

## BINDING OF ERYTHROMYCIN TO *ESCHERICHIA COLI* RIBOSOMES

HIROSHI TERAOKA

Shionogi Research Laboratory, Shionogi & Co., Ltd.,  
Fukushima-ku, Osaka, Japan

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This paper concerns a precise study on the nature of the binding of erythromycin to *Escherichia coli* (Q13) ribosomes. (1) An equimolar amount of erythromycin bound to 50S ribosomal subunits at extremely low concentrations and this binding was not affected by the addition of 30S ribosomal subunits. (2) The binding of erythromycin to ribosomes was not significantly influenced by decreasing the  $Mg^{++}$  concentration to  $10^{-4}M$ , although it was dependent upon the concentration of  $K^+$  or  $NH_4^+$  as previously reported. Other components required for protein synthesis showed little effect on the binding. (3) Washing the ribosomes with  $NH_4Cl$  did not influence their erythromycin-binding capacity or their dependence on  $K^+$  or  $NH_4^+$ . The addition of  $Li^+$  caused a remarkable inhibition of the antibiotic binding and this inhibition was reversed by the concomitant addition of  $NH_4^+$ . (4) The erythromycin-binding ability of ribosomes from *Bacillus subtilis*, gram-positive bacteria, was roughly the same as that of those from gram-negative *E. coli*. In contrast to bacterial ribosomes, rabbit reticulocyte ribosomes bound erythromycin hardly at all.

In the earlier papers from this laboratory it was reported that erythromycin binds to *Escherichia coli* ribosomes and that this binding resulted in a characteristic change in the ability of ribosomes for poly A dependent polylysine synthesis<sup>1,2</sup>. This was confirmed by the isolation of <sup>14</sup>C-erythromycin bound ribosomes by Sephadex column chromatography<sup>3</sup> and by adsorption on Millipore filter<sup>4</sup>. The binding of this antibiotic was also confirmed with ribosome preparations from gram-positive bacteria such as *Bacillus subtilis*<sup>5</sup> and *Staphylococcus aureus*<sup>6</sup>. TAUBMAN *et al.*<sup>5</sup> revealed that erythromycin binds exclusively to 50S ribosomal subunits of *B. subtilis*, a subunit specificity likewise observed for *S. aureus* ribosomes<sup>6</sup> and for *E. coli*<sup>7</sup>. We have previously reported the essential requirement of monovalent cations, such as  $K^+$  or  $NH_4^+$ , for the binding of erythromycin to *E. coli* ribosomes<sup>8</sup> as shown also for ribosomes from *B. subtilis*<sup>9</sup> and *S. aureus*<sup>10</sup>. Furthermore, a recent study in this laboratory suggested that the binding affinity of *E. coli* ribosomes for erythromycin depends upon the ribosome conformation which is reversibly altered with changes in the concentration of  $K^+$  or  $NH_4^+$ , the rate of the alteration being largely dependent upon temperature<sup>11</sup>.

In this report, further investigations on the binding of erythromycin to ribosomes are presented and the results are consistent with our previous notion that the ability

of ribosomes to bind erythromycin is dependent upon the ribosome conformation.

### Materials and Methods

Ribosomes were prepared according to the method of NIRENBERG<sup>12)</sup> from *E. coli* Q13, *E. coli* QE201 (erythromycin-resistant mutant derived from Q13 as described previously<sup>13)</sup>) and *B. subtilis* (PCI 219) respectively. Rabbit reticulocyte ribosomes were prepared as described in the previous paper<sup>14)</sup>: 105,000×g supernatant fraction (S-100) and tRNA were obtained from *E. coli* Q13 as described in the previous paper<sup>2)</sup>. The 30S and 50S subunits of the ribosomes were prepared by sucrose gradient centrifugation after dissociation by the addition of phosphate<sup>15)</sup>. <sup>14</sup>C-Erythromycin was prepared by short term incubation of washed mycelium of *Streptomyces erythreus* with 1-<sup>14</sup>C-propionate according to KANEDA *et al.*<sup>16)</sup> Paper chromatographic analysis of this preparation showed that approximately 87%, 4% and 9% of total radioactivity was found in the spots of erythromycin A, B and C, respectively. Antimicrobial assay employing *B. subtilis* (PCI 219) showed that the radioactive preparation which corresponded to approximately 0.61 μg of erythromycin A gave 13,000 cpm under the experimental conditions described below.

