BINDING OF TETRACYCLINE TO THE 30S RIBOSOMES AND TO POLYURIDYLIC ACID

Robert H. Connamacher[1] and H. George Mandel

Department of Pharmacology
The George Washington University School of Medicine
Washington, D.C.

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Tetracycline has been shown by Franklin (1963) and by Laskin (1964) to inhibit the polyuridylic acid directed polypeptide synthesis in cell-free systems of Escherichia coli. Suarez and Nathans (1965) and Hierowski (1965) have shown that the drug partially inhibits the attachment of amino acid acyl sRNA to ribosomes, but only at a concentration above that needed for inhibition of the polypeptide synthesis. We have found that tetracycline binds specifically to the 30S ribosomes of E. coli and Bacillus cereus both in vivo and in vitro and to polyuridylic acid in vitro.

METHODS

B. cereus was grown in a casamino acid-salts medium, E. coli in a nutrient broth-glucose medium. For in vivo studies, tetracycline was added 25 minutes before harvesting the cells. Cells were frozen until used.

Bacteria were sonicated in 2.5 volumes of a Tris-Mg^{++} buffer pH 7.8. They were centrifuged for 10 minutes at 3000 x g to remove whole cells, 10,000 x g to sediment cell wall (sediment microscopically cell-free), 20,000 x g (20 min.), 30,000 x g (30 min.), and 105,000 x g (90 min.). Ribosomes were washed once with buffer. In dialysis experiments with B. cereus, the 105,000 x g supernatant was dialyzed overnight against buffer.

Five ml. of a suspension of cell wall (over 100 mg.), ribosomes (10-20 mg., by $O.D._{260}$ assay) or soluble fraction (10-20 mg., assay by Lowry

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et al., 1951) were placed in dialysis bags and dialyzed for 20 hrs. against 10 ml. buffer containing enough tritiated tetracycline to make a final equilibrium concentration of 1-3 μg/ml (2-6 μM, 0.42 μc/μg). One tenth ml. of the inside and outside solutions were counted in a Nuclear-Chicago scintillation counter and the inside to outside count ratio (I/O ratio) calculated.

Five ml. sucrose gradients (5-20% sucrose) were layered with 0.5 to 0.75 mg of ribosomes. The gradients were spun for 90 min. (10^{-2} M Mg^{++}) or 150 min. (10^{-4} M Mg^{++}). Fractions of 0.2 ml. were collected, diluted to 1.1 ml. with water, absorbance read at 260 mμ, and the entire sample radioassayed.

Chromatograms on Whatman 3MM paper were run in 70% aqueous isopropanol. Paper electrophoresis at pH 3.5 (formate buffer) employed a Savant Electrophoresis Apparatus at 3000 volts for 30 min. Radioactivity was measured on a Vanguard strip counter.

We are indebted to Dr. D. Buyske of Lederle Laboratories for both tritiated and non-radioactive tetracycline. Additional tritium-labeled drug was purchased from the New England Nuclear Corp. Polyuridylic acid was acquired from Calbiochem, Inc. Poly U-^{14}C came from Miles Laboratories, UDP from Sigma Laboratories.

RESULTS

1. Dialysis binding: Tetracycline bound to the B. cereus ribosomal fraction (I/O ratio 1.15) and to a lesser degree to the supernatant fraction (I/O 1.08). Lowering the Mg^{++} concentration from 10^{-2}M to 10^{-4}M increased the binding of tetracycline to I/O of 1.38 and 1.11 respectively. Cell wall bound tetracycline only to a very slight extent.

