BIKEN JOURNAL Vol. 9, 45-50, 1966

STUDIES ON THE MECHANISM OF ACTION OF COLISTIN III. PRECIPITATION OF *ESCHERICHIA COLI* RIBOSOMES WITH COLISTIN

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(Received December 17, 1965)

SUMMARY A purified preparation of ribosomes of *Escherichia coli* strain B could be precipitated with colistin and other polymyxin group antibiotics. The precipitation was counteracted by divalent cations. Maximum precipitation was observed at pH 8.3. No significant difference in the extent of precipitation at 4°C and at 37°C was observed. The biological significance of this precipitation is discussed in relation to the mechanism of polymyxin group antibiotics.

INTRODUCTION

During investigations on the mechanism of action of colistin (polymyxin E), evidence was obtained that acid soluble ribocompounds are released by it from *E. coli* cells into the medium (NAKAJIMA and KAWAMATA, 1965 b). It was also suggested that depolymerization of cellular ribonucleic acid (RNA) occurred with colistin (NAKAJIMA and KAWAMATA, 1965 b). Thus, the effect of colistin on *E. coli* ribosomes was studied. In this paper the precipitation of *E. coli* ribosomes with colistin and related peptide antibiotics are presented.

MATERIALS AND METHODS

1. *Organism*

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

2. *Antibiotics*

The preparation of colistin and other related antibiotics used are as described previously (NAKAJIMA and KAWAMATA, 1965 a). Streptomycin sulfate was a product of Kaken Chemical Co., Ltd. Tokyo. and dihydrostreptomycin sulfate was from Kyowa Hakko Kogyo Co., Ltd. Tokyo.

3. *Preparation of ribosomes*

E. coli strain B was grown at 37°C in flasks, containing 100 ml of broth (pH 7.2), using a rotary shaker operated at 130 rpm. Cultures were grown to the begining of the stationary phase (about 5 hours incubation). Then the bacterial cells were collected by centrifugation at 4,000 rpm (2,000 $\times g$) at 2°C for 20 minutes and the precipitated cells were washed once with cold 10^{-2} M tris (hydroxymethyl) aminomethane (tris) containing 10^{-2} M Mg acetate (pH 7.4) and centrifuged again at 4,000 rpm (2,000 $\times g$) for 20 minutes. The washed cell pellet was stored in a deep freezer at −20°C before use. Cells (7 g, wet weight) were ground (4°C) with 20 g of alumina (Wako Pure Chemical Co., Ltd.) for 5 to 8 minutes. The mixture was extracted with 3 volumes of 10^{-2} M tris buffer (pH 7.4) containing 10^{-2} M Mg acetate. After the addition of 10 μg/ml of deoxyribonuclease

(DNase) (once crystallized, Worthington Biochemical Co.) the extract was centrifuged at 3,000 rpm (1,250 $\times g$) at 2°C for 5 minutes. The precipitate was re-extracted with 10^{-2} M tris containing 10^{-2} M Mg acetate (pH 7.4) as previously. The supernatant fractions from the two centrifugations were combined and further centrifuged twice at 7,000 rpm (6,500 $\times g$) at 2°C for 10 minutes. The supernatant fraction thus obtained was centrifuged at 105,000 $\times g$ (40,000 rpm) at 2°C for 120 minutes and the precipitated ribosomal fraction was resuspended in 20 to 30 ml of buffer (10^{-2} M tris containing 10^{-2} M Mg acetate pH 7.4 using a tephlon hand homogenizer.

Further purification of the ribosomal fraction was achieved by two repetitions of the centrifugation procedure, first at 8,000 rpm (8,500 $\times g$) for 10 minutes and then at 40,000 rpm (105,000 $\times g$) for 120 minutes.

The purified sample of ribosomal suspension in tris containing 10^{-2} M Mg acetate (pH 7.4) was stored in crushed ice in an ice box. Before experiments a portion of the suspension was dialyzed three times for 12 hours each time at 4°C in cellophane tubing against tris (pH 7.4) containing 10^{-4} M Mg acetate.

4. *Estimation of precipitation of E. coli ribosomes with colistin*

To a 1 ml portion of dialyzed sample of ribosomal suspension in tris (pH 7.4) containing 10^{-4} M Mg acetate which had an optical density of 5.0 at 260 mμ, 0.2 ml of an aqueous solution of colistin or another antibiotic of the desired concentration was added and 0.8 ml of 10^{-2} M tris (pH 7.4) to give a final volume of 2.0 ml. Then the mixture was kept at 4°C for 30 minutes in a test tube. The optical density of the specimen at 420 mμ was measured with a Hitachi spectrophotometer (EPU-2).

