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INHIBITION OF RIBOSOMAL TRANSLOCATION BY AMINOGLYCOSIDE ANTIBIOTICS

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Summary. The translocation of AcPhe-tRNA in a purified system and that of peptidyl-tRNA in a crude, complete polypeptide synthesizing system containing endogenous E. coli polysomes are inhibited by antibiotics of the neomycin, kanamycin and gentamicin groups. The extent of inhibition varies with the different antibiotics, but it correlates well with the capacity of each antibiotic to inhibit polypeptide chain elongation. Thus, the inhibition of translocation by these antibiotics is clearly significant for their inhibitory effect on polypeptide synthesis.

Introduction. It is well known that aminoglycoside antibiotics of the neomycin, kanamycin and gentamicin groups act on the ribosome and cause both inhibition of polypeptide synthesis and errors in the translation of the genetic message (misreading) (1,2). It has traditionally been assumed that the inhibition of polypeptide synthesis results, at least in part, from the interference of the antibiotics with the process of aminoacyl-tRNA binding to the ribosome. The dearth of detailed information on the mechanism of inhibition of polypeptide synthesis, as well as our recent finding that hygromycin B (an aminoglycoside antibiotic of a different group) induces both misreading and inhibition of ribosomal translocation (3), have led us to examine the action of these drugs on individual steps of the elongation cycle of protein synthesis. We have found that they significantly inhibit the translocation of peptidyl-tRNA.

Methods. Preparation of 1 M NH_4Cl-washed E. coli MRE600 ribosomes and polysomes, EF-G, EF-T (EF-Ts + EF-Tu), and S100 extracts (supernatants from 100.000 x g spin) has been described elsewhere (4-7). [^{14}C]Phe-tRNA (950 cpm/pmol) and Ac-[^{14}C]Phe-tRNA (890 cpm/pmol) were prepared from purified $tRNA^{Phe}$ (a gift from Dr. G.D.Novelli, Oak Ridge National Laboratory, USA). [^{3}H]Phe-tRNA (1680 cpm/pmol) was prepared from a deacylated tRNA mixture (General Biochemicals, Chagrin Falls, Ohio, USA) and used without further purification. [^{3}H]Puromycin and other labelled materials were from The Radiochemical Centre, Amersham, England. Other antibiotics were gifts from the following sources: gentamicin C1, gentamicin C1a, sisomycin and verdamicin, Schering Corp. Bloomfield, New Jersey, USA; kanamycin A and kanamycin B, Bristol Labs., Syracuse, N.Y., USA; tobramycin, Lilly Indiana of Spain, Madrid; ribostamycin, Meiji Seika Kaisha, Yokohama, Japan; neamine, neomycin B, neomycin C and bluensomycin, Upjohn Co., Kalamazoo, Mich., USA; paromomycin and viomycin, Parke-Davis Co., Detroit, Mich., USA.

Ribosomes were activated in the presence of poly(U) prior to each

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experiment (8). Nonenzymic binding of Ac-[^{14}C]Phe-tRNA to the ribosomal A-site was carried out at 20 mM magnesium acetate and in the presence of deacylated tRNA^{Phe} (8). Other methods are specified in the legends to Tables and Figures.

