Ribosome–Tetracycline Interactions†

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ABSTRACT: The interaction between the antibiotic tetracycline (TC) and the *E. coli* 70S ribosome has been studied by several techniques. Equilibrium binding data obtained using fluorescence methods suggest that there is only one strong binding site for the drug on the ribosome with binding constant $K_{ap} = 5.8 \times 10^4$ M^{-1}. Dose-response studies show that binding of a single drug molecule is sufficient to inhibit a single ribosome. Binding of tetracycline does not significantly shift the distribution between 70S ribosomes and 30S + 50S subunits. The presence of antibiotic, however, does increase the thermal stability of the 70S ribosome as judged by an increase in the melting temperature (T_m) of the RNA in the ribosome. In a series of congeneric tetracyclines there is a good correlation between the ability to confer thermal stability to the ribosome and the relative potency in inhibiting in vitro polypeptide synthesis. The data suggest that Mg^{2+} chelation is not important in the mechanism of action of tetracycline but that the drug may act by inhibiting ribosome conformational flexing. Temperature-jump relaxation studies reveal a two-step mechanism characterized by a slow binding of the drug to the ribosome followed by a faster conformational rearrangement viz., 70S + TC (slow) $\rightleftharpoons$ $complex_1$ (fast) $\rightleftharpoons$ $complex_2$. The corresponding kinetic constants have been calculated. The overall kinetic binding constant $K_{ap} = 4.4 \times 10^4$ M^{-1} calculated from kinetic data is in good agreement with the constant obtained by equilibrium methods.

Tetracycline is a broad spectrum antibiotic in widespread clinical use. The primary effect on the growth of sensitive microorganisms is an inhibition of protein synthesis on ribosomes. This inhibition is accomplished by blocking the binding of aminoacyl-tRNA to the ribosomal A site, both in vitro (Pestka and Nirenberg, 1966; Suzaka et al., 1966) and in vivo (Cundliffe and McQuillen, 1967), although other biochemical effects are elicited at higher concentrations (Laskin, 1967; Laskin and Last, 1971). Since tetracycline chelates mono- and divalent cations, it has been suggested (Albert, 1958) that chelation may be important in the inhibition of ribosome function. However, no correlation between the chelating ability of tetracycline congeners and their biological action has been found (Gale et al., 1972).

Binding of tetracycline to ribosomes of *B. megaterium* (70 S) and rat liver (80 S) is reported to be extensive; literally hundreds of molecules of drug may bind to one ribosome (Maxwell, 1968). However, less than one of these bound tetracyclines remains after sedimentation in sucrose, suggesting there may only be a single primary site for binding. The *E. coli* ribosome appears to be somewhat selective. Last (1969) found only 1–4 drug molecules bound to 70S ribosomes even at very high input ratios. Werner et al. (1975) have presented binding data (their Table 9) which suggests that there may only be a single binding site for oxytetracycline on the *E. coli* 70S ribosome and that neither free subunit has a strong binding site. Thus the interaction faces of the 30S–50S couple or an induced conformational change may be required for functional binding.

So far no quantitative binding studies have been reported. Also unresolved is the question of whether a single bound drug will inhibit ribosome function (Laskin and Last, 1971). In addition, little is known about the kinetic aspects of drugs binding to ribosomes. The present paper is concerned with a physicochemical characterization of these processes in the tetracycline ribosome system and will thus provide information hitherto unavailable on the mechanism and dynamics of antibiotic-ribosome interactions.

Materials and Methods

Cells and Ribosomes. *E. coli* MRE 600 (¾ log) was purchased from the Grain Processing Corp., Muscatine, Iowa. Ribosomes were prepared according to Staehelin et al. (1969) and stored in small aliquots of 1000 A_{260} units/mL at −70 °C. In most preparations analytical sucrose gradients revealed about 10% of "loose couples" or "B type" ribosomes (see Debey et al. (1975) for nomenclature) and 90% "tight couples" or "A-type" ribosomes. Ribosome concentrations were estimated by assuming that a 0.1% solution had an absorbance at 260 nm of 15 and that the 70S molecular weight is 2.6×10^6.

