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INTERACTION OF KANAMYCIN AND RELATED ANTIBIOTICS WITH THE LARGE SUBUNIT OF RIBOSOMES AND THE INHIBITION OF TRANSLOCATION

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SUMMARY

The binding of [^{3}H]kanamycin to E. coli ribosomes and ribosomal subunits was studied by equilibrium dialysis and Millipore filter methods. The 70S ribosome bound ca. two molecules up to the antibiotic concentration of 10 μM, and more at higher concentrations. Each ribosomal subunit was observed to possess one major binding site, and the affinity of the small ribosomal subunit was greater than that of the large subunit. The binding of [^{3}H]kanamycin to ribosomes and ribosomal subunits was reversed by neomycin or gentamicin, but not by streptomycin and chloramphenicol. Kanamycin, neomycin and gentamicin interfered with the binding of [^{14}C] tuberactinomycin O. Translocation of N-Ac-Phe-tRNA was markedly inhibited by kanamycin, neomycin or gentamicin, but not by streptomycin.

Kanamycin, an aminoglycoside antibiotic, was reported to interact with the small subunit of ribosomes and cause codon misreading (1 - 3). In a kanamycin-resistant mutant of E. coli, the resistance was attributed to alteration of S12 (3 - 5). On ther other hand, Suzuki et al.(6) showed that kanamycin affects peptidyl transferase reaction.

We have recently found that kanamycin, neomycin, and gentamicin interact with the large subunit of E. coli ribosomes as well as with the small subunit, and inhibit translocation much more markedly than transpeptidation in an in vitro system. The results are presented in this communication.

MATERIALS AND METHODS

Kanamycin was labelled with tritium by exposure to tritium gas, following the method of Wilzbach (7), and purified by ion-exchange resin chromatography, using Amberlite IRC-50. [^{3}H]Kanamycin, used in the present experiment, gave a single spot by paper chromatography, using a solvent system (acetone : 10% CH_3COONH_4 : 10% NH_4OH = 30 : 9 : 1, v/v), and was microbiologically active. The specific activity was 6.3 Ci/mole. The preparation and characteristics of [^{14}C]tuberactinomycin O employed were described in a previous paper (8).

Washed ribosomes and ribosomal subunits were obtained from E. coli Q13 by the method described previously (8). EF-G was purified by the procedure of Arai et al. (9). N-acetyl-[^{14}C]phenylalanyl-tRNA was prepared by the technique of Haenni and Chapeville (10). The binding of [^{3}H]kanamycin or [^{14}C]tuberactinomycin O to ribosomes and ribosomal subunits was determined by equilibrium dialysis and by Millipore filter method as described previously (8).

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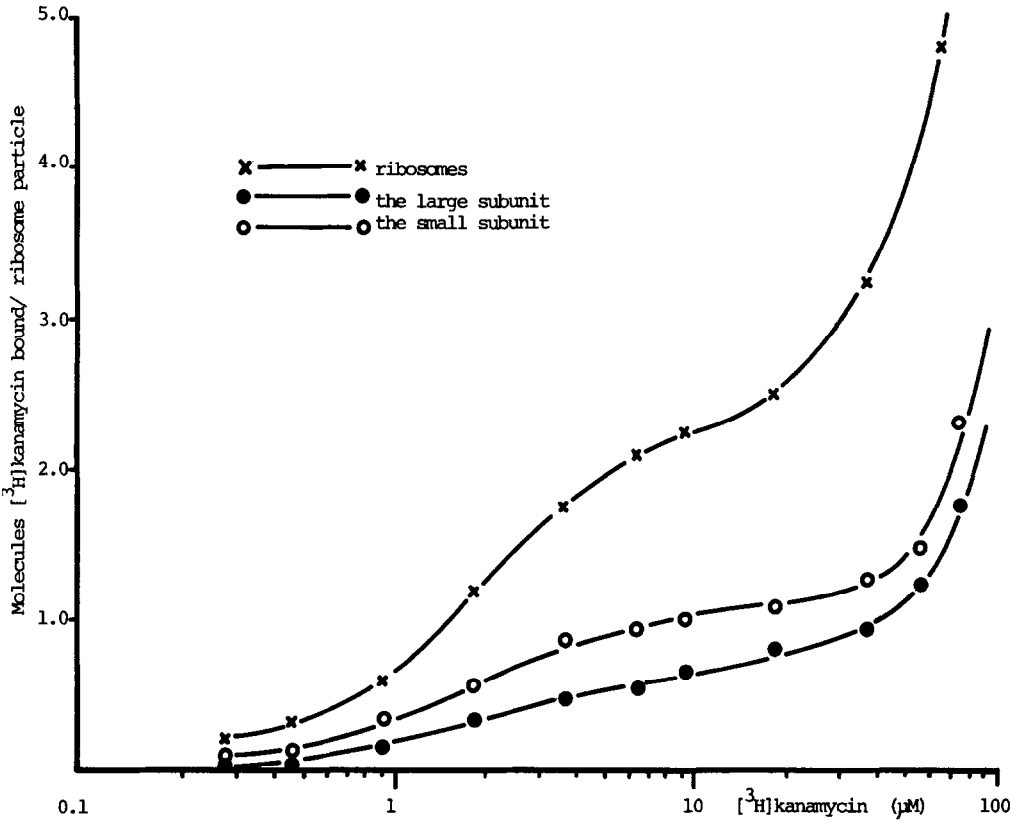

