# Interaction of Tetracyclines with Ribosomal Subunits from *Escherichia coli*. A Fluorometric Investigation†

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ABSTRACT: The three tetracycline derivatives, used in these experiments inhibit growing bacterial cultures and cell-free synthesis of protein in the following order: oxytetracycline strongest, tetracycline methiodide medium, and tetracyclinenitrile lowest. Interaction of these derivatives with 70S ribosomes, 50S and 30S subunits results in a considerable enhancement of the fluorescence of the tetracycline molecules. The analysis of the fluorescence curves allows to differenciate between the strong interaction by which about one molecule binds to the 50S and about three molecules bind to the 30S subunit and the weak interaction by which more than 100 molecules can interact with the 70S ribosome or subunits. The results indicate that upon interaction with the 50S subunit in the strong mode oxytetracycline gains a double fluorescence increase caused by $Mg^{2+}$ chelation bridges in addition to other ribosomal binding forces. Tetracycline–nitrile interacts in the strong binding mode only with the 30S subunit and the 70S ribosome and can thus be used to discriminate the strong binding sites at the 30S and 50S subunits and the strong and weak interactions at the 30S particle. The weak association with the 50S and 30S subunits and the strong interaction with the 50S subunit reflect well the differences in the inhibitory potencies of the three tetracycline derivatives.

Tetracyclines inhibit protein synthesis by inactivating ribosomal functions (Vazquez, 1964; Wolfe and Hahn, 1965). From interference of the antibiotics with initiation and elongation steps in cell-free systems of peptide synthesis, it was concluded that tetracyclines block the incoming site (A site) of the 30S ribosomal subunit for aminoacyl-tRNA (see Pestka, 1971, for summarizing references). Type and intensity of the inhibitory effects in the systems were markedly concentration dependent: at low tetracycline concentrations no preferential inhibition of any specific ribosomal site could be detected (Igarashi and Kaji, 1970). The relative concentration ratios of tetracycline to ribosome used in the *in vitro* systems were generally much higher than the corresponding values in the cells. Thus the available biological tests of ribosomal functions lack sensitivity in the most important range of inhibitor concentrations and tetracycline to ribosome concentration ratios. At present it is not clear what are the details of the mode of binding to ribosomes with respect to the biological significance of the tetracycline action.

Binding studies with labeled tetracycline revealed that the drug exhibits two types of binding: one type of strong interaction by which about one molecule or less is bound per ribosome and one other type of weaker, reversible association by which up to 300 molecules can bind per ribosome (Maxwell, 1968; Connamacher and Mandel, 1965; Day, 1966a,b). From a fluorescence analysis of tetracycline binding to 70S ribosomes (White and Cantor, 1971), which was published while this work was under way (Fey *et al.*, 1971), White and Cantor concluded that the strong binding of tetracyclines to 70S ribosomes involves chelation to the rRNA phosphate groups *via* $Mg^{2+}$.

In this work interaction of three tetracycline derivatives with 70S ribosomes and 30S and 50S ribosomal subunits were studied fluorometrically down to inhibitor concentrations of $10^{-7}$ M and tetracycline to ribosome ratios of 0.1. The derivatives used differ by one to two orders of magnitude in their biological activities. They were found to exhibit nearly identical absolute fluorescence emission intensities which are enhanced by $Mg^{2+}$ to the same degree. The tetracyclines are nearly free in solution at 0.1 mM $Mg^{2+}$ and chelated to $Mg^{2+}$ at 10 mM $Mg^{2+}$. These properties allowed us to study in more detail the interaction of these antibiotics with ribosomes and to ask: (1) whether $Mg^{2+}$ ions are of importance in the strong binding at the 30S or at the 50S subunit, (2) whether the strong and weak binding sites at the two ribosomal subunits can be discriminated, (3) whether the strong or weak interactions with the 50S or 30S ribosomal subunits are of biological significance.

## Materials and Methods

Oxytetracycline and the derivatives, tetracyclinenitrile and tetracycline methiodide, were kindly supplied by Dr. Schmid-Thomé and Dr. Summ, Hoechst, Frankfurt. *Escherichia coli* (MRE 600) cells were a gift from Biochemica Boehringer, Tutzing. The bacteria were harvested in the last third of the exponential growth phase and stored at −35°. Aluminum oxide was purchased from Serva, Heidelberg; DNase I (bovine pancreas) from Worthington; other reagents from E. Merck, Darmstadt.

