BINDING OF TOBRAMYCIN TO *ESCHERICHIA COLI* RIBOSOMES: CHARACTERISTICS AND EQUILIBRIUM OF THE REACTION

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A sample of [^{3}H] tobramycin (5,000 Ci/Mole) has been synthetized and incubated with the bacterial ribosome and its subunits. The results obtained show that this antibiotic has two types of binding sites. The primary one is probably responsible for the inhibition of protein synthesis whereas the secondary one is probably related to the misreading and reading through of the messenger RNA.

It has been demonstrated recently[1,2] that, at a low concentration, gentamicin, a powerful aminoglycoside antibiotic, is able to inhibit protein synthesis to a large extent whereas at higher concentrations this drug catalyzes misreading and reading-through of the messenger RNA. This biphasic process has probably some correlation to the presence of two types of binding sites on the ribosome: one site responsible for inhibition of the chain elongation; the other, for reversing inhibition, induction of misreading and reading-through of mRNA.

Binding experiments performed by MISUMI *et al.*[3] with kanamycin, neomycin and gentamicin on washed ribosomes and ribosomal subunits also showed that these drugs can bind to the ribosome, 50 S as well as to the 30 S subunit.

The present paper deals with the binding of tobramycin (Tob), an antibiotic related in structure to kanamycin and gentamicin, to the bacterial ribosome and its subunits.

Evidence is presented which shows that, as predicted, this drug has two types of selective binding sites on the ribosome itself as well as on the 50 S subunit, while it has only one type of binding site on the 30 S subunit.

Materials and Methods

Chemicals

Tritiated borohydride was from C.E.A. and had a specific radioactivity of 25,000 Ci/mole. Tobramycin was obtained from Eli Lilly; [^{3}H] tobramycin ([^{3}H] Tob) was synthetized by a method described previously for the synthesis of derivatives of 3′-deoxyparomomycin[3]: the amino-functions of tobramycin were protected by ethoxycarbonylation and the secondary hydroxyl groups by acetylation. The derivative thus synthetized was oxidized by the dimethylsulfoxide-pyridine-trifluoroacetic acid complex to the corresponding 6″ aldehyde which was then purified by column chromatography on silicic acid for an overall yield of 45%. Nine mg of this aldehyde were dissolved in 1 ml of methanol, added to $4 \cdot 10^{-6}$ mole of BT4Na (25,000 Ci/mole) in 1 ml of 0.01 M NaOH and stirred for 1 hour at room temperature. Methanol was then removed under vacuum and the residue treated with 1 ml of a water solution of barium hydroxide (0.5 M) at 100°C for 18 hours. Dry ice (1 g) was added to the medium; the precipitate was eliminated by filtration. The filtrate thus obtained was submitted to lyophilization. Purification of [^{3}H] Tob was performed by bidimentional thick-layer chromatography on silicic acid

plates with a mixture of methanol - 20% ammonia (3: 1) as eluant.

The specific radioactivity of synthetized [^{3}H] Tob was determined by the microbiological method: a known quantity of [^{3}H] Tob was dissolved in water and tested as an antibiotic against an *Escherichia coli* K12 strain. Results were compared against those obtained using a standard solution of an authentic sample of tobramycin.

Biological material

Tight and run off ribosomes were prepared by zonal centrifugation as described previously[5] from an S_{30} fraction of synchronized *E. coli* MRE 600. The ribosomal fractions thus obtained were concentrated with polyethyleneglycol. The ribosomal subunits (30 S and 50 S) were, again, prepared as described previously[5] by zonal centrifugation in the presence of a 0.4 M sodium chloride solution. The ribosomes and their subunits remained stable for months at -20°C in buffer A (10 mM tris-HCl, 10 mM $Mg(OAc)_2$, 100 mM NH_4Cl, 6 mM β-mercaptoethanol, pH 7.8).

One A_{260} unit corresponds to 24 p mole/ml of 70 S, 69 p mole/ml of 30 S and 35 p mole/ml of 50 S.