Buffers and Chemicals. All chemicals were reagent grade; sucrose used in ribosome preparations was Mann Ultra Pure. The tetracycline congeners (all as the hydrochloride salt) were donated by the following companies: Lederle (demethylchlortetracycline, chlortetracycline, tetracycline, minocycline), Wallace Laboratories (methacycline), and Pfizer (oxytetracycline). Because tetracycline solutions undergo both oxidative and photochemical degradation, all solutions were freshly prepared immediately prior to use and kept in the dark.

All the studies reported here except melting curves were done in buffer I: 150 mM NH_4Cl, 50 mM Tris[1]-HCl, pH 7.5, 10 mM $Mg(C_2H_3O_2)_2$. Because the presence of tetracycline caused precipitation of ribosomes at high temperatures in 10 mM Mg^{2+}, the melting curves were obtained in buffer I at 1 mM Mg^{2+}.

In Vitro Protein Synthesis. Poly(U) directed polyphenylalanine synthesis was performed according to Staehelin and Maglott (1971). The ID_{50} for tetracycline congeners is that concentration of the drug which leads to 50% inhibition of this polyphenylalanine synthesis. The rate of synthesis was generally linear over at least 20 min, but initial rates for the inhibition studies were calculated over a 10-min time course.

Temperature-Jump Measurements. The *T*-jump instrument is a split beam modification of the original Eigen–DeMaeyer design (Crothers, 1971). Tetracycline fluorescence was used to measure the concentration changes following a

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[1] Abbreviations used: rRNA, ribosomal RNA; A_{260} unit, that amount of ribosome dissolved in 1 mL which gives an absorbance at 260 nm of 1.0 in a 1-cm pathlength; *T* jump, temperature jump; TC, tetracycline; poly(U), poly(uridylic acid); Tris, tris(hydroxymethyl)aminomethane.

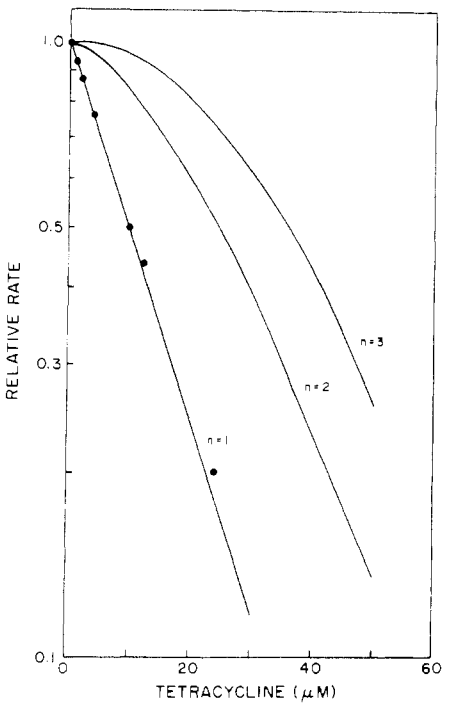

FIGURE 1: Relative rates of polyphenylalanine synthesis as a function of tetracycline concentration. The initial rates were measured in the standard assay system and normalized with respect to the control sample containing no drug. The curves for $n = 1$–3 where n is the number of drug molecules required to inactivate one ribosome are calculated from eq 1.

temperature jump from 20.4 to 24.2 °C. The 366-nm excitation was isolated from a 200-W Hg–Xe lamp by a Schoffel grating monochromator. The emission was detected at right angles on an EMI 9659 QB photomultiplier. Rayleigh scattering from the ribosome suspension at the same wavelength as the incident beam was not allowed to reach the photomultiplier by employing an Oriel G-772-4750 long pass filter in the emission light path.

Melting Curves. Ribosome thermal transitions were studied by monitoring the RNA absorbance at 260 nm as a function of temperature. The melting temperature (T_m) is defined as the temperature at which the absorbance has risen halfway between its initial (A_i) and final (A_f) values. The extent of transition is $B = (A_t - A_i)/(A_f - A_i)$ where A_t is the absorbance at a particular temperature. The absorbance–temperature profiles were plotted automatically for up to four samples using a Gilford 2400-S spectrophotometer.

Static Fluorescence and Light Scattering. A thermostated Aminco-Bowman SPF instrument equipped with a 150-W Hg–Xe lamp was used for both fluorescence and 90° scattering measurements. For tetracycline fluorescence measurements the excitation and emission wavelengths were 370 nm and 510 nm, respectively. Scattering of ribosome suspensions was measured with both monochromators set to 436 nm (an emission maximum for Hg). The Mg^{2+}-dependent transition from 30S and 50S subunits to 70S ribosomes was determined using light scattering as described by Debey et al. (1975).

