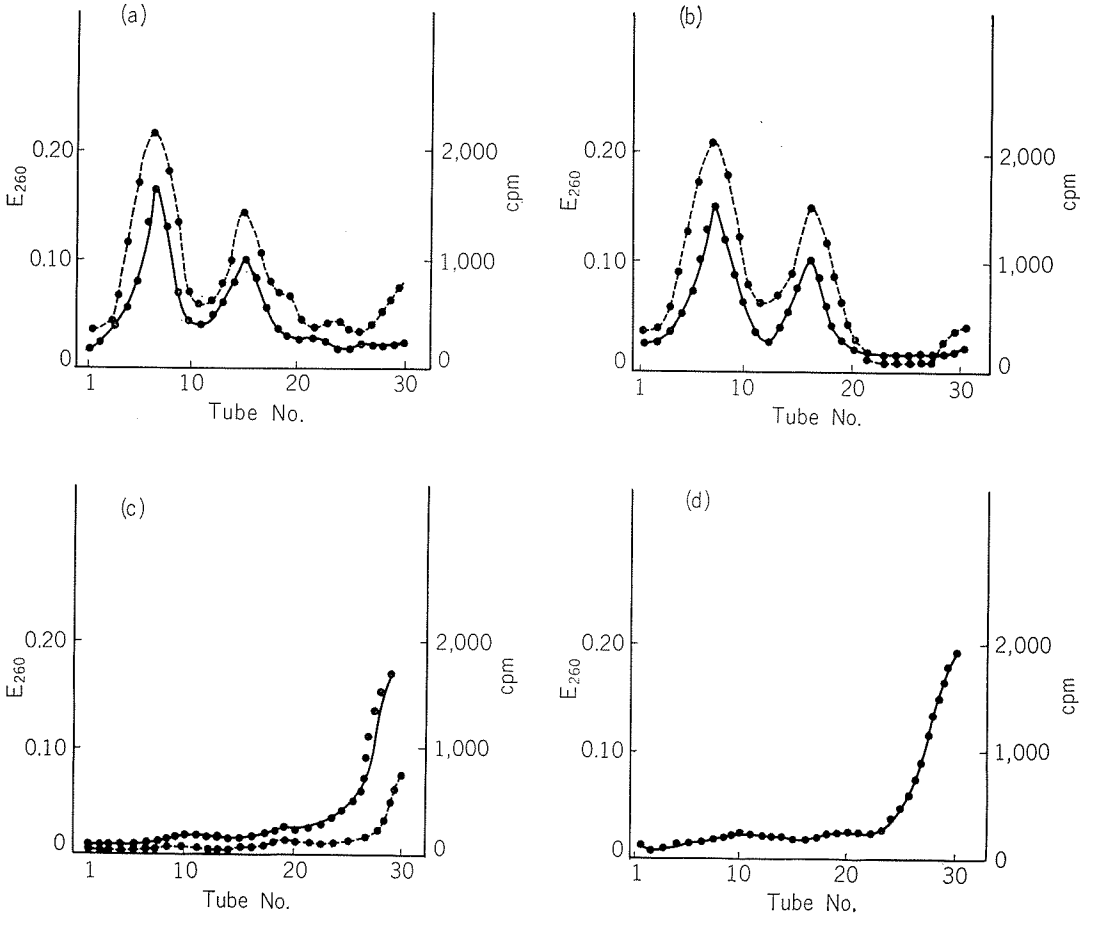


FIGURE 3 Effect of pancreatic RNase on colistin-ribosome complex

After dialyzing of *E. coli* ribosomes against buffer containing $10^{-4}$M Mg acetate at 4°C for 18 hours in the presence of 3 $\mu$g/ml of tritiated colistin, the sample was divided into three portions which were treated with 25 $\mu$g/ml of pancreatic DNase(b) or RNase(c) or was untreated (a) at 37°C for 15 minutes. The analysis of colistin-ribosome complexes was carried out by sucrose density gradient centrifugation as described in the text. As a control, *E. coli* ribosomes were treated with 25 $\mu$g/ml of pancreatic RNase for 15 minutes at 37°C in the buffer(d). ●——●: O.D.$_{260}$ of each fraction and ●------●: the radioactivity of $^{3}$H-colistin A.

The results illustrated in Fig. 3 clearly demonstrate that the RNase depolymerized not only the ribosomal RNA's of both the 30 *S* and 50 *S* particles but also caused the disappearance of $^3$H-colistin A from these particles. It is concluded from these results that $^3$H-colistin A was actually bound to the ribosomal RNA but not to the ribosomal protein. This supports the assumption that the RNase I released from 30 *S* ribosomes by forming a complex of colistin with ribosomes is capable of attacking both 30 *S* and 50 *S* ribosomes.

## DISCUSSION

It was reported in the preceding papers that colistin activated " latent " RNase (NAKAJIMA and KAWAMATA, 1966b) by forming a complex with the ribosomes of *E. coli* (NAKAJIMA and KAWAMATA, 1966a). In the present paper evidence is reported that colistin treatment resulted in the self-degradation of a purified sample of 30 *S* ribosomes at 37°C but not of 50 *S* ribosomes when incubated in $10^{-2}$M tris buffer (pH 7.4) containing $10^{-4}$M Mg acetate. Externally added $^3$H-RNA was decomposed to acid soluble compounds by incubation of radioactive RNA and colistin with 30 *S* ribosomes but not 50 *S* ribosomes. These results indicate that colistin activates " latent " RNase, namely, " RNase I ", associated with 30 *S* ribosome particles.