*Isolation of Enriched 70S Ribosomes, 50S and 30S Subunits.* Enriched 70S ribosomes were prepared according to Traub *et al.* (1971). As judged from runs in the analytical ultracentrifuge mainly 70S ribosomes were obtained contaminated by less than 20% of 50S and no 30S subunits. These 70S ribosomes were highly active (5–8 nmol of phenylalanine was incorporated per nmol of 70S ribosomes in 30 min).

Enriched 70S material was dialyzed for at least 4 hr in 10-ml samples against the dissociation buffer (0.01 M potassium phosphate–0.001 M $MgCl_2$–0.006 M mercaptoethanol, pH 7.6, at 4°); dialysis factor $\geq 10^6$. The samples were freed from aggregates by centrifugation for 10 min at 10,000*g* and

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TABLE I: Inhibitory Potencies of Tetracycline Derivatives.[a]

| | Tetracycline | Tetracycline Methiodide | Tetracyclinenitrile |
|---|---|---|---|
| *In vitro* peptide synthesis | | | |
| $K_i$ (M $\times 10^{-6}$) | 7.6 | 56 | 895 |
| Growth inhibiting concentrations ($\mu$g/ml) | | | |
| *Escherichia coli* | 1.25 | 250 | 250 |
| *Staphylococcus aureus* P 209 | 0.3 | 50 | 125 |

[a] Values taken from Summ and Christ (1967).

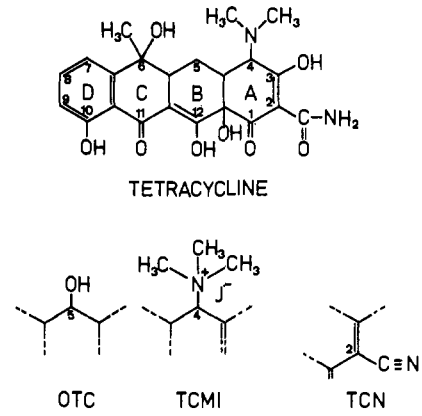


FIGURE 1: Chemical formulas of tetracycline, oxytetracycline (OTC), tetracycline methiodide (TCMI), and tetracyclinenitrile (TCN).

the $A_{260}$ was determined. Ultracentrifuge analysis proved complete dissociation of 70S ribosomes. The conditions of separation and purification of 30S and 50S ribosomes in the MSE B XIV zone rotor were chosen according to Eickenberry *et al.* (1970). Before use the subunits were dialyzed against standard buffer: 0.01 M Tris–0.05 M KCl–0.01 or 0.0001 M $Mg^{2+}$ (pH 7.2) at 20°; (dialysis factor $10^6$). After the following 10-min centrifugation at 10,000*g*, the concentration was determined. Typical recoveries of subunits at this stage were about 60% which is close to the value reported by Eickenberry *et al.* (1970). Molecular concentrations of ribosomes or subunits were calculated on the basis of the following relations: 10 mg (70, 50, or 30S) = 170 $A_{260}$; $10^{-6}$ M solutions thus contain 48, 32, or 16 $A_{260}$ per ml of these particles. An equimolar mixture at $1 \times 10^{-6}$ M corresponds to about 100 $A_{260}$ per ml.

*Preparation of Reassociated 70S Particles.* Pure subunits were mixed in equimolar concentrations, the $Mg^{2+}$ concentration was adjusted to 0.01 M and the mixture was incubated for 10 min at 37°. The reassociated 70S ribosomes were further isolated by sedimentation through a sucrose cushion as described for enriched 70S ribosomes. Analytical ultracentrifugation showed nearly quantitative reassociation.