Fig. 1. The dependence on [^{3}H]kanamycin concentration for its binding to ribosomes and ribosomal subunits.

RESULTS

The interaction of kanamycin and related antibiotics with ribosomes and ribosomal subunits:

The binding of [^{3}H]kanamycin to ribosomes and ribosomal subunits of E. coli was observed by equilibrium dialysis and Millipore filter methods. The binding reached an equilibrium within 5 minutes and was relatively temperature-independent in a range of 0 to 37°C. 10 mM Mg^{2+} was required for the optimal binding (data are not shown).

The equilibrium dialysis experiments were carried out over a range of the [^{3}H]antibiotic concentrations, and the concentration dependence on [^{3}H]kanamycin for its binding is presented in Fig. 1. In the [^{3}H]antibiotic concentration range of 0.3 to 10 μM, there was a progressive increase of the binding up to 2.2 molecules per ribosome, and more kanamycin bound to the ribosome at higher antibiotic concentrations, indicating that the ribosome has more than two binding sites for the antibiotic.

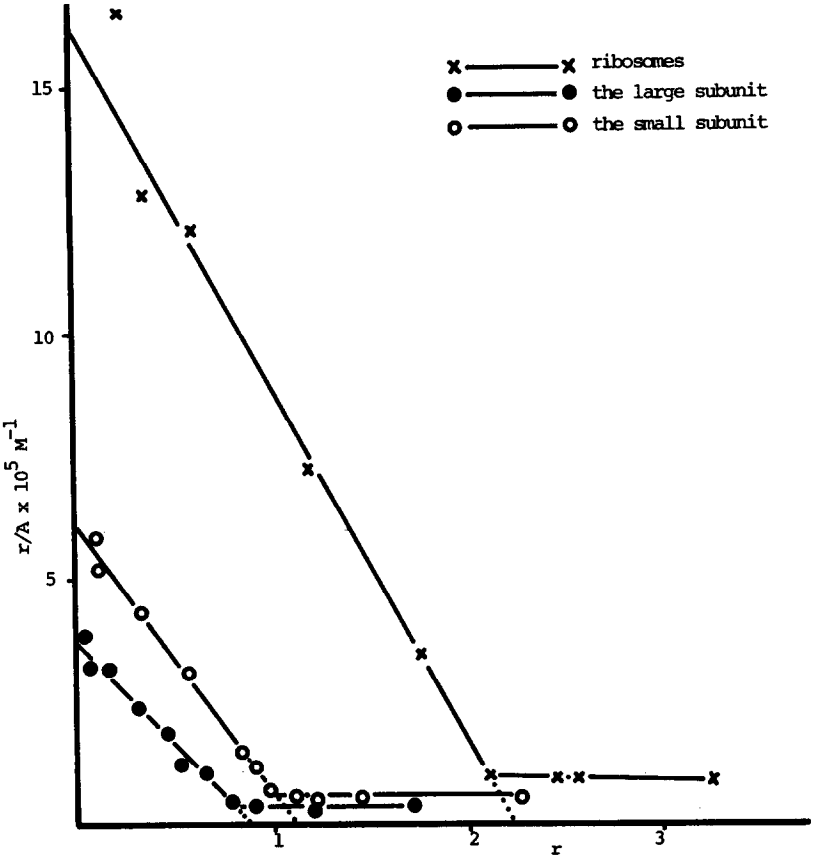

Fig. 2. Scatchard plots of equilibrium binding data for ribosomes and ribosomal subunits.