Equilibrium dialysis

To determine the properties of the reversible complex [^{3}H] Tob-biological particule, a Dianorm apparatus, whose rotating cells had a total volume of 500 μl, was used. Two compartments were formed by a semipermeable membrane (Visking) which had been soaked, beforehand at room temperature in the following buffer: 10 mM Tris-HCl pH 7.6, 100 mM NH_4Cl, 10 mM $Mg(OAc)_2$. 200 μl of the biological preparation (R_T) were introduced in one compartment of the cell and 200 μl of increasing concentrations (from $5\cdot10^{-7}$M) of tobramycin in the other. In addition, a similar quantity ($2\cdot10^{-8}$ mole: $8\cdot10^5$ dmp) of the synthetized [^{3}H] Tob was introduced into the cell with the tobramycin solution.

When equilibrium was reached (20 hours at 4°C), 100 μl of solution was pipetted from each compartment. The concentrations of bound antibiotic (C_B) and free antibiotic (C_F) as given by equations (1) and (2):

$$C_B = \frac{Ci(D_P - D_L)}{D_P + D_L} \quad (1)$$

$$C_F = \frac{Ci\,D_L}{D_P + D_L} \quad (2)$$

were determined by liquid scintillation (Kontron MR 300 instrument) in 5 ml of ACS scintillation liquid.

D_P and D_L are the d.p.m. in ribosomal and antibiotic compartments respectively.

Ci is the initial tobramycin concentration in the cell (the quantity of [^{3}H] Tob introduced and non-specific binding to the membrane and the cells are taken in account).

Results are the average of three or four experiments and are represented directly as:

$$\frac{D_P - D_L}{D_P + D_L} = f(Ci)$$

and following the SCATCHARD representation:

$$\frac{\bar{\nu}}{C_F} = f(\bar{\nu})$$

where $\bar{\nu} = \dfrac{C_B}{R_T}$

R_T is the total ribosomal or subunits concentrations used in the experiments.

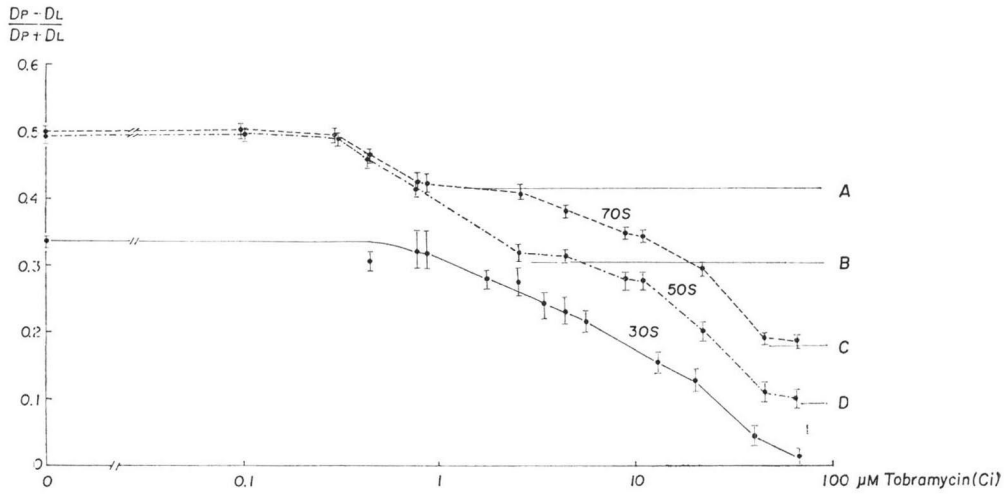
Results

A sample of [^{3}H] Tob of high specific radioactivity (5,000 Ci/mole) was synthetized and used as a marker to study the equilibrium of the binding of Tob to the ribosome and its subunits. Fig. 1 presents the results of these binding experiments. They were carried out by equilibrium dialysis using the isotopic dilution method[6]. The biological material used was from an *E. coli* MRE 600 strain sensitive to Tob. The concentrations of [^{3}H] Tob used were from 0.1 μM to 100 μM.