*Fluorescence Binding Assay.* The measurements were performed with a Zeiss PMOII with fluorescence attachment which allows excitation at 366 nm. Tetracycline and ribosome stock solutions were equilibrated at 20° for 30 min. For every "complex sample" (tetracycline + ribosomes or subunits) two corresponding controls (free tetracycline and free ribosomes) were exposed to identical conditions. Ribosomes (about 0.001 mM) showed a weak emission band at 500 nm and some light scattering. This contribution did not exceed 1–10% of that of the tetracyclines but had to be eliminated. Readings of the emission intensities of a buffer blank and of a series of fluorescein standards in 0.1 N NaOH with respect to one another and to a glass standard (Zeiss F53-10560) were taken at the beginning of each series of experiments. For every measurement (30, 60, and occasionally 90 min after mixing ribosomes and tetracyclines), the samples were placed in the light beam and fluorescence emission was determined 15 sec later. This was necessary since the tetracyclines (especially when complexed to ribosomes) are uv sensitive. All readings were taken with a constant excitation slit width. In a series of samples with varying ratios of tetracyclines to ribosome only the inhibitor concentration was varied to keep constant the stray light contribution from the ribosomes.

## Results

The tetracycline derivatives oxytetracycline, tetracycline methiodide, and tetracyclinenitrile (Figure 1), exhibit correlated effects on growing bacteria and on bacterial *in vitro* systems of phenylalanine incorporation into poly(phenylalanine). They differ in their biological effects by two orders of magnitude (Table I).

Oxytetracycline exhibits an excitation maximum at 375 nm in standard buffer and a fluorescence emission of maximum intensity at 520 nm. Excitation at 366 nm used in these experiments is only slightly less effective. Upon excitation at this wavelength tetracycline methiodide and tetracyclinenitrile showed emission maxima at 525 nm. The fluorescence intensities $I$ of the three derivatives increase by 40% for a pH increase from 7.25 to 7.85. Because tetracyclines rapidly lose stability with increasing pH, measurements were made at pH 7.25. The direct temperature dependence of the fluorescence turned out to be negligibly small in the temperature range between +2 and +20°. For all measurements a temperature of 20° was chosen because ribosomes and tetracyclines are more stable at 20° than at 37°. The half-life of the drug is 7 days at 22° and 26 hr at 37° (Walter and Heilmeyer, 1969).

The dependence of the emission intensities on $Mg^{2+}$ concentration of the three derivatives was studied first since the interaction with undissociated 70S ribosomes was measured at 10 mM $Mg^{2+}$ and the interaction with the subunits at 10 mM $Mg^{2+}$ and 0.5 mM $Mg^{2+}$. The relative fluorescence intensity of oxytetracycline was found to be about five times higher at 10 mM than at 0.1 mM $Mg^{2+}$ (Figure 2). Maximum intensity is reached at $Mg^{2+}$ concentrations of 10 mM. White and Cantor (1971) obtained from fluorescence intensity changes of $3.85 \times 10^{-7}$ M tetracycline at pH 7.8 in function of the concentration of $Mg^{2+}$ a binding constant of $2.5 \times 10^{-3}$. The mathematical analysis of the curve shown in Figure 2 revealed a nearly identical binding constant for oxytetracycline. The binding of $Mg^{2+}$ to tetracycline and oxytetracycline is thus found to be relatively weak. At a constant concentration of, *e.g.*, oxytetracycline of $2 \times 10^{-6}$ M, nearly all drug molecules ($\geq 96\%$) are magnesium chelated at 10 mM $Mg^{2+}$, about 30% at 0.5 mM and nearly none ($\leq 4\%$) at 0.1 mM $Mg^{2+}$.

All three compounds exhibit nearly identical absolute emission intensities for a given inhibitor concentration at 10 mM $Mg^{2+}$ and at 0.1 mM $Mg^{2+}$ (Figure 3). The curves for all compounds have identical slopes. Thus the quantum yields of the three compounds free or chelated to $Mg^{2+}$ are nearly equal.

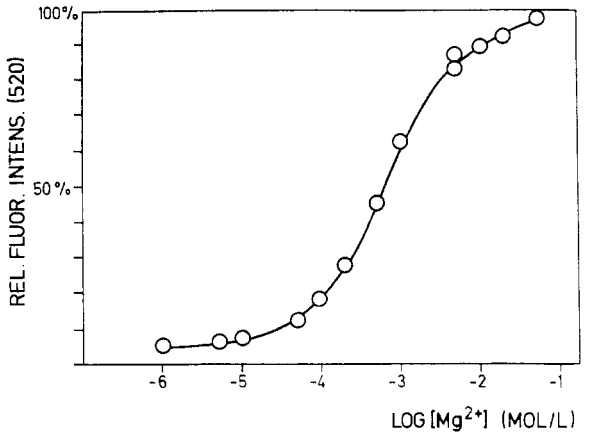


FIGURE 2: Mg dependence of oxytetracycline fluorescence. The drug concentration was $2 \times 10^{-6}$ M in 0.01 M Tris-HCl buffer (pH 7.2) at 20°. $Mg^{2+}$ concentration as shown on the abscissa.