In the binding curve with the small ribosomal subunit, over [^{3}H]kanamycin concentration range from 0.3 to 8 μM the binding increased with increasing antibiotic concentrations up to a plateau level of ca. 1.0 molecule per subunit, and additional molecules were attached to the subunit over an antibiotic concentration of 40 μM. With the large ribosomal subunit, a similar tendency of the binding was observed. The binding reached a plateau of ca. 0.8 molecule per subunit at antibiotic concentrations from 8 to 40 μM, and more kanamycin bound to the subunit at concentrations above 60 μM. The results indicated that the affinity of kanamycin to the small ribosomal subunit is higher than that to the large subunit. Similar results were obtained by Millipore filter technique (data are not shown).

The Scatchard plots [11] of data for equilibrium binding of kanamycin to ribosomes and ribosomal subunits are illustrated in Fig. 2. There appeared to

Table 1. Effects of some antibiotics on the binding of [^{3}H]kanamycin to ribosomes and ribosomal subunits.

Addition of antibiotics	Ribosomes	Ribosomal subunits	
	70S	30S	50S
None	228	97	95
Kanamycin	23	10	12
Neomycin	22	9	11
Gentamicin	30	12	26
Streptomycin	207	87	90
Viomycin	125	71	57
Chloramphenicol	225	89	90

The number represents pmoles of [^{3}H]kanamycin bound to 100 pmoles of the ribosome or ribosomal subunit.

Equilibrium dialysis was performed at 4°C for 20 hours with gentle shaking by the method described previously (8). The assay mixture, in 0.1 ml, contained: 10 mM Tris-HCl, pH 7.6, 10 mM $Mg(OAc)_2$, 80 mM NH_4Cl, 6 mM 2-mercaptoethanol, 1 μM *E. coli* ribosome or ribosomal subunit, 10 μM [^{3}H]kanamycin, and 100 μM cold antibiotic. In the case of the large subunit, four times higher concentrations of radioactive and cold antibiotics were used, because of less affinity for kanamycin. In these conditions, ca. two molecules of kanamycin bound per ribosome and ca. one molecule per subunit.

be a linear relationship between r and r/A, where r is the number of molecules of bound [^{3}H]kanamycin per ribosome or subunit, and A is the molar concentration of free [^{3}H]kanamycin. The 70S ribosome seemed to possess two major binding sites with an apparent association constant of approximately 7.4×10^5 M^{-1}, and more binding sites with lower association constants. The number of binding sites on each ribosomal subunit was one with stronger affinity (association constant 4.0×10^5 M^{-1} for the large subunit and 5.5×10^5 M^{-1} for the small subunit), and more with less affinity. The results indicated that kanamycin binds not only to the small ribosomal subunit but also to the large subunit, and each subunit possesses one major binding site.

Effects of some antibiotics on the binding of [^{3}H]kanamycin to ribosomes and ribosomal subunits were examined by equilibrium dialysis technique (Table 1). The binding of radioactive material to the ribosome and its subunits was diluted to ca. 1/10 by the addition of ten times higher concentration of cold kanamycin. The result indicated that the observed binding was specific for kanamycin.

Neomycin was found to reverse the binding of [^{3}H]kanamycin to ribosomes and ribosomal subunits at the same level with cold kanamycin, suggesting that both antibiotics possess the same binding sites on the ribosome. A similar tendency of somewhat less degree of inhibition was observed with gentamicin. Less degree of interference was demonstrated with viomycin. Streptomycin and chloramphenicol did not significantly affect the interaction of [^{3}H]kanamycin and ribosomes.

Table 2. Effects of aminoglycoside antibiotics on the binding of [^{14}C] tuberactinomycin O.

Addition of antibiotics	Ribosomes	Ribosomal subunits	
	70S	30S	50S
None	89	58	79
Kanamycin	28	7.8	24
Neomycin	30	9.6	29
Gentamicin	32	10	32

The number represents pmoles of [^{14}C]tuberactinomycin O bound per 100 pmoles of the ribosome or ribosomal subunit.

The experiment was carried out by the procedure described in the legend of Table 1, in which [^{3}H]kanamycin was replaced by [^{14}C]tuberactinomycin O. The same concentrations of radioactive and cold antibiotics were employed with the large subunit as in the case of the ribosome and small subunit (8).

Effects of aminoglycoside antibiotics on the binding of [^{14}C]tuberactinomycin O (8), an antibiotic closely related to viomycin, were studied (Table 2). Kanamycin, neomycin, and gentamicin were observed to reverse the binding to ribosomes and both ribosomal subunits, indicating that these aminoglycosides interact with the large ribosomal subunit as well as with the small subunit.