Binding Studies. To ascertain the extent of binding, two methods were employed to separate free and ribosome bound tetracycline from equilibrium mixtures. (1) The 0.45-μm nitrocellulose filters (Millipore Corp.) pass free tetracycline but retain ribosomes and ribosome bound tetracycline at concentrations up to at least 10 A_{260} units/mL. This allows a quick separation of free and tightly bound drug. (2) Bound tetracycline was separated from free by sedimenting ribosomes at 50 000 rpm in a Spinco 50 Ti rotor for 4 h in buffer I. Comparison of the fluorescence intensity of the ribosome free supernatant with a standard curve yields the concentration of free drug and, by subtraction, the amount bound to the ribosomes in the pellet. Quantitative equilibrium binding information was obtained by fluorescence titration experiments as described below.

Results

Effect of TC on in Vitro Polyphenylalanine Synthesis. The initial rate of polyphenylalanine synthesis is linear over at least 20 min. As the concentration of tetracycline is increased, the initial rate of synthesis is still linear but the relative rate decreases in proportion to the number of ribosomes surviving the action of the drug. Thus one can interpret these changes in rates in terms of a "survival curve" analogous to photochemical inactivation (Dertinger and Jung, 1970). If N_0 is the total number of ribosomes and N is the number surviving the action of the drug, then assuming that binding of drug molecules is statistically distributed and independent of each other one may write

$$\frac{N}{N_0} = e^{-VD} \sum_{k=1}^{n-1} \frac{(VD)^k}{k!} \tag{1}$$

where n is the mean number of drug molecules or "hits" required for inactivation, D is the dose, and V is a parameter representing the magnitude of the sensitivity to the drug. It is evident that, when only a single drug is required for inactivation ($n = 1$), the equation reduces to an exponential first-order process:

$$\frac{N}{N_0} = e^{-VD} \tag{2}$$

A survival curve is then simply a plot of those units which remain functional after treatment with the drug. In the present case the relative rates of polyphenylalanine synthesis are a direct measure of N/N_0. The curve shown in Figure 1 is first order suggesting that binding of one tetracycline inactivates one ribosome. For comparison the curves calculated for higher numbers of binding events to bring about inactivation are shown also in Figure 1 and demonstrate pronounced shoulders which are not observed in the data. This does not necessarily indicate that only one drug binds per ribosome, only that interaction with one drug molecule is sufficient to inhibit one ribosome.

The effect of Mg^{2+} and tetracycline on polyphenylalanine synthesis is shown in Figure 2. As shown by other workers (e.g., Hierowski, 1965) the amount of synthesis falls off on either side of a Mg^{2+} optimum. The presence of 10 μM tetracycline shifts the Mg^{2+} optimum slightly upward, and at high Mg^{2+} levels the drug is no longer inhibitory. Hierowski (1965) did not observe a shift in the Mg^{2+} optimum in the presence of tetracycline perhaps because an "S-30" fraction was used and not purified ribosomes.

Equilibrium Binding Studies. The equilibrium binding properties of the tetracycline–ribosome interaction were quantitated by employing the visible fluorescence of the drug. The intrinsic binding constant was obtained in the first instance by extrapolating to infinite ribosome concentration using an equation modified from Bloomfield et al. (1974).

$$\frac{1}{(F_{ap} - F_f)} = \frac{1}{\Delta F C_R^\circ B_{ap} K_{ap}} + \frac{1}{\Delta F} \tag{3}$$

Here F_f and F_{ap} are the measured fluorescence intensities of free tetracycline and tetracycline at varying concentrations of 70S ribosomes, ΔF is the fluorescence difference between free and completely bound tetracycline, B_{ap} is the apparent

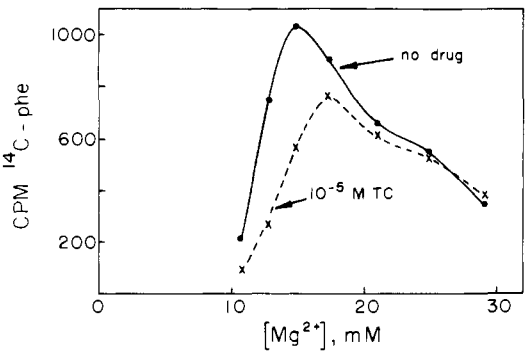

FIGURE 2: The Mg^{2+} dependence of the extent of polyphenylalanine synthesis in the presence (X) and absence (●) of 10^{-5} M tetracycline. The standard assay system (Materials and Methods) was used at the indicated Mg^{2+} concentrations.