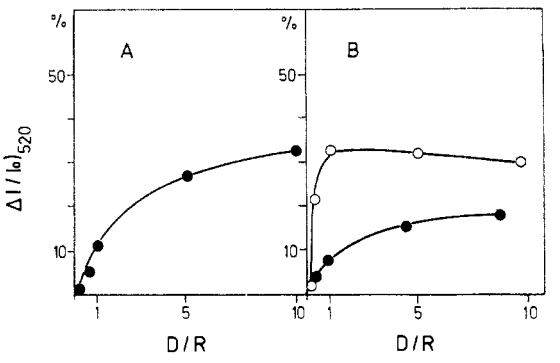


FIGURE 4: Relative fluorescence enhancements ($\Delta I/I_0$) of oxytetracycline by ribosomes or subunits. Standard conditions for fluorescence measurements: constant concentration of ribosomal particles $1 \times 10^{-6}$ M in 0.01 M Tris–0.06 M KCl–0.01 M $Mg^{2+}$ (unless otherwise stated), pH 7.2 at 20°. $D/R$ = molar concentration ratio drug to ribosomes. (A) 70S ribosomes; (B) 50S subunits (○–○) and 30S subunits (●–●). $\Delta I = I_c - I_0$; $I_0 = I_d + I_r - I_b$; $I_c$ = fluorescence intensity of the complex in % of the emission intensity of the fluorescein standard = 100%; $I_d$ = fluorescence intensity of free drug at the given concentration of $Mg^{2+}$; $I_r$ = fluorescence intensity of free ribosomes; $I_b$ = fluorescence intensity of the solute (buffer).

Association of ribosomal particles with oxytetracycline at 10 mM $Mg^{2+}$ results in a considerable enhancement of the emission intensity of the drug (Figure 4). If the relative fluorescence enhancement ($\Delta I/I_0$) (for definition, see legend of Figure 4) is plotted *vs.* the molar concentration ratio ($D/R$) of total drug to ribosome or subunits characteristic curves are obtained for 70S ribosomes, 50S, and 30S subunits. The relative fluorescence enhancements increase continuously with $D/R$ up to 10 for 70S and 30S particles and reach maximum values of 30% for 70S and 18% for 30S particles. With the 50S subunit a maximum of the relative enhancement of 30% is already reached for $D/R$ values of 1. Thus the maximum relative fluorescence enhancement for the 70S complex is closer to the value of the 50S complex than to the value of the 30S complex and always definitely lower than the sum of both. This was reproducible for different ribosome and subunit preparations.

Figure 5 shows the same experimental results for 50S particles in a plot of the absolute intensities (all calculated with respect to one fluorescein standard) *vs.* $D/R$. The curves for the fluorescence intensity of the complex ($I_c$) and $\Delta I$ (intensity of complex − intensity of the sum of the components $I_0$) start off slightly curved and converge only later to a straight line with a steeper slope than the straight line for $I_0$. Thus the plateau in the $\Delta I/I_0$ *vs.* $D/R$ plot does not indicate that oxytetracycline binding to ribosomes has reached saturation, but that on further binding of drug molecules to ribosomes or subunits the interaction must occur in the same well-defined binding mode. For 70S ribosomes the slope remains constant for raising ratios up to $D/R$ = 200. This corresponds to a certain quantum yield $q_b$ for each bound oxytetracycline molecule. The value of $q_b$ is proportional to the slope of the straight part of the $I_c$ *vs.* $D/R$ curve. $\Delta I/I_0$ values >0 simply signify that the quantum yield $q_b$ of a tetracycline molecule bound in this mode is greater than the quantum yield $q_f$ of a free drug molecule. When tetracycline binding reaches saturation, the curve $I_c$ *vs.* $D/R$ converges to the curve $I_0$ *vs.* $D/R$ and $\Delta I/I_0$ will decrease.