Results and Discussion. The action of aminoglycoside antibiotics on translocation was determined by examining their effects on the EF-G plus GTP-dependent reaction of ribosome-bound Ac-[^{14}C]Phe-tRNA with puromycin (Fig. 1). Among the neomycin group of antibiotics, neomycin B and C were the most potent inhibitors; ribostamycin (which lacks one of the neosamine residues of the neomycins) and paromomycin (a mixture of two antibiotics identical to neomycins B and C, except that one -NH_2 group is replaced by an -OH group) were less active and neamine (which lacks one neosamine and the D-ribose residues of the neomycins) was the weakest inhibitor. The kanamycins tested (kanamycins A and B and tobramycin) were inhibitory, but not as strongly as neomycin B or C. The gentamicins (gentamicin C1, C1a, sisomicin and verdamicin) were all equally active, and showed an unexpected pattern of inhibition. Maximal inhibition (about 60%) occurred between 3 and 10 μM and higher concentrations were less inhibitory. This pattern was also observed with Ac-[^{14}C]Phe-tRNA·ribosome complexes purified from free Ac-[^{14}C]Phe-tRNA by Sepharose 6B chromatography and, consequently it was not due to the antibiotics enhancing Ac-[^{14}C]Phe-tRNA binding to the ribosomes and promoting increased Ac-[^{14}C]Phe-puromycin synthesis (not shown). Moreover, a similar multiphasic concentration effect has been observed by P.C.Tai (personal communication) on the inhibition of polypeptide chain extension by natural polysomes. These findings support the suggestion (2) that the gentamicins may have more than one binding site on the ribosome. Streptomycin and its chemically related antibiotic bluensomycin caused weak inhibition (40%) which did not vary in a wide range of concentrations (1 - 400 μM). Weak inhibition of translocation by high concentrations of streptomycin (0.5 mM) in a system similar to ours has previously been reported (10).

We verified that the inhibition of Ac-[^{14}C]Phe-puromycin synthesis was indeed due to an interference with translocation and not with peptide bond formation. Table I shows that neither neomycin B, neamine, kanamycin A, tobramycin, gentamicin C1, streptomycin nor bluensomycin significantly modified the reaction of P-site-bound Ac-[^{14}C]Phe-tRNA with puromycin.

Table I also shows that, except for streptomycin, which caused over 50% inhibition, all the aminoglycosides tested were weak inhibitors of the GTP plus EF-T-dependent binding of [^{3}H]Phe-tRNA to the ribosome (column A). Moreover, when purified [^{14}C]Phe-tRNA^{Phe} was used in this assay, the weak

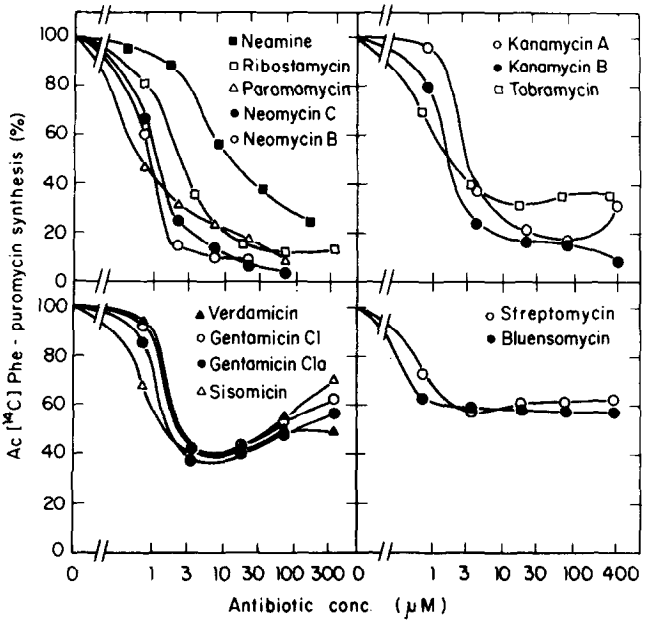

Fig. 1. Effect of antibiotics on the EF-G plus GTP-dependent synthesis of Ac-[^{14}C]Phe-puromycin. Translocation of Ac-[^{14}C]Phe-tRNA and puromycin reaction was conducted in mixtures (20 μl) containing: 20 mM NH_4Cl, 50 mM KCl, 12 mM magnesium acetate, 14 mM Tris-HCl pH 7.8, 1 mM dithiothreitol, 10 A_{260} units/ml of ribosomes containing 9 pmol/A_{260} unit of A-site-bound Ac-[^{14}C]Phe-tRNA, 60 μg/ml EF-G, 0.2 mM GTP, 0.5 mM puromycin and antibiotic as specified. Reaction was started by addition of EF-G plus GTP and by heating the tubes to 30°. After either 2 or 4 min of incubation at this temperature, the mixtures were analyzed for Ac-[^{14}C]Phe-puromycin synthesized (9). Values in controls without EF-G and GTP were subtracted.

inhibition disappeared (except that caused by streptomycin, which was nevertheless strongly reduced) (Table I, column B) indicating that the effect was probably due to enhanced competition between [^{3}H]Phe-tRNA and other tRNAs caused by increased misreading, rather than to direct inhibition of the binding process.