TABLE I: Binding of Tetracycline to 70S Ribosomes in Buffer I.[a]

Tetracycline/ ribosome (molar input ratio)	No. of tetracycline bound per ribosome	
	Nitrocellulose filtration	Sedimentation
2	1.2	
4	0.6	
7	1.1	
10	0.9	
13	1.5	
8.0		1.0
11.0		1.0
42		1.5

[a] In the nitrocellulose filtration experiments free and ribosome bound tetracycline are separated by filtration through 0.45-μm Millipore HAWP filters. In sedimentation experiments the ribosome bound drug is in the pellet while free tetracycline remains in the supernatant.

number of binding sites per ribosome, K_{ap} is the intrinsic binding constant, and $C_R°$ is the total molar concentration of ribosomes (this formulation is valid under conditions such that $B_{ap}(C_R° - C_B) \sim B_{ap}C_R°$, where C_B is the concentration of bound tetracycline). A plot of $1/(F_{ap} - F_f)$ vs. $1/C_R°$ is shown in Figure 3, from which a value of 5.8×10^4 M^{-1} for $B_{ap}K_{ap}$ is calculated (range 4.4×10^4 to 7.2×10^4 for three determinations).

In order to interpret the temperature-jump experiments described below and to calculate the binding constant K_{ap}, a determination of the number of binding sites, B_{ap}, is necessary. To measure the amount of bound antibiotics at a particular ribosome concentration directly, two methods were used to separate free and bound tetracycline (see Materials and Methods). Table I shows that using either nitrocellulose filtration or sedimentation only about one tetracycline is strongly bound even at input ratios up to 42 drug molecules per 70S ribosome. Consequently the binding constant K_{ap} is 5.8×10^4 M^{-1}.

Equilibrium binding parameters were also obtained from fluorescence titration data by using a Scatchard plot (Scatchard, 1949). Figure 3 (insert) shows such a plot of r/C_F vs. r where r is the fraction of sites which are occupied ($r = C_B/C_R°$) and C_F is the concentration of free drug. Although there is typically some scatter in the points, the calculated constants ($B_{ap} = 0.6$; $K_{ap} = 4.1 \times 10^4$) are in reasonable agreement with those obtained by the method above. Hence the two alternative approaches give a self-consistent picture of the tetracycline-ribosome binding equilibrium.

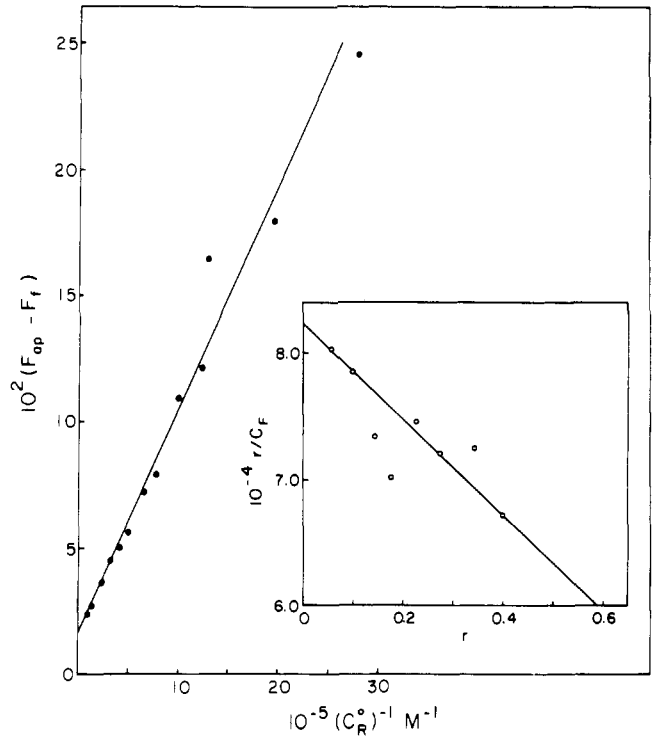

FIGURE 3: Double-reciprocal plot of fluorescence vs. concentration according to eq 1. The value for $K_{ap}B_{ap}$ is 5.8×10^4 M^{-1} at $T = 25$ °C in buffer I. Tetracycline fluorescence was detected at 510 nm with 370-nm excitation. Insert: Scatchard plot of fluorescence titration data $r/C_F = K_{ap}(B_{ap} - r)$.