Poly A and GTP were obtained from Sigma Co. ATP was purchased from Schwarz BioResearch Inc. Phosphoenolpyruvate and pyruvate kinase were obtained from C. F. Boehringer & Soehne GmbH.

The binding of erythromycin to ribosomes was measured by the adsorption of the binding-complex on Millipore filter<sup>4)</sup>. The reaction mixture contained ribosomes (2.8~4.0 A<sub>260</sub> units as indicated in respective legends), 2,700 cpm of <sup>14</sup>C-erythromycin (175 μμmoles), 50 mM Tris-HCl (pH 7.8), 16 mM magnesium acetate and 70 mM KCl in a final volume of 125 μl. The reaction mixture was incubated at 37°C for 15 minutes unless otherwise specified. After incubation, the reaction tube was immediately immersed in an ice bath and the reaction mixture diluted with 5 ml of cold buffer containing 0.025 M Tris-HCl (pH 7.8), 0.06 M KCl and 0.02 M magnesium acetate. This diluted mixture was poured onto a Millipore filter (HA, 0.45 μ, pre-washed with the same buffer) and the filter was washed 5 times with 3-ml portions of the cold buffer. No detectable amount of erythromycin was liberated from the erythromycin-ribosome complex during the washing process. The bound <sup>14</sup>C-erythromycin on the filter was counted with a Beckman liquid scintillation counting system using conventional toluene scintillation solution. Under these assay conditions, adsorption of the ribosomes on the Millipore filter was not affected by addition or omission of the various components in the reaction mixture described above.

### Results

#### (1) Stoichiometric Binding of Erythromycin to *E. coli* Ribosomes

As shown in Fig. 1, a linear relationship was observed between the amount of ribosomes and the total count of <sup>14</sup>C-erythromycin bound to the ribosomes at sufficient concentration of <sup>14</sup>C-erythromycin.

As shown in Table 1, the specific activity of the <sup>14</sup>C-erythromycin-ribosome complex decreased proportionally on making increasing additions of nonlabeled erythromycin A to the incubation mixture containing fixed amounts of ribosomes and <sup>14</sup>C-erythromycin. On the basis of these observation, it may safely be assumed that the same amount of erythromycin is bound to a fixed quantity of ribosomes throughout the range of erythromycin concentration employed, and so the specific activity of the original preparation of <sup>14</sup>C-erythromycin could be calculated from combinations of the experimental data on the radioactivities bound to the ribosomes and the amounts of nonlabeled erythromycin added. The values obtained from the possible combina-

Fig. 1. The binding of erythromycin to ribosomes.

Incubation mixture contained the components described under "Materials and Methods" except that indicated amounts of ribosomes and 875  $\mu\text{moles}$  of  $^{14}\text{C}$ -erythromycin (13,500 cpm) were added.

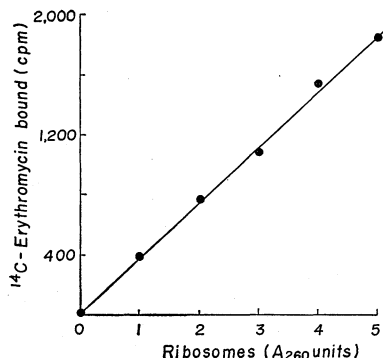
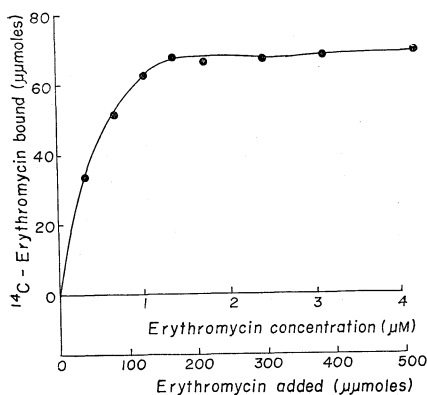


Fig. 2. Effect of erythromycin concentration on the binding of erythromycin to ribosomes.

Incubation mixture contained 3.0  $A_{260}$  units of ribosomes and the components described under "Materials and Methods" except that indicated amounts of  $^{14}\text{C}$ -erythromycin were added.



was calculated using a specific activity of 2,700 cpm per 175  $\mu\text{moles}$ .

As shown in Fig. 2, the amount of erythromycin bound to a fixed amount of ribosomes reached a maximum at a very low concentration of antibiotic and no detectable increase was observed with further increment in the antibiotic concentration. If one  $A_{260}$  unit of 70S ribosomes of *E. coli* corresponds to approximately 25  $\mu\text{moles}$  as shown by TISSIERES *et al.*<sup>15)</sup>, then the result obtained in this experiment is consistent with the previous observation that approximately one molecule of erythromycin binds to a ribosome from *B. subtilis*<sup>17)</sup> or *S. aureus*<sup>9)</sup> at low concentration of the antibiotic.