Inhibition by aminoglycoside antibiotics of translocation of N-acetyl-Phe-tRNA on the ribosome:

N-Acetylphenylalanyl-puromycin formation by the ribosome with N-acetyl-[^{14}C] phenylalanyl-tRNA and puromycin in the absence of EF-G and GTP was used as a model system for peptidyl transferase reaction; and the effect of aminoglycoside antibiotics was examined (Table 3). The puromycin reaction was not significantly affected by kanamycin, neomycin, gentamicin, or streptomycin up to the antibiotic concentration of 10 μM. A slight inhibition by kanamycin, neomycin or gentamicin was observed at high antibiotic concentration of 100 μM. In a simultaneous experiment, blasticidin S, an inhibitor of peptidyl transferase (12), definitely blocked the reaction.

Translocation of N-acetylphenylalanyl-tRNA from the acceptor site to the donor site was observed by the puromycin reaction enhanced by the addition of EF-G and GTP (13, 14). The stimulated reaction was profoundly prevented by kanamycin, neomycin or gentamicin; but not significantly affected by streptomycin (Table 3). The results indicated that the former group of aminoglycosides inhibits translocation of peptidyl-tRNA on the ribosome.

DISCUSSION

The in vitro translocation of aminoacyl-tRNA or peptidyl-tRNA on the ribosome has been prevented by kanamycin, neomycin or gentamicin; but not significantly

Table 3. Effects of aminoglycoside antibiotics on N-acetylphenylalanyl-puromycin synthesis in the absence or presence of EF-G and GTP.

Antibiotics		N-Ac-[^{14}C]Phe-puromycin formed	
		without EF-G & GTP	enhanced by EF-G & GTP
None		100 % (637 cpm)	100 % (1714 cpm)
Kanamycin	0.1 μM	97	72
	1.	95	32
	10.	95	17
	100.	85	3
Neomycin	0.1	95	68
	1.	93	27
	10.	90	12
	100.	86	2
Gentamicin	0.1	97	74
	1.	95	34
	10.	94	15
	100.	88	2
Streptomycin	0.1	102	97
	1.	97	95
	10.	96	92
	100.	93	81
Blasticidin S	100.	16	6

The assay for peptidyltransferase reaction and translocation of peptidyl-tRNA was carried out by acetylphenylalanyl-puromycin synthesis.

The reaction mixture, in 0.2 ml, contained: 50 mM Tris-HCl, pH 7.6, 15 mM $Mg(OAc)_2$, 60 mM NH_4Cl, 6mM 2-mercaptoethanol, 50 μg poly[U], 60 μg N-acetyl-[^{14}C]Phe-tRNA, and 6 A_{260} units washed ribosomes. It was incubated at 37°C for 10 minutes, and cooled in an ice-bath; and then 0.2 mM puromycin and the antibiotic were added to the mixture with or without 100 μg EF-G and 0.1 mM GTP. It was further incubated at 37°C for 10 minutes. The reaction was terminated by addition of 0.5 ml of 0.2 M potassium acetate, pH 5, and extracted with 1.5 ml ethyl acetate. The radioactivity of the solvent layer was assayed in Bray's scintillator.

affected by streptomycin. Kanamycin, neomycin or gentamicin have been observed to interact with both small and large ribosomal subunits. On the other hand, the binding of streptomycin is limited to the small subunit (15, 16). The findings suggest that the inhibition of translocation by kanamycin, neomycin, and gentamicin is attributed to the interaction with the large ribosomal subunit. Contrary to the observation by Suzuki et al. (6), in the present in vitro system, kanamycin, neomycin, and gentamicin affect translocation much more markedly than transpeptidation. The discrepancy may be due to the difference of experimental systems employed.

Kasugamycin, streptomycin, kanamycin, and other aminoglycosides block initiation of protein synthesis, although the detailed mechanism and degree of inhibition are different with each antibiotic (17 - 19). Tai, Wallace, and

Davis (20) have investigated the effect of streptomycin in a natural messenger system, and concluded that inhibition of protein synthesis by streptomycin is caused by the inhibition of peptide chain initiation, but not by the codon misreading. However, the significance of inhibition of translocation by kanamycin, neomycin, and gentamicin for the inhibition of total protein synthesis remains to be determined.

The relationship of in vitro inhibition of translocation by kanamycin and related antibiotics to the in vivo effect remains open to discussion. Although aminoglycoside antibiotics affect various phases of protein synthesis, the lethal action of miscoding-promoting aminoglycosides may be attributed to codon misreading. The bactericidal effect of streptomycin or kanamycin is recovered by the presence of peptide chain elongation inhibitors: chloramphenicol, mikamycin A, blasticidin S, erythromycin or tetracycline; but not by puromycin (21 - 23). It indicates that normal ribosomal cycle or continued protein synthesis is essential for the lethal action, and codon misreading, which requires continued peptide chain elongation, may be the primary action, leading to cell death.

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