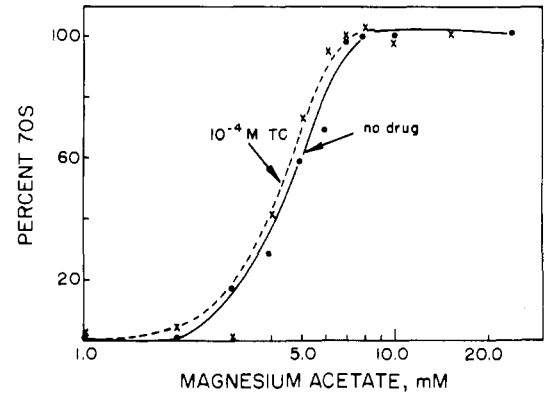

FIGURE 4: The percentage of 70S ribosomes in buffer I at the indicated Mg^{2+} concentrations. Temperature = 20 °C. The data were obtained from equilibrium light-scattering measurements according to Debey et al. (1975) as described in Materials and Methods. The solid line is 5 A_{260} units per mL of ribosome and the dashed line is the same plus 0.1 mM tetracycline.

The Effect of Tetracycline on the Subunit Equilibrium. Both in vivo and in vitro the 70S ribosome is in reversible equilibrium with free subunits, the distribution depending on the Mg^{2+} concentration and other solution conditions (Zitomer and Flaks, 1972; Debey et al., 1975). It seems plausible that an inhibitor of ribosome function might alter the subunit association reaction. To test this possibility, the extent of association as a function of Mg^{2+} was monitored by the light scattering technique (Debey et al., 1975). Figure 4 shows that, even in the presence of 0.1 mM tetracycline (~700 TC/ribosome), the subunit reaction is not significantly perturbed.

Unfolding of the Ribosome. The unfolding or denaturation of the ribosome can conveniently be measured by monitoring the absorbance of the RNA (260 nm) as a function of tem-

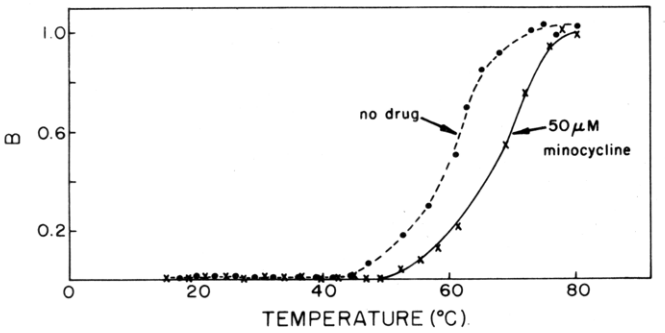

FIGURE 5: Thermal denaturation profiles of ribosomes in the presence (X) and absence (●) of 50 μM minocycline. The RNA absorbance at 260 nm was measured as a function of temperature and plotted as described in Materials and Methods. Each of the tetracycline congeners shifted the transition midpoint to higher temperatures (see Table II). Minocycline caused the largest shift in T_m and it is shown alone for illustrative clarity.

TABLE II: The Effect of Tetracycline Congeners on the Unfolding of Ribosomes.

Congener	T_m[a]	ID_{50}[b]
Tetracycline	62	14.8
Oxytetracycline	63	12
Demethylchlortetracycline	68	6.8
Chlortetracycline	64	5.8
Methacycline	66	4.8
Minocycline	68	3.2
No drug	61.5	

[a] T_m = midpoint of thermal transition. [b] ID_{50} = concentration required for half-maximal inhibition of polyphenylalanine synthesis under standard conditions.