It is well known that the ribosomes from *E. coli* contain three enzymes with RNase activity. These included (1) so-called " latent " RNase (RNase I), which is an endonuclease located exclusively in the 30 *S* ribosomal fraction (ELSON, 1958, 1959; SPAHR and HOLLINGWORTH, 1961; TAL and ELSON, 1963; COMMACK and WADE, 1965; GESTELAND, 1965, 1966), (2) potassium-activated phosphodiesterase (RNase II), which acts only in the presence of $K^{1+}$ and $Mg^{2+}$ ions and is quite distinct from RNase I (SPAHR and SCHLESSINGER, 1963; SPAHR, 1964; SINGER and TOLBERT, 1965) and (3) polynucleotide phosphorylase, which in the presence of phosphate decompose RNA (LITTAUER and KORNBERG, 1957; SEKIGUCHI and COHEN, 1963). " Debris " RNase (ANRAKU and MIZUNO, 1965), " acid-soluble " ribosomal RNase (ANDERSON and CARTER, 1965) and RNase released from spheroplasts of *E. coli* (NEU and HEPPEL, 1964a, b, c) are all very similar to RNase I. These enzymes differ, however, from RNase I in chromatographic behavior and substrate affinity (GESTELAND, 1966). RNase I is inactive as long as it remains attached to ribosomes and disruption of ribosomes by urea or EDTA (BOLTON *et al.*, 1959; Elson, 1958, 1959; SPAHR and HOLLINGWORTH, 1961) releases active RNase. With regard to the mechanism of activation by these agents, urea is known to decompose ribosomes by breaking hydrogen bonds while EDTA does so by chelating with $Mg^{2+}$ion.

Ribosomes require a bivalent cation such as $Mg^{2+}$ or $Ca^{2+}$ ion to preserve their integrity (CHAO and SCHACHMAN, 1956; CHAO, 1957). These cations interact with the negatively charged RNA phosphate groups (HAMILTON and PETERMAN, 1959; RODGERS, 1964) and probably play an essential role in maintaing the structure of the ribosomes (T'SO *et al.*, 1958). NEWTON (1955) claimed that loci combining with polymyxin in bacteria are polyphosphates. We reported that colistin interacts with nucleic acids and ribosomes (NAKAJIMA and KAWAMATA, 1965a, 1966a) to form water-insoluble complexes perhaps by binding to the phosphate radicals in the macromolecules and the formation of these complexes was counteracted by a higher concentration of $Mg^{2+}$ or $Ca^{2+}$ ion. Similar effects of these ions were observed in the activation of the RNase (NAKAJIMA and KAWAMATA, 1966b) and the release of UV-absorbing materials (NAKAJIMA and KAWAMATA, 1965b).

These observations suggest that colistin combines competitively with these divalent cations for phosphate radicals of ribosomal RNA and cosequently results in a structural change of the ribosomes. This structural

change must be essential for the activation of ribosomal RNase by colistin, although the actual mechanism of the release of the RNase from ribosomes still remains unknown.

To demonstrate the actual localization of colistin in *E. coli* cells, $^3$H-colistin A (polymyxin $E_1$) was prepared and purified by silicic acid-celite column chromatography. It was found by differential centrifugation that *in vivo* when *E. coli* cells were treated with $^3$H-colistin A and then were disintegrated mechanically, the radioactivity of $^3$H-colistin A was distributed largely in two fractions, viz., the ribosomal fraction and the cell wall fraction. The concentration of colistin in the ribosomal fraction of *E. coli* was much greater than that in the cell wall fraction. According to NEWTON (1955), polymyxin was found associated equally with (a) the cell wall fraction (sedimented at $10{,}000 \times g$, in 20 minutes) and (b) the small particle fraction (supernatant at $100{,}000 \times g$, for 15 minutes) of *Ps. aeruginosa*. The discrepancy of his results with ours might be based upon the differences of bacterial species and the techniques employed.

The mode of binding of colistin with ribosomes of *E. coli* was further investigated by *in vitro* dialysis experiments and colistin was found to combine with both 30*S* and 50*S* ribosomal particles but not with 70*S* ribosomes, which indicates that the binding of colistin with ribosomes was extremely dependent on $Mg^{2+}$ion unlike that of tetracycline, which binds preferentially with 30*S* and 70*S* ribosomes (CONNAMACHER and MANDEL, 1965). This specificity of binding of colistin to different sizes of ribosome particles and the activation of ribosomal RNase will provide a useful approach to studies on the detailed structure and biological function of the ribosomes in bacteria.

The first step in the action of colistin and other polymyxin group antibiotics, therefore, should be the interaction of the antibiotic with phospholipids contained in the bacterial cell surface. The possible resulting alteration of the surface barrier would lead to the release of some important cellular substances. The penetration of the antibiotic further into the bacterial cells, however, will result in the formation of complexes with both RNA and DNA in the cells. As indicated in the results, this antibiotic is localized chiefly in the ribosome fraction. The activated RNase thus produced, therefore, will lead to the degradation not only of ribosomal RNA but also of other types of RNA. All the changes described above caused by the antibiotic in the cells are direct causes of bacteria death and the mechanism of the action of the antibiotic can be explained as the consequence of the over all effects of the reactions.

To verify this assumption it would be useful to do experiments with mutant bacteria which have no ribosomal RNase. Experiments with colistin resistant *E. coli* would also provide evidence for this assumption if it could be demonstrated that there is a difference in the attitude of the ribosomes or the cell surface to the antibiotic in resistant bacteria. Investigations on this are now in progress.

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