5. *Determination of ribonucleic acid (RNA) and protein in ribosome preparations*

The amount of RNA in ribosomal suspensions was determined by the method of MEJBAUM (1939) and protein was determined by the method of LOWRY *et al.* (1951).

RESULTS

1. *Sucrose density gradient analysis of the purified sample of E. coli ribosomes*

The purified sample of ribosomes from *E. coli* B was prepared by the method described above. Then 0.2 ml of this purified ribosomal suspension, with an optical density of 30 at 260 mμ was layered on the top of 4.4 ml of a sucrose gradient (exponentially 5–20 per cent) containing 10^{-2} M tris (pH 7.4) and 10^{-4}M Mg acetate in a Hitachi RPS-40 swinging bucket tube (Beckman cellulose nitrate tube). The tube was centrifuged at 37,000 rpm for 90 minutes at 2°C. The bottom of the tube was then punctured with a needle for blood transfusion (1 mm diameter). Fractions of seven drops each were taken to measure the optical density at 260 mμ (7 drops) in 3 ml of distilled water.

The purified preparation gave two components corresponding to 50 *s* and 30 *s* (Fig. 1).

2. *Precipitation of ribosomes with colistin*

The precipitation of ribosomes with colistin was investigated by the method described

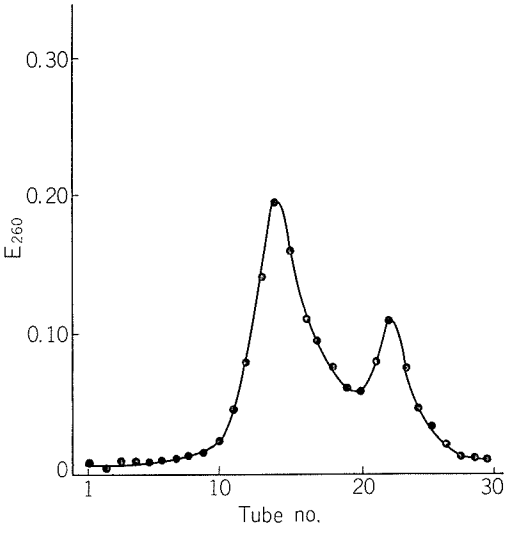

FIGURE 1 Sedimentation analysis of ribosomes from *Escherichia coli* B.
The ribosome preparation was centrifuged on a sucrose gradient (5–20%) containing 10^{-2} M tris (pH 7.4) and 10^{-4} M Mg acetate at 37,000 rpm, for 90 minutes at 2°C. The method of analysis is described in the text.

TABLE 1 *Precipitation of E. coli ribosomes with polymyxin group antibiotics*

Polymyxins*	Polymyxin-ribosome complex (E_{420})
Polymyxin B	0.424
Polymyxin B_1	0.402
Polymyxin B_2	0.449
Polymyxin D_1	0.436
Colistin A (Polymyxin E_1)	0.405
Colistin B (Polymyxin E_2)	0.448
Colistin sulfate	0.410
Deacylcolistin	0.368
Streptomycin**	0.120
Dihydrostreptomycin**	0.082
Bacitracin**	0.00

* 100 μg/ml ** 500 μg/ml
Polymyxin group antibiotics (100 μg/ml) and other basic antibiotics (500 μg/ml) were incubated with *E. coli* ribosomes (E_{260}=2.5) at 4°C for 30 minutes. The resulting turbidity was measured at 420 mμ.

above. As illustrated in Table 1, 100 μg/ml of colistin and other polymyxin group antibiotics including deacylcolistin resulted in the precipitation of the same amount of ribosomes.

Streptomycin and dihydrostreptomycin, however, caused precipitation only when 5 times or more of the amount of colistin was used, and no precipitation was observed with bacitracin.

Next precipitation was carried out with amount of colistin corresponding to a final range of concentrations of 0 to 500 μg per ml. After completion of the precipitation, each specimen of suspension was centrifuged at 3,000 rpm for 15 minutes and the supernatant fraction was analyzed for RNA and protein.