perature. The midpoint of the transition (T_m) is then an index of the thermal stability under the particular conditions employed. As shown in Figure 5 and Table II, the binding of tetracycline and its congeneric derivatives alters the thermal stability of ribosomes. In addition, for the series of tetracycline congeners there is a good correlation between the ability to confer thermal stability to the ribosome and the relative potency in inhibiting in vitro polypeptide synthesis (Table II). That is, the most potent drug (minocycline) is most effective at raising the T_m of the ribosomes. Only demethylchlortetracycline does not follow the pattern, being of intermediate pharmacologic potency but as effective as minocycline at raising the transition temperature. Notably, the three congeners which have a methyl group on the C ring of the molecule (tetracycline, chlortetracycline, and oxytetracycline) are relatively ineffective at raising the T_m as compared to those which have a hydrogen atom (demethylchlortetracycline and minocycline) or are planar $=CH_2$ (methacycline). This is probably the result of a stereospecific binding interaction which is somewhat inhibited by the presence of a bulky group ($-CH_3$) out of the plane of the ring system. The crystal structure and ring nomenclature for tetracycline are those given by Stezowski (1976).

Temperature-Jump Studies. Little information on the rates and molecular mechanisms of drug–ribosome interactions is available. Because such reactions are generally quite rapid, I have used the temperature-jump relaxation technique to study the kinetics of tetracycline binding to the 70S ribosome. Following a rapid temperature perturbation, two well-resolved relaxation times can be extracted from the variation in the fluorescence intensity with time (Figure 6). In the simplest interpretation, the concentration dependence (Figure 7) of

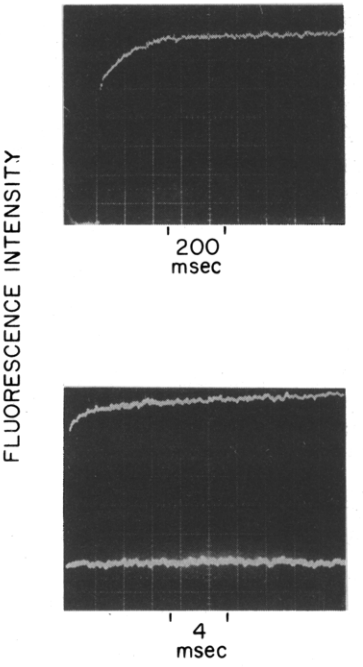

FIGURE 6: Oscilloscope traces of the change in tetracycline fluorescence following a rapid temperature jump. The vertical axis is an arbitrary scale of fluorescence intensity and the horizontal axis is a time scale in milliseconds. The upper and lower traces show τ_{slow} and τ_{fast}, respectively. The conditions are 3.1×10^{-6} M 70S, 2×10^{-5} M tetracycline in buffer I, final temperature = 25.2 °C.

these relaxation times suggests that the following mechanism applies:

$$TC + 70S \underset{K_1}{\overset{slow}{\rightleftharpoons}} complex \underset{K_2}{\overset{fast}{\rightleftharpoons}} complex \qquad (4)$$

That is, a slow bimolecular absorption of the drug onto the ribosome is followed by a rapid structural rearrangement of the antibiotic–ribosome complex. Standard techniques (Eigen and DeMaeyer, 1963) allow derivation of two equations for the concentration dependences of the relaxation times for this mechanism:

$$1/\tau_{slow} = k_1(TC + 70S) + k_{-1}\left(\frac{1}{1 + K_2}\right) \qquad (5)$$

$$1/\tau_{fast} = k_2 + k_{-2} \qquad (6)$$

where

$$K_1 = k_1/k_{-1} \qquad (7)$$

$$K_2 = k_2/k_{-2} \qquad (8)$$

and the overall equilibrium constant is then

$$K_{ap} = K_1(1 + K_2) \qquad (9)$$

Equations 5 and 6 predict the concentration behavior shown by the experimental data in Figure 7 and thus the mechanism shown is an adequate description on the kinetics. There is no closed analytical solution to eq 5 and 6, but the rate and equilibrium constants have been obtained from the data by taking advantage of mathematical relationships between the elements of the determinant which define the relaxation spectrum (see Crothers, 1971, for details). These constants are given in Table III.

