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EQUILIBRIUM AND KINETIC STUDIES ON THE INTERACTION OF TETRACYCLINES WITH CALCIUM AND MAGNESIUM

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The interaction of Ca^{2+} and Mg^{2+} with three Tetracycline antibiotics (tetracycline, chlorotetracycline, and oxytetracycline) has been investigated. Spectrophotometric measurements have been used to determine the apparent association constant for this interaction as a function of pH. It is shown that the results are consistent with a model in which the metal ion can form complexes with both the fully-deprotonated and mono-protonated forms of the Tetracycline. The temperature-jump relaxation method has been used to measure the kinetics of formation of the complexes of Mg^{2+} with the Tetracyclines. The results are compared with those of previous studies of Mg^{2+} complex formation reactions and it is shown that the data is consistent with the normal dissociative model. A possible role for metal ion chelation in the mechanism of antibacterial action of the Tetracyclines is discussed.

1. Introduction

The Tetracyclines are broad spectrum antibiotics of considerable clinical usefulness. The mechanism of the antibacterial action of the Tetracyclines is associated with the inhibition of protein synthesis on ribosomes. Thus it has been demonstrated, both in vitro [1] and in vivo [2] that Tetracyclines block the binding of amino-acyl-tRNA to the ribosomal A site.

Since the Tetracyclines chelate certain divalent cations, including magnesium [3], it has been suggested [4,5] that chelation may be important in the inhibition of ribosome function. Whilst most workers have attempted to exclude the involvement of chelation [6,7] the existence of a correlation between chelating ability and biological effectiveness has been reported [8]. In addition to their usefulness as antibacterial agents, certain of the Tetracyclines, notably the 7-chloro derivative, have been extensively used as a fluorescence probe for calcium [9,10].

The identity of the ligand atoms involved in chelation has been the subject of much controversy. (The basic structure of the Tetracyclines is shown in fig. 1). For example, Conover [11] proposed chelation through oxygen coordination to the BCD chromophore

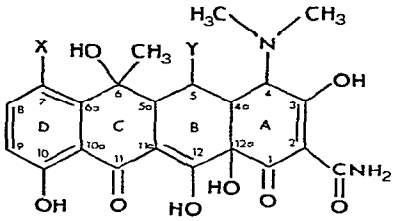

Fig. 1. Structure of the Tetracycline antibiotics (tetracycline, X = Y = H; oxytetracycline, X = H, Y = OH; chlorotetracycline, X = Cl, Y = H).

(at C_{11} and C_{12}) and this proposal has been supported by extensive circular dichroism measurements [12,13] and other studies [14,15]. On the other hand, Baker et al. [16] proposed A-ring coordination through oxygen atoms of the tricarbonylmethane group (at C_2 and C_1 or C_3) and this proposal has in turn been supported by NMR measurements in DMSO solutions [17]. A-ring coordination through the dimethylamino group at C_4 has also been proposed [19], as has simultaneous coordination to both the A-ring and BCD chromophores [18,9].

Both the stoichiometry and extent of protonation

of the metal complexes formed in solution is also rather unclear. Successive ionizations of the protonated Tetracycline, H_3L^+ yields species which may be designated as H_2L, HL^- and L^{2-}. It has been suggested [8] that the Tetracyclines can form MHL and ML simultaneously with some metals but only ML[8] *or* MHL [4,8,9] with others. In addition, the formation of 1:2 complexes, ML_2 has been reported [4,19] and Stezowski has suggested [15] that M_2L may exist.

In an attempt to resolve some of these differences, especially with regard to the type of complex formed, the apparent association constant for the interaction of Mg^{2+} and Ca^{2+} with three Tetracyclines has been measured as a function of pH. The data is consistent with the simultaneous existence of MHL and ML for each of the Tetracyclines investigated. The kinetics of interaction of Mg^{2+} with the Tetracyclines, measured by the temperature-jump relaxation method, are shown to be consistent with this model.