The results (Fig. 2) indicate that 92.3 per cent of the RNA and 97.8 per cent of the protein were precipitated at a concentration of 100 μg/ml of colistin, provided magnesium was not added to the mixture. When ribosomal suspensions were mixed with various amounts of colistin in the presence of 10^{-2}M $MgCl_2$, a higher concentration of colistin (300 μg/ml) was required for complete precipitaton of

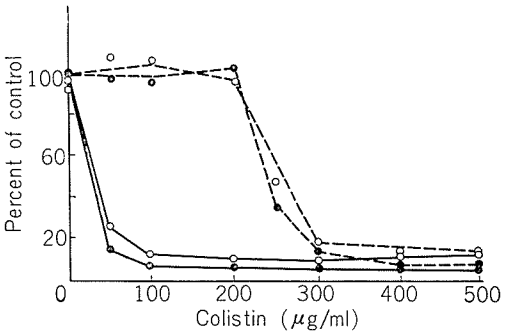

FIGURE 2 Ribonucleic acid and protein remaining in the supernatant fraction after centrifugation (3,000 rpm, 15 minutes) of the insoluble complex of *E. coli* ribosomes with various concentrations of colistin in tris buffer (pH 7.4).
(●) Ribonucleic acid, (○) protein
Solid line : No added magnesium
Broken line : 0.01 M $MgCl_2$ added
An aliquot (2 ml) of ribosome-colistin suspension was acidified by addition of cold perchloric acid (PCA) to a final concentration of 0.5 N. The acid insoluble fraction was suspended in 0.5 N PCA and heated at 90°C for 15 minutes. After centrifugation, the supernatant fraction was analyzed for RNA by Mejbaum's method (1939). The hot PCA 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).

the ribosomes.

In the preceding paper (NAKAJIMA and KAWAMATA, 1965 a) the effect of cations on the formation of a complex between colistin and nucleic acid was reported. In the present experiment Mg^{2+} was also found to have an inhibitory effect on the precipitation.

Therefore, studies were made on the effects of neutral salts on the precipitation. Samples of 0.2 ml of an aqueous solution of 1 mg/ml of colistin were mixed with 0.1 ml of 0.2 M salt solution and 0.7 ml of 10^{-2}M tris (pH 7.4) buffer in a final volume of 2 ml mixtures were stood at 4°C for 30 minutes and then the resulting turbidities were measured. As indicated in Table 2, Mg^{2+} was the most potent inhibitor of the precipitation, followed by Ca^{2+}. Monovalent cations such as Li^{1+}, K^{1+}, Na^{1+} had no effect but sodium citrate also

TABLE 2 *Effects of neutral salts on the formation of a colistin-ribosome complex*

Salt (0.01 M)	Colistin-ribosome complex (E_{420})
H_2O	0.360
NaCl	0.358
KCl	0.306
LiCl	0.350
$MgCl_2$	0.032
$MgSO_4$	0.036
$CaCl_2$	0.150
Na-citrate	0.040

100 μg/ml of colistin and *E. coli* ribosomes ($E_{260}=2.5$) were incubated at 4°C for 30 minutes in the presence of 0.01 M neutral salts.

antagonized the precipitation.

3. *Effect of pH on the precipitation*

To investigate the effect of pH on the precipitation, 0.9 ml samples of ribosomal suspension in tris (pH 7.4) containing 10^{-4} M Mg acetate and 0.1 ml of an aqueous solution of 10 mg/ml of colistin were mixed with 1 ml of 0.02 M buffer solutions of various pH values in a total volume of 2 ml and kept at 4°C for 30 minutes. Then the resulting turbidity was measured. As seen in Fig. 3, the maximum turbidity was obtained at pH 8.3.

4. *Effect of temperature on precipitation*

To investigate the effect of temperature on precipitation 1 ml of the ribosomal suspension in 10^{-2} M tris (pH 7.4) containing 10^{-4} M Mg acetate was mixed with aqueous solutions of colistin at final concentrations of colistin ranging from 0 to 500 μg/ml and the total volume was adjusted to 2 ml with 10^{-2} M tris (pH 7.4) containing 10^{-4} M Mg acetate. The mixtures were kept for 30 minutes in a water bath either at 4°C or 37°C. Then the resulting turbidity was measured. As seen in Fig. 4 there was no great difference in the precipitations at the two temperatures although slighty higher values were obtained at 37°C.