As is evident from Fig. 2, the addition of 75  $\mu\text{moles}$  erythromycin, which is approximate equimolar to ribosomes in the reaction mixture, caused the formation of as much as 50  $\mu\text{moles}$  erythromycin-ribosome complex. This fact indicates that *E. coli* ribosomes have very high affinity for the antibiotic.

Table 1. Effect of the addition of non-radioactive erythromycin A on the specific activity of  $^{14}\text{C}$ -erythromycin-ribosome complex

$^{12}\text{C}$ -Erythromycin added ( $\mu\text{moles}$ )	$^{14}\text{C}$ -Erythromycin bound (cpm)
0	950
100	607
200	435
400	293
800	166

Incubation mixture contained 2.8  $A_{260}$  units of ribosomes and indicated amounts of  $^{12}\text{C}$ -erythromycin A in addition to the components described under "Materials and Methods".

Table 2. Effect of 30S subunits on the binding of erythromycin to 50S subunits

Ribosomal subunits added ( $A_{260}$ unit)	$^{14}\text{C}$ -Erythromycin bound	
	cpm	$\mu\text{mole}$
30 S (1.0)	1	0.1
50 S (1.0)	449	29.1
50 S (2.0)	991	64.2
30 S (1.0)+50 S (1.0)	499	32.4
30 S (1.0)+50 S (2.0)	998	64.8

Incubation mixture contained the components described under "Materials and Methods" except that indicated amounts of 30S and 50S ribosomal subunits were used in place of 70S ribosomes.

tions of experimental data were very close to each other. Their average was 2,700 cpm per 175  $\mu\text{moles}$ , a value which agreed very well with that obtained from microbiological assay in which 2,700 cpm of  $^{14}\text{C}$ -erythromycin corresponded to 166  $\mu\text{moles}$  of erythromycin A. Throughout this paper the amount of  $^{14}\text{C}$ -erythromycin

Table 2 shows that a 50S ribosomal subunit could bind one molecule of erythromycin as has been observed for 70S ribosomes. The 30S subunits bound no detectable amount of the antibiotic and the addition of 30S subunits did not affect the binding of erythromycin to 50S subunits.

(2) Effects of the Components Required for Polypeptide Synthesis  
on the Binding of Erythromycin to Ribosomes

In an attempt to detect the effects of components in poly A directed polylysine synthesizing system on the binding of erythromycin to ribosomes, S-100, poly A, ATP, phosphoenolpyruvate, pyruvate kinase, GTP, and 2-mercaptoethanol were added to the binding reaction mixture. As shown in Table 3, the addition of these components showed no detectable effect on this binding.

(3) The Time Course of the  
Binding of Erythromycin  
to Ribosomes

The rate of binding of erythromycin was studied at three different temperatures (Fig. 3). As expected from the previous paper<sup>11)</sup>, at 37°C the maximum binding was observed within approximately 5 minutes of incubation. At 25°C, however, much longer incubation was needed for the maximum binding of the antibiotic. At 0°C, a significant but small amount of the antibiotic was bound to ribosomes even after 15 minutes incubation.

(4) Influence of pH

The amount of erythromycin bound to the ribosomes remained essentially constant at pHs between 7.0 and 8.0.

(5) Effect of  $Mg^{++}$  Concentration

The ability of ribosomes to bind erythromycin was not significantly altered by decreasing the concentration of  $Mg^{++}$  to  $10^{-4}M$  or by the addition of phosphate, which induces dissociation of 70S ribosomes into 50S and 30S subunits.

(6) Effect of Monovalent Cations

In the previous paper<sup>11)</sup>, we suggested that the binding affinity of *E. coli* ribosomes

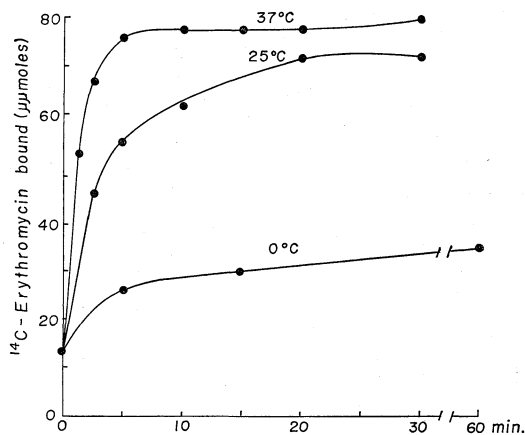
Table 3. Effect of components of poly A directed polylysine synthesizing system on the binding of erythromycin to ribosomes