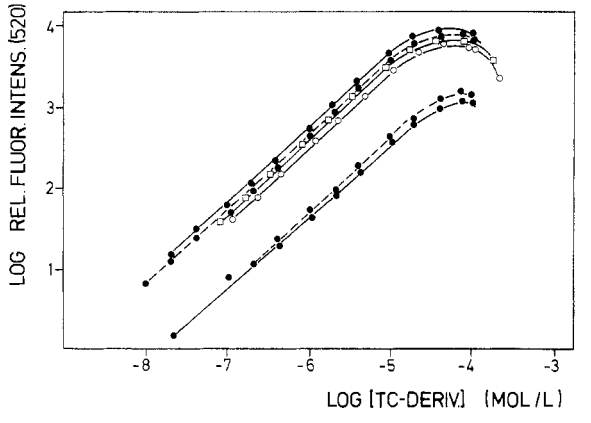


FIGURE 3: Fluorescence intensities of tetracycline derivatives *vs.* drug concentration. Upper four curves at $10^{-2}$ M $Mg^{2+}$, lower two curves at $10^{-4}$ M $Mg^{2+}$. Tetracycline (●—●), oxytetracycline (●– –●), tetracycline methiodide (□—□), and tetracycline nitrile (○–○) in $10^{-2}$ M Tris buffer (pH 7.2) at 20°.

The fluorescence changes with $D/R$ for the three derivatives upon addition of the 50S and 30S subunits were measured at 10 and 0.5 mM $Mg^{2+}$ (Figures 6 and 7).

The maximum relative fluorescence enhancements for the 50S subunit were found to be considerably higher at low levels of $Mg^{2+}$. This effect was much less pronounced in the case of the 30S subunit.

The plateau values of the relative fluorescence enhancements at 0.5 mM $Mg^{2+}$ upon complex formation with the 50S subunit at $D/R$ 1:1 are highest for oxytetracycline (145%), medium for tetracycline methiodide (75%) and zero for tetracyclinenitrile. Analogous experiments with the 30S subunits (Figure 7) reveal at $D/R$ ratios above 5, that oxytetracycline causes a relative increase by 40%, tetracycline methiodide by 30% whereas tetracyclinenitrile has no effect. However, in the $D/R$ regions between 0.1 and 1 tetracyclinenitrile shows a considerable fluorescence enhancement at high and also at low levels of $Mg^{2+}$ indicating interaction with the 30S ribosomal subunit. The curve for tetracyclinenitrile in Figure 7B should be viewed at as being shifted to the left with respect to the curves for the other two derivatives. This means slightly too high tetracyclinenitrile concentrations in this series of measurements for the concentrations of 30S subunits present in an active binding state. Such scale shifts are difficult to avoid completely when only biologically active subunit prep-

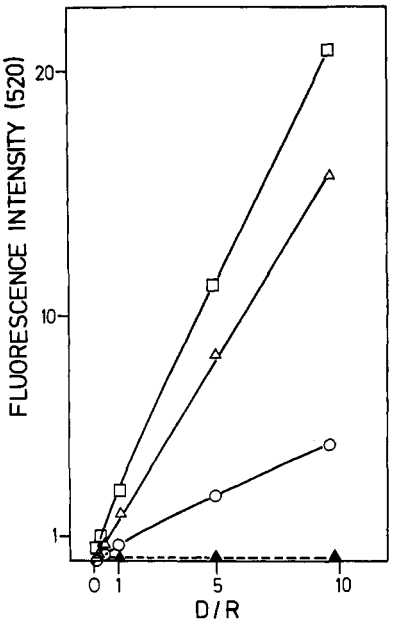


FIGURE 5: Absolute fluorescence intensities of raising concentrations of oxytetracycline in the presence of 50S ribosomal subunits. Standard conditions and abbreviations as for Figure 4. Fluorescene: Δ–Δ is free oxytetracycline, ▲–▲ ribosomal subunit, and □–□ oxytetracycline + 50S subunit. (○–○) Difference between complex and free drug ($\Delta I$).

arations are admitted for the fluorescence test. Active ribosomal subunit populations are not completely homogenous in their conformational state (Kurland, 1970).