We studied the significance of the inhibition of translocation for the mechanism of polypeptide synthesis inhibition in a more physiological system: E. coli endogenous polysomes supplemented with S100 extract and ^{14}C-amino acids. (Previous studies have shown that initiation of polypeptide synthesis does not occur in this system (11)). Table II shows that in this system all the antibiotics tested significantly inhibited polypeptide elongation, the strongest inhibitors being neomycins B and C and kanamycin B. Next, we determined the position of peptidyl-tRNA in the polysomes inhibited during synthesis by neomycin B by measuring its reactivity with [^{3}H]puromycin. As controls we used tetracycline- or viomycin-blocked polysomes. Since during

Table I. Effect of aminoglycoside antibiotics (0.1 mM) on the reaction of P-site-bound Ac-[^{14}C]Phe-tRNA with puromycin and on the GTP plus EF-T-dependent binding of labelled Phe-tRNA to ribosomes

Antibiotic	Ac-[^{14}C]Phe-puromycin synthesized	Labelled Phe-tRNA bound	
		(A)	(B)
	(% activity)		
None	100	100	100
Neomycin B	98	78	98
Ribostamycin	---	63	100
Neamine	108	69	97
Kanamycin A	107	69	105
Tobramycin	95	60	106
Gentamicin C1	96	79	101
Streptomycin	95	45	87
Bluensomycin	91	---	---

To measure the reaction of puromycin with P-site-bound Ac-[^{14}C]Phe-tRNA, ribosomes with A-site-bound Ac-[^{14}C]Phe-tRNA were preincubated at 30° for 5 min in the presence of EF-G and GTP (3,8), supplemented with the indicated antibiotic and 0.5 mM puromycin, and incubated at 30° for 2 min. Ac-[^{14}C]Phe-puromycin was then determined by the method of Leder and Bursztyn (9). Binding of either [^{3}H]Phe-tRNA containing other tRNAs (A) or purified [^{14}C]Phe-tRNA^{Phe} (B) to ribosomes complexed with poly(U) and P-site-bound deacylated tRNA^{Phe}, in the presence of GTP and EF-T, was performed as previously described (3). Incubation at 30° for 45 sec was followed by the nitrocellulose filter assay. Binding in the absence of EF-T was less than 10% of that in the presence of the factor. One hundred per cent represents either 4.0 pmol Ac-[^{14}C]Phe-puromycin synthesized or 4.4 pmol [^{3}H]Phe-tRNA bound or 7.2 pmol [^{14}C]Phe-tRNA^{Phe} bound, all per A_{260} unit of ribosomes.

polypeptide chain elongation viomycin specifically inhibits translocation (12) and tetracycline blocks aminoacyl-tRNA binding (13, review), these antibiotics retain peptidyl-tRNA in either the ribosomal A- or P-site, respectively. Fig. 2 shows that the peptidyl-tRNA of tetracycline-inhibited polysomes reacted rapidly with [^{3}H]puromycin, while less than half of that of the viomycin- or neomycin B-blocked polysomes reacted rapidly; the remainder reacted slowly. It is suggested that neomycin B, like viomycin, retains peptidyl-tRNA in the ribosomal A-site but, in addition, allows residual polypeptide chain elongation (Table II), which transitorily moves the peptidyl-tRNA to the ribosomal P-site and causes the slow reaction with [^{3}H]puromycin.