Addition of other components	<sup>14</sup> C-Erythromycin bound (cpm)
None	1,005
tRNA	1,032
S-100	980
Poly A	1,015
ATP, GTP, Phosphoenolpyruvate and pyruvate kinase	1,029
2-Mercaptoethanol	1,001
Each component added together	975

To the reaction mixture (containing 2.8  $A_{260}$  units of ribosomes) described under "Materials and Methods", tRNA (45  $\mu g$ ), S-100 (80  $\mu g$  protein), poly A (10  $\mu g$ ), ATP (0.375  $\mu mole$ ), GTP (0.0375  $\mu mole$ ), phosphoenolpyruvate (1.25  $\mu mole$ ), pyruvate kinase (3.75  $\mu g$ ) and 2-mercaptoethanol (1.25  $\mu mole$ ) were added as indicated.

Fig. 3. Time course of the erythromycin binding to ribosomes.

The reactions were carried out as described under "Materials and Methods" except that incubations were carried out varying periods of time and at different temperatures as indicated. Incubation mixture contained 3.0  $A_{260}$  units of ribosomes.



for erythromycin depends upon the ribosome conformation which is reversibly altered with changes in the concentration of  $K^+$  or  $NH_4^+$ . As expected from the previous paper, the binding of erythromycin was strongly dependent upon the concentration of  $K^+$  or  $NH_4^+$ . As shown in Fig. 4, the amounts of erythromycin bound to a fixed amount of ribosomes increased as the concentration of  $K^+$  was raised, reaching a maximum at approximately  $0.05 M K^+$  in the presence of  $1.4 \times 10^{-6} M$  erythromycin.  $NH_4^+$  was a little more efficient than  $K^+$ , the maximum binding of erythromycin being observed at approximately  $0.03 M NH_4^+$  under the same conditions. At  $0.02 M$

Fig. 4. Effect of monovalent cations on the binding of erythromycin to ribosomes.

The reactions were carried out at varying concentrations of  $K^+$ ,  $NH_4^+$ ,  $Na^+$  or  $Li^+$  in place of  $0.07 M KCl$  by the use of  $2.8 A_{260}$  units of ribosomes. Other conditions were the same as described under "Materials and Methods".

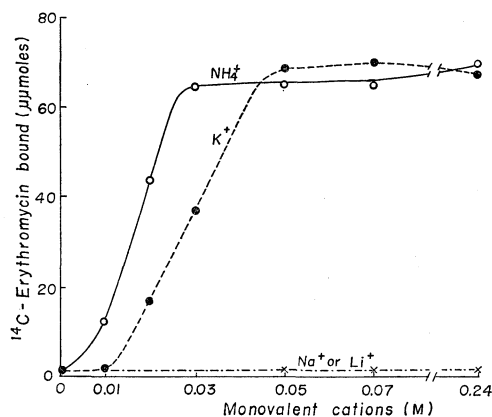
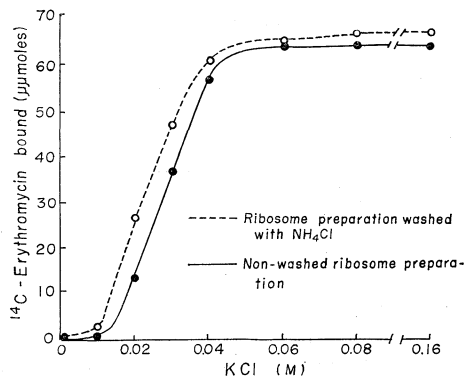


Fig. 5. Effect of  $NH_4Cl$  washing on the erythromycin binding ability of ribosomes.

Ribosomes were preincubated at  $37^\circ C$  for 15 minutes in the buffer containing  $0.24 M NH_4Cl$  with other components of usual reaction mixture, then sedimented by centrifugation (at  $105,000 \times g$  for 2 hours) through the buffer containing  $0.24 M NH_4Cl$  at  $25^\circ C$ . The sedimented ribosomes were resuspended in tris-magnesium buffer containing  $0.06 M KCl$ . The erythromycin binding abilities of these  $NH_4Cl$ -washed ribosomes and non-treated control ( $2.8 A_{260}$  units of ribosomes respectively) were tested at various concentrations of  $KCl$  as described in Fig. 4.