2. Materials and methods

The Tetracycline derivatives were obtained from Sigma Chemical Co., as the hydrochloride salt, and used without further purification. Because Tetracyclines undergo both photochemical and oxidative degradation [15], all solutions were freshly prepared immediately prior to use and kept in the dark whenever possible. Analar reagent magnesium and calcium nitrate were obtained from BDH Chemicals Ltd. and standardized volumetrically by titration with disodium EDTA. The solutions for the kinetic and equilibrium measurements were prepared using doubly distilled water. An ionic strength of 0.15 M was mainatined with KNO_3 and solutions were buffered with Tris, Triethylamine or ammonia + HNO_3 (concentrations approximately 10^{-2} M). The kinetic and equilibrium behaviour was independent of the nature and concentration of the buffer used.

Association constants were measured spectrophotometrically by making use of the different absorption spectra of free and complexed Tetracyclines. The apparent association constant at given pH (in the range 6.5–8.5) was measured by adding aliquots of concentrated $Mg(NO_3)_2$ or $Ca(NO_3)_2$ to a known volume of the Tetracycline derivative (generally $\approx 5 \times 10^{-5}$ M) in a 1 cm cuvette maintained at 23.5 ± 0.2°C. No pH change occurred during the titration. Measurements were generally made at 390 nm and the data were analysed by the method of Scatchard [20].

Values for the pK's of the Tetracyclines were also measured spectrophotometrically, from the pH dependence of absorption spectra. pK_2 and pK_3 were determined (at 23.5°C, $I = 0.15$ M) by measuring the optical density at 390 nm of a solution of the Tetracycline (generally $\approx 5 \times 10^{-5}$ M) in the pH ranges 5.4–7.2 and 9.2–10.3 respectively. In the determination of pK_3 the adjustment of the solution to high pH was made immediately prior to measurement in order to minimize the slow, time-dependent degradation of the Tetracycline. All spectrophotometric measurements were made on a Cary 118 spectrophotometer.

The kinetic data were obtained at 23.5°C ($I = 0.15$ M) in a temperature-jump spectrometer (Messenlagen Studiengesellschaft). Concentration changes following the temperature jump were monitored by transmittance at 390 nm. After equilibration at 18.5°C, a temperature-jump of 5 ± 0.2° was made by means of a calibrated high voltage discharge (20 KV). The resultant relaxation trace was analysed by comparison with an electronically generated exponential of known time constant; values of τ^{-1} are the average of at least five individual determinations.

3. Results

3.1. Equilibrium measurements

The macroscopic pKa values of the Tetracyclines in aqueous solution are approximately 3.3, 7.4 and 9.4 [23,24]. A fourth pKa of 10.7 has also been reported [4]. These values correspond to successive ionizations of the protonated species H_3L^+

$$H_3L^+ \underset{3.3}{\overset{pK_1}{\rightleftharpoons}} H_2L \underset{7.4}{\overset{pK_2}{\rightleftharpoons}} HL^- \underset{9.4}{\overset{pK_3}{\rightleftharpoons}} L^{2-} .$$

In the pH range 5.4–7.2 only the species HL^- and H_2L exist in significant amounts and contribute to the measured absorbance. Then,

$$\frac{[TC]_0}{A_\lambda - \epsilon_{H_2L} \cdot [TC]_0} = \frac{1 + K_2[H]}{\epsilon_{HL^-} - \epsilon_{H_2L}}, \quad (1)$$

where $[TC]_0$ = total Tetracycline concentration, A_λ = absorbance at wavelength, λ, and the ϵ values are

molar extinction coefficients.

A value for ϵ_{H_2L} was obtained from the limiting value of A_λ at low pH and K_2 was determined from the plot of eq. (1) (fig. 2a).