Fig. 1. Effect of increasing concentration of [^{3}H] tobramycin by the isotopic dilution method on the *E. coli* ribosome and its subunits.

The experiments were carried out in 10 mM Tris-HCl pH 7.6, 100 mM NH_4Cl, 10 mM $Mg(OAc)_2$ as described in materials and methods.

0.4 μM of ribosomes (----), 50 S (-·-·-) or 30 S (—) were dialysed against 0.02 μM of [^{3}H] Tob and increasing concentrations of tobramycin.



With 70 S particle, over a [^{3}H] Tob concentration range of 0.3 μM to 0.8 μM, there was a decrease in radioactivity which leveled off in a plateau; a new decrease began at 2.5 μM and continued until 45 μM where bound radioactivity again leveled off in plateau C. This plateau corresponded to non-specific binding.

With the 30 S subunit, only one steady decrease in bound radioactivity was observed before to a plateau representing non-specific binding was reached.

The SCATCHARD plot of $\bar{u}/C_F$ against μ is linear for identical binding sites, with a slope equal to $-1/K_D$ (K_D: dissociation constant of the complex studied) and the abscissa intercept equal to n (number of binding sites per particule).

When results of Fig. 1 were represented by this method, curves of Fig. 2 could be obtained which clearly showed two types of selective binding sites for the 70 S and 50 S particules. However the 30 S subunits possessed only one type of binding site with the approximate values of K_D and n shown in Table 1.

More accurate values for these constants could be obtained, especially for the primary binding sites located on the 70 S and the 50 S particules, by substracting non-specific binding, represented in

Table 1. Dissociation constants K_D and number of binding sites per particules determined from SCATCHARD analysis of Fig. 2.

Particles	K_DM	n	K_DM	n
70 S	0.2×10^{-6}	1	10×10^{-6}	30
50 S	0.2×10^{-6}	1	6×10^{-6}	15
30 S	—	—	3.3×10^{-6}	7

Table 2. Dissociation constant K_D and number of binding sites per particule determined from SCATCHARD representation of Fig. 3.

Particles	K_DM	n	K_DM	n
70 S	4.2×10^{-7}	1	7×10^{-6}	7
50 S	5×10^{-7}	1	4.8×10^{-6}	7
30 S	—	—	3.3×10^{-6}	7

Fig. 2. SCATCHARD analysis of the specific binding of tobramycin in the concentration range 0.1 to 100 μM to the bacterial ribosome (△), 50 S (●) and 30 S (○) subunits.

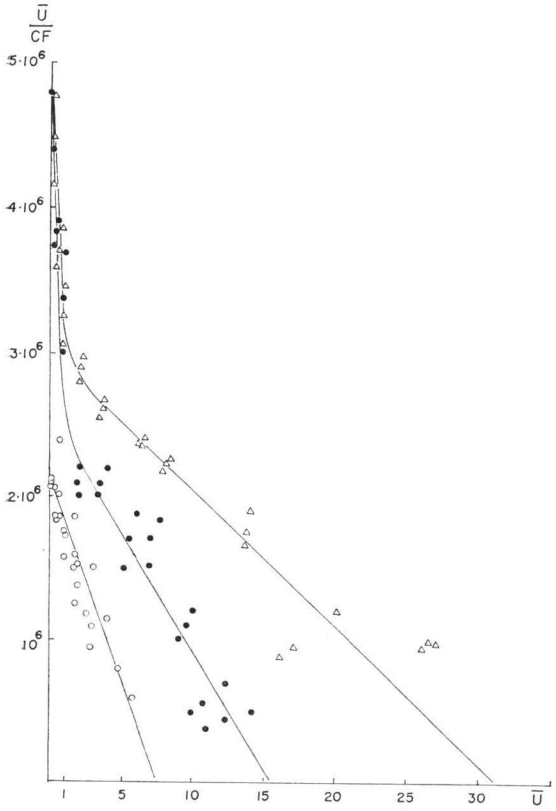


Fig. 3. SCATCHARD analysis of the specific binding of tobramycin in the concentration range 0.1 to 100 μM to the bacterial ribosome (A) and the 50 S subunit (B) by substracting non-specific binding represented by (C) and (D) in Fig. 1.