$KCl$  where only a little binding of erythromycin was observed, the binding of erythromycin to ribosomes increased with the erythromycin concentration (Table 4). This fact supports the previous suggestion that

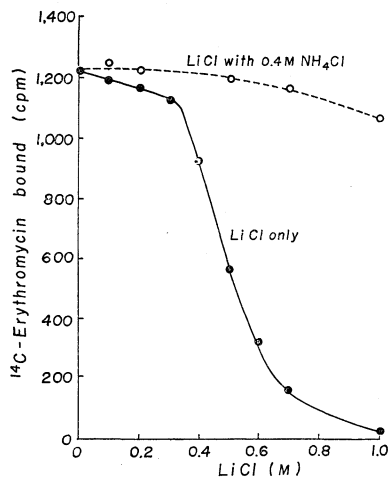
Table 4. Effect of erythromycin concentrations on the binding at low concentration of  $KCl$

$^{14}C$ -Erythromycin concentration ( $\mu M$ )	$^{14}C$ -Erythromycin bound	
	(cpm)	( $\mu\mu mole$ )
0.66	93	6.0
1.33	174	11.2
3.3	366	23.7
6.6	453	29.4
13.3	661	42.9

Incubation mixture contained  $2.8 A_{260}$  units of ribosomes and the components described under "Materials and Methods" except that the concentration of  $KCl$  was decreased to  $0.02 M$  and various amounts of  $^{14}C$ -erythromycin were added as indicated.

Fig. 6. Inhibitory action of  $Li^+$  on the binding of erythromycin and reversal of the inhibition by the concomitant addition of  $NH_4^+$ .

Incubation mixture contained indicated concentrations of  $LiCl$  with or without  $0.4 M NH_4Cl$  in addition to the components (containing  $3.2 A_{260}$  units of ribosomes) described under "Materials and Methods".



$K^+$  or  $NH_4^+$  induce a change in the affinity of ribosomes for the antibiotic.

To exclude the possibility that a factor which affects the affinity of ribosomes for erythromycin may be released from ribosomes at higher concentration of  $K^+$  or  $NH_4^+$ , ribosomes were sedimented by ultracentrifugation after the treatment with  $NH_4Cl$  solution. The erythromycin binding capacity of these  $NH_4Cl$ -washed ribosomes was compared with that of non-treated ribosomes (Fig. 5). No detectable difference was observed between these two preparations in the final extent of binding and dependence on  $K^+$  concentration.

As shown in Fig. 4,  $Na^+$  or  $Li^+$  could not replace  $K^+$  or  $NH_4^+$  in enabling the binding of erythromycin to ribosomes, as was also the case in cell-free protein synthesis.

As shown in Fig. 6, the addition of  $LiCl$  showed a remarkable inhibition on the binding of erythromycin in the routine assay medium (0.07 M  $KCl$ ). As indicated by the dotted line, however, this inhibition was almost completely reversed by the concomitant addition of 0.4 M  $NH_4Cl$ .

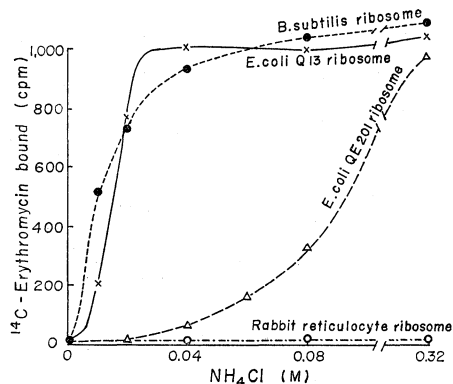
#### (7) Binding of Erythromycin to Ribosomes from *B. subtilis* and Rabbit Reticulocyte

It has been shown that *B. subtilis*, a strain of gram-positive bacteria, is hundreds times more sensitive to erythromycin than a gram-negative *E. coli*, and that the antibiotic shows little toxicity to mammals<sup>19</sup>. It was therefore of interest to compare the erythromycin binding affinity of ribosomes from these different sources. By changing the concentration of  $K^+$  or  $NH_4^+$  at a fixed concentration of erythromycin, the ability of these ribosomes to bind erythromycin was tested as described in the previous paper<sup>18</sup>.