2. Sucrose gradient centrifugation: Tetracycline was found to bind to 70S ribosomes of B. cereus and E. coli exposed to the drug. In sucrose gradients containing 10^{-4}M Mg^{++}, tetracycline bound to the 30S ribosome but not to

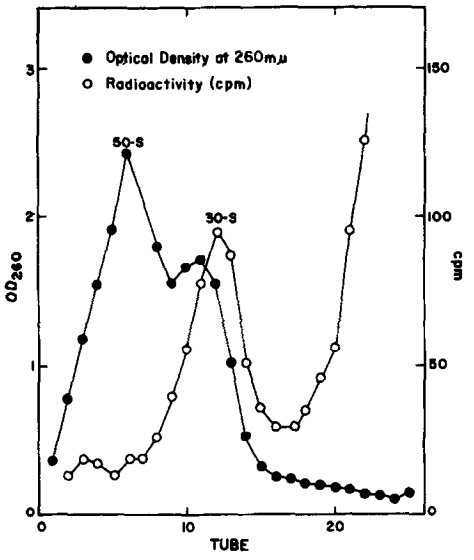

Fig. 1. *In vivo* binding of ^{3}H-tetracycline to the 30S ribosome of *B. cereus*. Sucrose gradient, 10^{-4} M Mg^{++}.

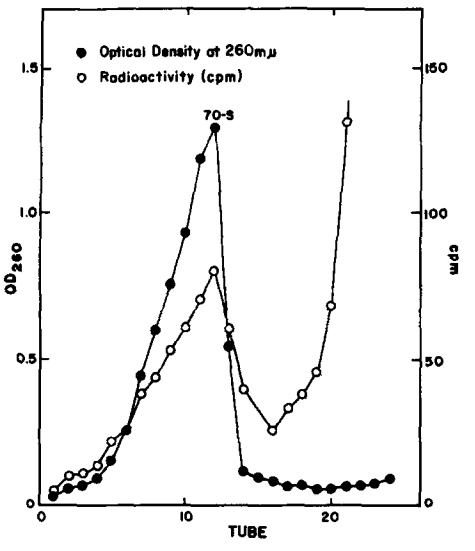

Fig. 2. *In vitro* binding of ^{3}H-tetracycline to the 70S ribosome of *E. coli*. Sucrose gradient, 10^{-2} M Mg^{++}.

the 50S ribosomes (Fig. 1). Binding to the 70S or 30S ribosomes also took place when tetracycline was added directly to ribosomes at 0° (Fig. 2), indicating that the substance bound was not a metabolic product of the drug. The binding compound was considered to be tetracycline rather than a possible contaminant since more than one source of tetracycline was used, each showing only one radioactive component in the solvent system of Urx *et al*. (1963), and since three-fold dilution of the labeled with the unlabeled drug resulted in a corresponding reduction in radioactive binding to the ribosomes. Although the total amount of tetracycline was low, about 0.42 mμmoles/mg. ribosomes, this binding was seen in more than 25 experiments.

Preincubation of the 30,000 x g supernatant with ATP, an ATP generating system, and amino acids reduced or even eliminated tetracycline binding (Fig. 3). Preincubated *E. coli* ribosomes (but neither preincubated or unpreincubated *B. cereus* ribosomes) were able to bind poly U-^{14}C, and this

binding was decreased only 20% by addition of tetracycline 15 min. prior to poly U. Tetracycline, therefore, does not act by preventing the attachment of mRNA to ribosomes. Treatment of a ribosomal suspension from B. cereus with 10^{-4} M EDTA 15 min. before the addition of tetracycline reduced the specific activity of the tetracycline-30S ribosomal peak by 20% so that Mg^{++} was not considered to be essential for the tetracycline binding.

3. In vitro binding of poly U and tetracycline: Tetracycline added to solutions of UDP and poly U was chromatographed on Whatman 3MM paper