With native and reassociated 70S ribosomes the influence on the fluorescence of the three tetracycline derivatives was measured only at 10 mM $Mg^{2+}$ to avoid dissociation of the ribosomes into subunits (Figure 8A,B). At a $D/R$ value of 10 the fluorescence enhancement of oxytetracycline is 30%, of tetracycline methiodide about 22%. Tetracyclinenitrile interacts with the 70S ribosome at low $D/R$ values as with the 30S subunits. Surprisingly corresponding experiments with 70S ribosomes reassociated from isolated 30S and 50S subunits show, that these particles behave like 50S particles and do not interact with tetracyclinenitrile.

## Discussion

Association of tetracyclines to bacterial ribosomes has been investigated by using radioactively labeled compounds (Maxwell, 1968; Day, 1966a,b; Connamacher and Mandel, 1965). Interaction was found with both the 30S and the 50S subunit. We have tried to determine the number of available binding sites at *E. coli* 70S ribosomes and subunits with tritiated tetracyclines, by means of equilibrium dialysis. During this process (16 hr at 1°) the 70S ribosomes had lost 90% of their activity (in cell-free synthesis of poly(phenylalanine)) and hence structural integrity. Thus these studies do not give precise information on the number of binding sites with respect to biological significance.

The fluorescence enhancement of tetracyclines upon association with ribosomes and ribosomal subunits permits binding to be followed quickly and directly. When the relative fluorescence enhancement $\Delta I/I_0$ is plotted *vs.* the molecular concentration ratio of total drug to total ribosomes or subunits ($D/R$), characteristic curves are obtained for the three tetracycline derivatives. Moreover the curves are different for

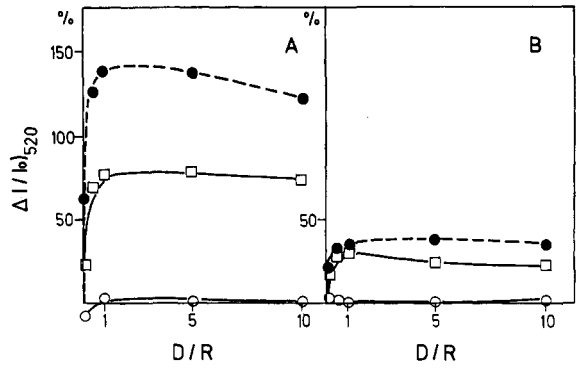


FIGURE 6: Fluorescence enhancements of tetracycline derivatives by addition of 50S ribosomal subunits at (A) $5 \times 10^{-4}$ M $Mg^{2+}$ and (B) $10^{-2}$ M $Mg^{2+}$. For standard conditions and abbreviations, see Figure 4. Oxytetracycline (●--●), tetracycline methiodide (□–□), and tetracyclinenitrile (○–○).

70S ribosomes, 50S, and 30S subunits. The characteristics of the fluorescence curves are: a region of a steep increase at $D/R$ ratios up to 1:1 for 50S and to about 3:1 for 30S and 70S particles and a plateau region at higher $D/R$ ratios. When the complexes of oxytetracycline with ribosomes were sedimented for 6 hr at high speed through 1.1 M sucrose, the fluorescence analysis revealed that after this process only a few molecules remain bound to the particles (G. Fey, unpublished results.) Day (1966a,b) suggested that on the average only one molecule of tetracycline is bound in a strong mode per 50S particle and about three times as much are bound to the 30S subunit. On the basis of these findings we take the region of steep increase in the $\Delta I/I_0$ *vs.* $D/R$ plots as reflecting the influence of the first strong and nearly irreversible binding type, whereas the plateau region as representing the second week and reversible mode of interaction.

White and Cantor (1971) concluded from a fluorescence analysis that $Mg^{2+}$ ions are involved in the strong interaction of tetracyclines with the ribosomes. When these authors studied the binding at a tetracycline to ribosome ratio of 1 and at 0.1 mM $Mg^{2+}$ (a concentration at which 70S ribosomes dissociate into subunits), they observed a threefold increase of the original fluorescence intensity of the drug. This fluorescence enhancement is comparable in magnitude with the value we have found upon interaction of oxytetracycline with the 50S subunit at 0.5 mM $Mg^{2+}$. The relative fluorescence enhancement $\Delta I/I_0$ is 150% (Figure 6). From this we can calculate the increase in the relative fluorescence intensities,