Discussion

Equilibrium Binding and Fast Kinetic Studies. Apparently because previous workers (Maxwell, 1968; Day, 1966 a,b) have

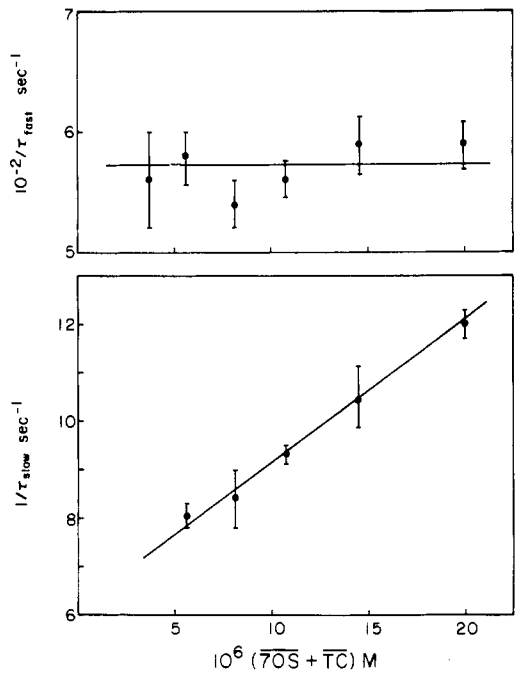

FIGURE 7: The concentration dependencies of τ_{fast} (upper panel) and τ_{slow} (lower panel). Each point represents an average of two to eight analyzed oscilloscope traces, the error bars being the standard deviation from the mean. The concentration variable (70S + TC) is the sum of the equilibrium concentrations of the two species calculated from the binding constant, K_{ap}.

described multiple binding of tetracycline to *B. megaterium* and rat liver ribosomes, there are no published quantitative studies using ribosomes from *E. coli*. The results from the dose-response studies (Figure 1) show that inhibition of a single *E. coli* ribosome is accomplished by interaction with a single drug. One cannot deduce from studies on this type if multiple sites exist, only that a single "hit" by the drug is effective. The fact that a *single* specific site exists is demonstrated by the binding studies shown in Table I and Figure 3; even at very high input ratios of drug to ribosome only one drug binds tightly with most of the tetracycline remaining free in solution.

Knowing the number of binding sites allows calculation of an intrinsic binding constant (see above) extrapolated to the condition of complete binding ($r \rightarrow 0$, where r is the fraction of sites which are occupied). In the kinetic experiments at an intermediate extent of binding ($r = 0.44$), the overall equilibrium constant calculated from the data (4.4×10^4 M^{-1}) is in good agreement with that obtained by equilibrium methods (5.8×10^4 M^{-1}). This lends confidence to the interpretation of the kinetics and suggests that the nature of the binding does not change with r just as one would expect for a single binding site per ribosome. Multiple or interacting binding sites, on the other hand, could show different intrinsic binding properties as a function of the extent of binding.

Using the K_{ap} it is possible to calculate the concentrations of free and bound drug at any input concentration of total ribosome and tetracycline. Such a calculation for tetracycline at 14.8 μM (equal to the ID_{50}) and 2.5 μM ribosome (concentration used in dose-response experiments) yields a value of $r = 0.4$. This is in reasonable agreement with the r value predicted ($r = 0.5$) by a single binding site model at a drug concentration which leads to half maximal inhibition (ID_{50}). Thus, the equilibrium binding and fast kinetic studies are in accord with the notion that a single drug molecule inhibits a single ribosome.

TABLE III: Kinetic and Equilibrium Constants for the Interaction of Tetracycline with 70S Ribosomes in Buffer I at 25.2 °C.

$$TC + 70S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{complex} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{complex}^1$$

k_1	$= 2.85 \times 10^5$ M^{-1} s^{-1}
k_{-1}	$= 15$ s^{-1}
k_2	$= 315$ s^{-1}
k_{-2}	$= 236$ s^{-1}
K_1	$= k_1/k_{-1} = 1.9 \times 10^4$ M^{-1}
K_2	$= k_2/k_{-2} = 1.33$
K_{ap}	$= K_1(1 + K_2) = 4.4 \times 10^4$ M^{-1}
T	$= 25.2$ °C

Indirect kinetic results for drug-ribosome interactions obtained by measuring the binding constant and dissociation rate ($k_1 = K_{ap}k_{-1}$) have been reported for streptomycin (Chang and Flaks, 1972) and erythromycin (Langlois et al., 1976), although this technique does not reveal the presence of mechanistic intermediates (the T-jump results, however, do demonstrate a conformational rearrangement of the drug-ribosome complex; see eq 4). Comparing the three drugs, tetracycline and streptomycin have an "on" rate of about 10^5 M^{-1} s^{-1} while erythromycin is about tenfold slower. These rates are at least four orders of magnitude slower than expected for a diffusion-controlled reaction. This is perhaps not surprising when one considers the very small solid angle and restricted orientation of the drug binding site on the ribosome. The rate-limiting dissociation step for both streptomycin and erythromycin is quite slow, on the order of 0.01 s^{-1}. Tetracycline, on the other hand, dissociates from the ribosome much more rapidly ($k_{-1} = 15$ s^{-1}) and consequently has a lower binding affinity.