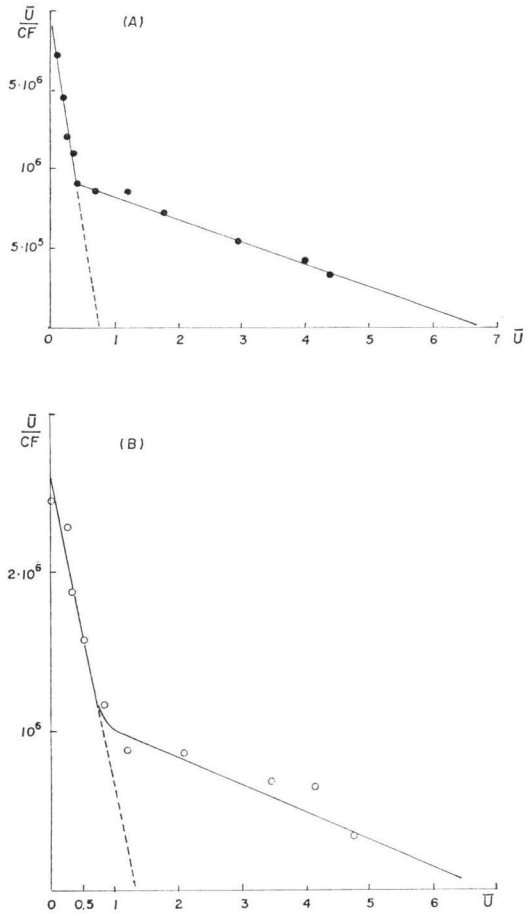


Fig. 1 by plateau *C* for 70 S ribosome and by plateau *D* for the 50 S subunit.

Upon linearization under these restrictive conditions curves of Fig. 3 were obtained. They allowed us to determine real characteristic constants (K_D and n) of the reversible complexes, [^{3}H] Tob-biological particule, which are shown in Table 2.

Discussion

The biphasic SCATCHARD curves obtained with the 70 S and 50 S particules reveal the presence of either two types of independent binding sites or of two types of sites induced by an anticooperative effect. It is not yet possible to distinguish between the two models. Experiments are underway to try to dissociate the complexes formed in order to make a more detailed analysis of the system.

The first class of sites shows a strong binding of one molecule of Tob per ribosome or per 50 S subunit. This type of site does not exist for the 30 S subunit. The second type of binding for ribosomes has an affinity about 50 times less than that of the first and could involve the binding of about 10 molecules of the antibiotic at numerous sites of similar affinity.

The graphs obtained after substraction of non-specific binding allow us to better visualize the first type of sites, but certainly lead to error in the interpretation of the second type, for these affinities are

relatively low and the substraction of non-specific binding leads to an underestimation of the molecules bound to the biological particule. The same type of secondary binding site is also present in the ribosomal subunits.

Our results correlate exactly with experiments reported by DAVIS and BÖCK[1,2] who demonstrated that a tight binding site was probably responsible for the inhibition of protein synthesis which occurs when concentrations lower than 1 μg/ml of gentamicin are reached in the medium. At higher concentrations of the drug, primary as well as secondary binding sites are occupied. There is a subsequent small increase in protein synthesis which coincides with the appearance of misreading and reading-through of mRNA. Our results also correlate with those of MISUMI *et al.*[3] who demonstrated a selective binding of kanamycin A, neomycin and gentamicin to the bacterial ribosome as well as to its subunits.

Based on these results, it is possible to propose the following model: at low concentrations, one molecule of tobramycin is bound to the high affinity binding site leading to inhibition of protein synthesis. At higher concentrations, several molecules of Tob are bound to the low affinity binding sites provoking the appearance of misreading.

Additional experiments are now under way which will better our knowledge of these binding sites and their function in protein synthesis.

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