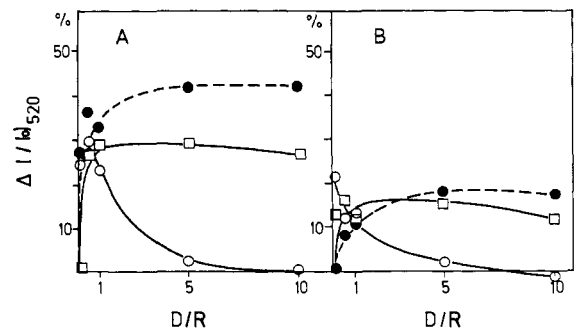


FIGURE 7: Fluorescence enhancements of tetracycline derivatives by 30S subunits at (A) $5 \times 10^{-4}$ M $Mg^{2+}$ and (B) $10^{-2}$ M $Mg^{2+}$. Standard conditions and abbreviations, see Figure 4. Symbols as in Figure 6.

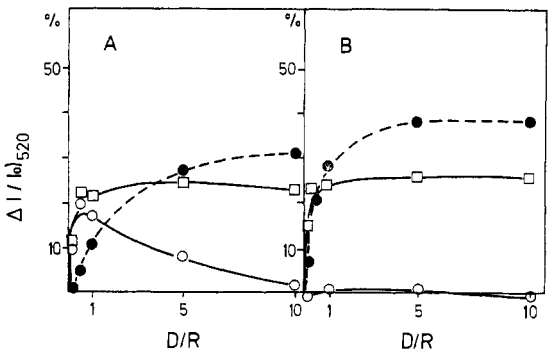


FIGURE 8: Fluorescence enhancements of tetracycline derivatives by (A) native 70S ribosomes and (B) reassociated 70S particles. Standard conditions and abbreviations, see Figure 4. Symbols as in Figure 6.

$I_c/I_0$ to be 2.5-fold. As can be seen from Figure 2 the transition of partially chelated (0.5 mM $Mg^{2+}$) to totally chelated (50 mM $Mg^{2+}$) oxytetracycline results in a threefold increase of the relative fluorescence intensity of the molecule. These facts indicate that a $Mg^{2+}$ chelation bridge might be involved in the strong binding of tetracyclines to the 50S subunit.

Binding studies with the 50S subunit at 10 mM $Mg^{2+}$ give evidence that oxytetracycline could gain an additional 30% fluorescence increase. Interactions other than chelation by $Mg^{2+}$ are therefore involved in this type of binding with the 50S particle. A similar two-step increase in the fluorescence intensity has been observed during the association of tetracyclines with membranes by Caswell and Hutchison (1971). The results were interpreted as a transition from free to $Mg^{2+}$-chelated and from $Mg^{2+}$-chelated to membrane-bound tetracycline.

The fluorescence enhancement for oxytetracycline upon association with the 30S subunit at 0.5 mM $Mg^{2+}$ is about 30% and at 10 mM $Mg^{2+}$ about 10% higher than the original fluorescence (Figure 7). Since at 10 mM $Mg^{2+}$ nearly all tetracycline molecules are in the chelated state they are already immobilized and can not gain as much fluorescence increase than the free molecules upon binding to the subunit.

The fluorescence of tetracyclinenitrile is enhanced only in the *D/R* region from 0.1 to 5 upon addition of 30S subunits or native 70S ribosomes. From a comparison of the fluorescence curves of the complexes of ribosomal particles with tetracyclinenitrile and oxytetracycline, we conclude that (a) the strong and weak binding sites at the 30S subunit and (b) the strong binding sites at the 30S and 50S subunits can be discriminated and must therefore be different.

Since tetracyclinenitrile does not inhibit cell-free synthesis of polypeptides in the low concentration range the strong binding site for tetracycline at the 30S subunit is probably not important for ribosomal function in cell-free systems. On the other hand, the relative fluorescence enhancements reflecting the weak binding mode with 50S or 30S subunits and the 70S ribosome agree well with the order of inhibitory potencies of the three tetracycline derivatives. This is also relevant for the strong binding site at the 50S subunit.

The differences in the fluorescence curves of the tetracycline complexes with the 70S native ribosome and the 70S reassociated particles (Figure 8) could probably be ascribed to a conformational difference between 70S native and 70S reassociated particles.

From this considerations it is evident that tetracycline derivatives could be of importance to elucidate specific sites at the ribosomal particles. Moreover tetracycline derivatives could become useful to probe structural and functional integrity of the ribosomes.

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