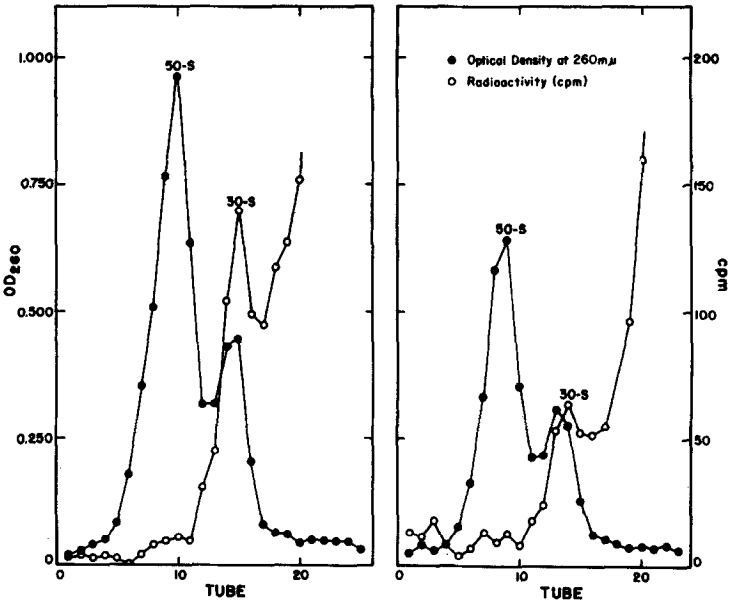

Fig. 3. Effect of incubation on the binding of tetracycline to E. coli ribosomes. Sucrose gradient, 10^{-4} M Mg^{++}
a) not incubated
b) incubated to remove mRNA

in an isopropanol-water system. Poly U prevented the usual mobility of tetracycline, even in the presence of 10^{-4} M EDTA. Electrophoresis at pH 3.5 of these mixtures showed also that poly U bound tetracycline strongly and prevented the usual migration of tetracycline towards the cathode. UDP showed very little binding with tetracycline.

DISCUSSION

These experiments show that tetracycline binds specifically to the 70S and 30S ribosomes of E. coli and B. cereus. The 30S ribosome has 3

known binding sites: one for binding the 30S to the 50S ribosome, one for binding of mRNA to the ribosome, and one for attachment of sRNA. Because tetracycline is able to bind to the 70S ribosome *in vivo* and *in vitro*, the 30S to 50S binding site apparently is not the one to which tetracycline binds. The small effect of tetracycline on binding of poly U to preincubated *E. coli* ribosomes confirms the conclusion of Suarez and Nathans (1965) that tetracycline does not affect the binding of mRNA to ribosomes. Suarez and Nathans (1965) and Hierowski (1965) have shown that the antibiotic is able to block the adsorption of amino acid charged sRNA to ribosomes. Our evidence suggests that tetracycline may inhibit protein synthesis by binding directly to mRNA, thus preventing the attachment of sRNA to the mRNA-ribosome complex.

The inability of *B. cereus* to bind poly U may explain the lack of poly U-directed polypeptide synthesis with ribosomes from those bacteria, although natural messenger is effective (Grünberger, 1964). Perhaps messenger of *B. cereus* is not destroyed during such preincubation[1]. Thus *B. cereus* ribosomes, to which mRNA is still attached after preincubation, would then bind tetracycline but not poly U. On the other hand, preincubated *E. coli* ribosomes would bind little tetracycline because mRNA has been released and now can accept poly U.

The increased binding of tetracycline to ribosomes in low Mg^{++} buffer and the failure of 10^{-4} M EDTA to greatly affect this binding either to ribosomes or to poly U suggest that the binding to RNA does not require Mg^{++}

SUMMARY

1. Tetracycline binds specifically to the 70S and 30S ribosomes of *B. cereus* and *E. coli*, both *in vivo* and *in vitro*. Incubation of *E. coli* ribosomes with ATP and amino acids reduces the amount of tetracycline bound.

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[1]We are indebted to Dr. W.C. Werkheiser for this suggestion.

2. Tetracycline binds strongly to poly U *in vitro*.

3. The binding of tetracycline to ribosomes and poly U occurs in the presence of EDTA, suggesting that this binding is not Mg^{++} dependent.

4. It is suggested that tetracycline binds directly to mRNA, thus inhibiting the binding of sRNA to mRNA.

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ABBREVIATIONS

UDP, uridine diphosphate; mRNA, messenger RNA; sRNA, soluble RNA; poly U, polyuridylic acid.

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