As shown in Fig. 7, the erythromycin binding affinity of ribosomes from *B. subtilis* was roughly the same as those from *E. coli*, while no detectable amount of the antibiotic was bound to rabbit reticulocyte ribosomes even at a high  $NH_4^+$  concentration where the maximum binding of erythromycin was observed with ribosomes from an erythromycin resistant *E. coli* mutant (QE201).

Fig. 7. Comparison of the erythromycin binding ability of ribosomes from *E. coli*, *B. subtilis* and rabbit reticulocyte.

Incubation mixture contained 3.0, 3.0, 4.0 and 2.8  $A_{260}$  units of ribosomes from *E. coli* Q13, *E. coli* QE201, *B. subtilis* (PCI-219) and rabbit reticulocyte respectively. The erythromycin binding abilities of these ribosomes were tested at various concentrations of  $NH_4Cl$  as described in Fig. 4.



#### Discussion

As reported previously, addition of erythromycin to the polylysine synthesizing system of *E. coli* cell-free extract induced complete inhibition of the synthesis of highly polymerized polylysine and simultaneous accumulation of di-, tri-, and tetralysine<sup>2</sup>. This characteristic accumulation of small lysine peptides has been demonstrated to be due to

the binding of erythromycin to ribosomes<sup>1,2</sup>). Preliminary studies indicated that the binding of erythromycin to ribosomes was not interfered with by the concomitant addition of chloramphenicol and that the non-enzymatic binding of aminoacyl-tRNA to ribosomes was not inhibited by the binding of erythromycin<sup>4</sup>). It was further shown that lincomycin, which like chloramphenicol has been considered to act on 50S ribosomal subunits, does not inhibit the binding of erythromycin to ribosomes<sup>20</sup>). Much larger amounts of chloramphenicol and lincomycin were required to inhibit the poly A directed polylysine synthesis in *E. coli* cell-free system than that required for erythromycin and the accumulation of large amounts of small lysine peptides was not observed even when excessive amounts of chloramphenicol or lincomycin were added to the *E. coli* cell-free system of polylysine synthesis<sup>20</sup>). Considered together with the results in Fig. 2, these facts suggest that erythromycin, in comparison with chloramphenicol or lincomycin, binds much more tightly to a specific site of *E. coli* ribosomes and induces highly specific changes in the abilities of the ribosomes.

It was suggested in a previous paper that the affinity of *E. coli* ribosomes for erythromycin depends upon the ribosome conformation, which is reversibly altered with changes in the concentration of  $K^+$  or  $NH_4^+$ <sup>11</sup>). As shown in Fig. 5,  $NH_4Cl$ -washing of ribosomes did not alter their ability to bind erythromycin. Since LiCl has been considered to induce alterations of ribosome structure<sup>21</sup>), the inhibition of the erythromycin binding caused by LiCl may be due to alteration of the ribosome structure. Our previous report suggested that the presence of  $K^+$  or  $NH_4^+$  may not always be necessary for the binding of erythromycin but that these monovalent cations may have an important role in the regulation of the conformational changes of ribosomes<sup>11</sup>). Therefore, the observation that the inhibition by LiCl of the binding of erythromycin to ribosomes was reversed by the concomitant addition of  $NH_4Cl$  may indicate that  $NH_4^+$  prevents the alteration of ribosome structure by LiCl which reduces the erythromycin binding ability of ribosomes. It is interesting to note that  $K^+$  or  $NH_4^+$ , indispensable for polypeptide synthesis, was also essential for the binding of erythromycin to ribosomes, while  $Na^+$  and  $Li^+$  were inactive in the binding as they are in polypeptide synthesis. Erythromycin bound exclusively to 50S ribosomal subunits and this binding was not affected at all by the presence of 30S subunits. This may indicate that 30S ribosomal subunits do not have a significant effect on the conformational change of 50S subunits.

Erythromycin is highly active against gram-positive bacteria compared with gram-negative bacteria. As shown in Fig. 7, however, the ribosomes prepared from these two bacterial groups seem to have roughly the same ability to bind erythromycin. Hence the difference in susceptibility to erythromycin of gram-positive and gram-negative bacteria may be due to a difference in some factor(s) other than ribosomes, possibly in the permeability of their cells to the antibiotic. It is also evident in Fig. 7 that erythromycin is scarcely bound to reticulocyte ribosomes even under conditions where the maximum amount of the antibiotic binds to the ribosomes from a highly erythromycin resistant mutant of *E. coli*. This result is in harmony with the fact that erythromycin exhibits very low toxicity to mammals, suggesting that there are great differences in the conformation of ribosomes of bacteria and mammals.

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