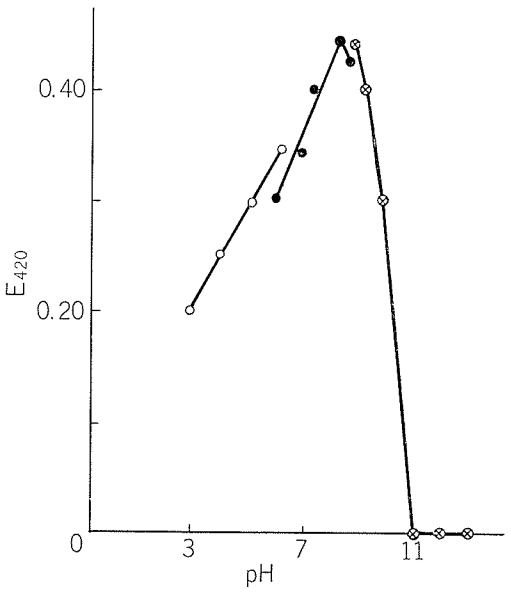

FIGURE 3 Influence of pH on the precipitation of *E. coli* ribosomes with colistin.
500 μg/ml of colistin and *E. coli* ribosomes ($E_{260}=2.5$) were incubated at 4°C for 30 minutes in the presence of 0.01 M buffer.
○——○ Glycine-HCl buffer
⊗——⊗ Veronal-NaOH buffer
●——● Sörensen-phosphate buffer

DISCUSSION

Precipitation of ribosomes with various basic antibiotics such as streptomycin or dihydrostreptomycin can be attributed to the polyanionic character of the ribosomes (MOSKOWITZ 1963).

Polymyxins are also basic peptide antibiotics with γ-primary amino radicals in the molecule which contribute to the binding with the phosphate radicals in DNA, RNA and phospholipids. Ribosomes are known to be the site of protein synthesis in the cell, conjugating with messenger RNA (WATSON, 1963). Precipitation of ribosomes with polymyxins, therefore, possibly brings about a disturbance in the normal function of the ribosomes as the site of protein synthesis. Indeed basic antibiotics, such as streptomycin, dihydrostreptomycin, neomycin

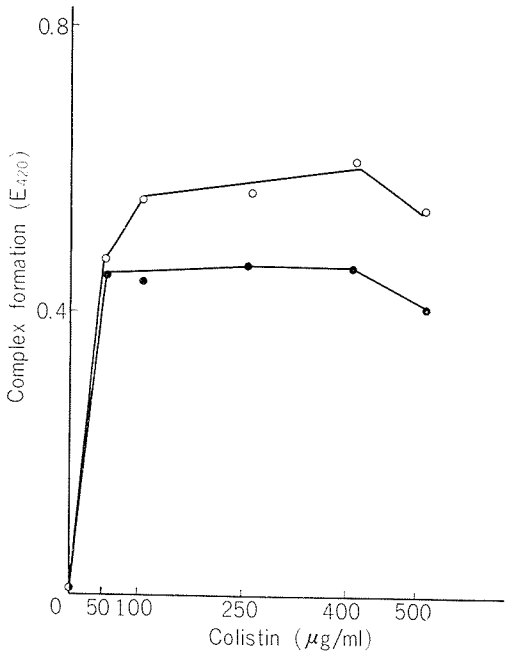

FIGURE 4 Effect of temperature on the precipitation of *E. coli* ribosomes with colistin.
E. coli ribosomes ($E_{260}=2.5$) and various concentrations of colistin were incubated for 30 minutes at 4°C (●——●) and at 37°C (○——○).

and kanamycin were found to cause extensive and specific alterations in the coding of synthetic polynucleotide *in vitro* (DAVIES *et al.* 1964).

Similar effects of polymyxins in a cell free system for polyphenylalanine synthesis are now under investigation.

On the other hand it has been reported that self-digestion of ribosomes occurs with 0.01 M ethylene diaminetetraacetate (EDTA) or 4 M urea (BOLTON, 1964). Recently we have also found that the materials released from *E. coli* cells with colistin are acid soluble, low molecular ribocompounds. In view of the fact that acid soluble ribocompounds are released by the action of colistin (MOHAN *et al.*, 1963, NAKAJIMA and KAWAMATA, 1965 b) it is probable that the structure and function of ribosomes are greatly altered when *E. coli* ribosomes are precipitated with colistin. RNase activity was also demonstrated in the ribosomal suspension after colistin treatment (NAKAJIMA and KAWAMATA, to be published). The relationships between the formation of complexes of polymyxin group antibiotics with DNA, RNA and phospholipids and the release of acid soluble, low molecular ribocompounds and the precipitation of ribosomes with these antibiotics presented in this paper await further investigations.

ACKNOWLEDGEMENTS

The authors are indebted to Prof. T. SUZUKI (Institute for Protein Research, Osaka University) for helpful discussion and for kindly providing us with various polymyxins.

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