Role of Mg^{2+} in Tetracycline Action. It has been postulated that the inhibitory effect of tetracycline may involve chelation of Mg^{2+} ions (Albert, 1958; White and Cantor, 1971; Fey et al., 1973). There are several ways in which this might be possible. The drug could simply act by nonspecifically chelating free Mg^{2+} ions thereby reducing the concentration available for stabilization of the ribosome. Tetracyclines do have a high tendency to form a complex with divalent ions in a 1:1 stoichiometry (Dürckheimer, 1975). However, at the concentrations employed both in vitro and in vivo the amount of drug is much less than 1% of the amount of Mg^{2+}, making direct reduction of the concentration of this cation an unlikely mechanism for tetracycline action.

It is also possible that a single magnesium ion simultaneously binds a tetracycline molecule and a negatively charged center on the ribosome. Put differently, Mg^{2+} ions may form a connecting bridge between the drug and the ribosome. It has been shown that tetracycline does not bind to DNA or albumin in the absence of divalent ions (Kohn, 1961) suggesting such a bridging role for metals. This experiment cannot be done directly with ribosomes because removal of Mg^{2+} destroys the organelles structural integrity, but, from relative fluorescence studies, Fey et al. (1973) concluded that Mg^{2+} bridges might be involved in the binding of tetracycline to the 50S subunit. However, the great excess of total divalent ions over tetracycline coupled with a moderate association constant (2.5×10^3 M^{-1}, White and Cantor, 1971) ensures almost complete complexation of this antibiotic with Mg^{2+} at concentrations which are normally employed. Hence it is likely that the metal-complexed form of the drug is the form that interacts with the ribosome. It is also plausible that such a complex forms an electrostatic bridge through the Mg^{2+} to a negative

charge on the ribosome, but this is only a consequence of the binding equilibrium and not the direct reason for inhibition by the drug.

A direct role for Mg^{2+} chelation in the inhibitory action of tetracycline could be complexation of essential ribosome bound Mg^{2+} ions. Since it is clear from the data in this paper that inhibition of a single ribosome occurs via a single tetracycline, then the chelation hypothesis would require a highly specific Mg^{2+} interaction at the drug sensitive site on the ribosome. Presumably the role played by Mg^{2+} in the ribosome is to maintain essential secondary structure of rRNA and to stabilize certain RNA–protein electrostatic interactions. Although no evidence for special classes of Mg^{2+} sites exists, one could imagine that disruption of a particular metal ion site would alter a singularly important interaction among the ribosomal components.

Two experimental results argue against this chelation mechanism for tetracycline action. First of all the drug does not appreciably alter the Mg^{2+}-dependent subunit association reaction. If Mg^{2+} essential to the subunit interaction was being chelated, the titration curve might be expected to have a midpoint at higher Mg^{2+} concentration than the control to offset this effect. This is not observed. Secondly, tetracycline, like Mg^{2+}, stabilizes the ribosome from thermal denaturation. Again, if chelation of essential bound Mg^{2+} was important, this would be expected to destabilize the ribosome, thereby lowering the T_m. In fact the opposite is found and the ability of the drug to stabilize the ribosome is nicely correlated with the inhibitory activity of a congeneric series of tetracyclines.

Stabilizing the ribosome structure may be an important component of the inhibitory action of the drug. Much evidence exists suggesting that the ribosome is conformationally flexible and that this flexibility is important in function (Tritton and Crothers, 1977; Schrier and Noll, 1971; Noll and Noll, 1976). Binding of aminoacyl-tRNA, which is blocked by tetracycline, may require a structural rearrangement which, because of increased stability, is antagonized by the drug. Notably, at high Mg^{2+} concentrations where polyphenylalanine synthesis is decreased even in the absence of drug, tetracycline has no inhibitory action. Since this inhibition by higher levels of Mg^{2+} may also reflect an "overstabilization" of structure thus preventing the conformational motions required for polypeptide synthesis, tetracycline no longer has the stabilizing increment necessary to provoke ribosomal inhibition.

Acknowledgments

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