Table II shows the results of similar experiments performed with other aminoglycosides, taking as 100 and 0% the synthesis of peptidyl-[^{3}H]puromycin observed with tetracycline and viomycin, respectively. In all cases, except with streptomycin, there was significant inhibition of peptidyl-[^{3}H]puromycin formation. Moreover, there was good general correlation between the degree of

Table II. Effect of aminoglycosides on polypeptide synthesis and peptidyl -[^{3}H]puromycin formation by polysomes

Antibiotic added	Concentration	Polypeptide synthesis	Peptidyl -[^{3}H]puromycin formation
	(mM)	(% activity)	
None	---	100	---
Tetracycline	0.2	---	100
Viomycin	0.1	---	0
Neomycin B	0.07	8	16
Neomycin C	0.07	7	-5
Paromomycin	0.07	49	61
Ribostamycin	0.07	33	69
Neamine	0.07	66	62
Kanamycin A	0.09	44	56
Kanamycin B	0.08	23	33
Tobramycin	0.07	34	51
Gentamicin C1	0.3	51	64
Gentamicin C1a	0.3	36	77
Sisomicin	0.4	33	59
Streptomycin	0.2	70	96

Polypeptide synthesis on purified E. coli polysomes in the presence of the indicated antibiotic was performed as described (3,12), using a mixture of sixteen ^{14}C-amino acids (10 μCi/μmol) plus ^{12}C-histidine, methionine, tyrosine and cysteine. Incubation was at 34° for 4 min. 100% represents the incorporation of 27 aminoacids per ribosome. Peptidyl-[^{3}H]puromycin synthesis was carried out essentially as indicated in the legend to Fig. 2, except that synthesis in 24 μl reaction mixtures was stopped 2 min after addition of [^{3}H]puromycin. Synthesis in reactions containing tetracycline and viomycin (5.7 and 3.1 pmol/A_{260} unit of polysomes) was equated to 100 and 0%, respectively.

inhibition of polypeptide synthesis and that of the puromycin reaction (Table II). It thus seems clear that the inhibition of translocation at least partly causes the inhibition of polypeptide chain elongation. It is still possible, however, that interference with other steps of the elongation cycle (specially aminoacyl-tRNA binding, Table I) may also contribute to the inhibition of polypeptide chain elongation. We would stress that the misreading induced by these antibiotics, even at high concentrations (1,2), requires the inhibition of translocation to be only partial (Table II).

It is noteworthy that hygromycin B (3) and all the aminoglycosides now found to inhibit translocation significantly in both natural and artificial mRNA-directed systems possess the 2-deoxystreptamine group, while streptomycin and bluensomycin do not (14). Moreover, streptomycin binds to one site on the 30S subunit (15,16), while it is unclear whether the inhibitors of translocation at the concentrations used in our experiments bind

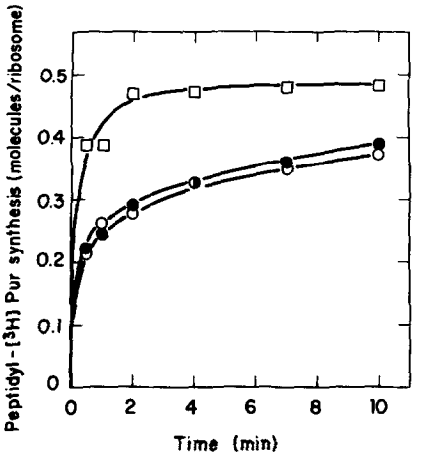

Fig. 2. Time course of peptidyl-[^{3}H]puromycin formation with polysomes blocked during synthesis by either 0.18 mM tetracycline (□), 0.06 mM neomycin B (●) or 0.09 mM viomycin (○). E. coli polysomes in 100 µl mixtures were allowed to incorporate unlabelled aminoacids at 34° for 6 min (3,12) at which time incorporation was stopped by adding 2 µl of solutions containing the antibiotics. After 1 min at 34°, 10 µl 0.1 mM [^{3}H]puromycin (720 cpm/pmol) was added, incubation was continued and portions (15 µl) were taken out at intervals and analyzed for cold-trichloroacetic acid precipitable material. Backgrounds obtained in controls without polysomes were subtracted.

to one or more sites on the 30S, 50S or on both subunits (2). Recently, a gentamicin-resistant mutant of E. coli has been shown to have an altered protein (L6) in the 50S subunit (17). Regardless of the localization of their binding site, the dual effect of the antibiotics on translocation and aminoacyl-tRNA recognition suggests a close relationship between the ribosomal components implicated in each of these functions (3).

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