At pH values $\geqslant$9.2 only the species HL^- and L^{2-} contribute to the measured absorbance. Then,

$$\frac{[TC]_0}{A_\lambda - \epsilon_{L^{2-}} \cdot [TC]_0} = \frac{1}{\epsilon_{HL^-} - \epsilon_{L^{2-}}} \cdot \left(1 + \frac{1}{K_3[H]}\right), \quad (2)$$

where $[TC]_0$ and A_λ have the meanings defined above. A value for $\epsilon_{L^{2-}}$ was obtained from the limiting value of A_λ at high pH and K_3 was determined from the appropriate plot (fig. 2b). The results for the Tetracycline derivatives examined are summarized in table 1. The values reported here are somewhat lower than those previously reported (consistent with the higher ionic strength of the medium used here) but the order of the basicity of these antibiotics on the basis of pK_3, is the same as that previously reported, viz:

tetracycline > chlorotetracycline > oxytetracycline .

The metal binding equilibria were studies by measuring the apparent association constant (K_{app}) as a function of pH in the range 6.5–8.5. The data has

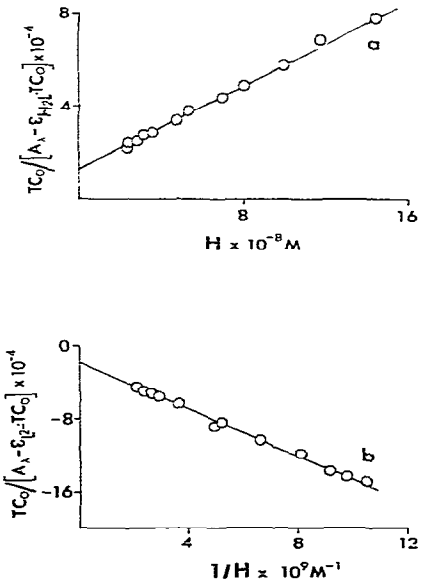

Fig. 2. Determination of pK_2 (a) and pK_3 (b) for Tetracycline. See text for details.

Table 1
pKa values for Tetracycline hydrochlorides in aqueous solution at 23.5°C (I = 0.15 M)

Derivative	pK_1	pK_2	pK_3
Tetracycline	(3.42) [a]	7.50(0.03)	9.13(0.04)
Chlorotetracycline	(3.26) [a]	7.35(0.04)	8.89(0.03)
Oxytetracycline	(3.26) [a]	7.35(0.04)	8.77(0.02)

[a] Values for pK_1 are from ref. [8].

been analysed in terms of a model involving simultaneous coordination of HL^- and L^{2-} to the metal ion, according to the scheme shown below,

$$\begin{array}{ccc} H_2L & & \\ \Big\Downarrow pK_2 & & \\ HL^- + M^{2+} & \overset{K_{MHL}}{\rightleftharpoons} & (MHL)^+ \\ \Big\Downarrow pK_3 & & \\ H^+ + L^{2-} + M^{2+} & \overset{K_{ML}}{\rightleftharpoons} & (ML)^0 \end{array} .$$

Kapp is related to the individual association constants, K_{ML} and K_{MHL}, by eq. (3)

$$K_{app} = \frac{K_{ML} + K_{MHL}K_3[H]}{1 + K_3[H] + K_2K_3[H]^2}, \quad (3)$$

which may be rewritten as,

$$\alpha K_{app} = K_{ML} + K_{MHL}K_3[H] , \quad (4)$$

with $\alpha = 1 + K_3[H] + K_2K_3[H]^2$, i.e. a function of pH. A plot of αK_{app} against $K_3[H]$ enables determination of K_{ML} and K_{MHL} from the intercept and slope respectively. If only MHL is formed ($K_{ML} = 0$) the intercept of this plot will be zero. If only ML is formed ($K_{MHL} = 0$), αK_{app} will be independent of $K_3[H]$.

The variation of K_{app} with pH for each of the systems investigated (fig. 3) is consistent with simultaneous formation of ML and MHL. The binding data were analysed as eq. (3) using a version of the CURFIT computer routine [21] which makes a least-squares fit to a non-linear function using the algorithm of Marquardt [21]. A simple linear least-squares fit to eq. (4) yielded identical results and the fitted plot of αK_{app} against $K_3[H]$ for Mg^{2+}/oxytetracycline is shown in fig. 4. The values obtained for K_{ML} and K_{MHL} are collected in table 2. The results are in general agreement with those previously reported in that

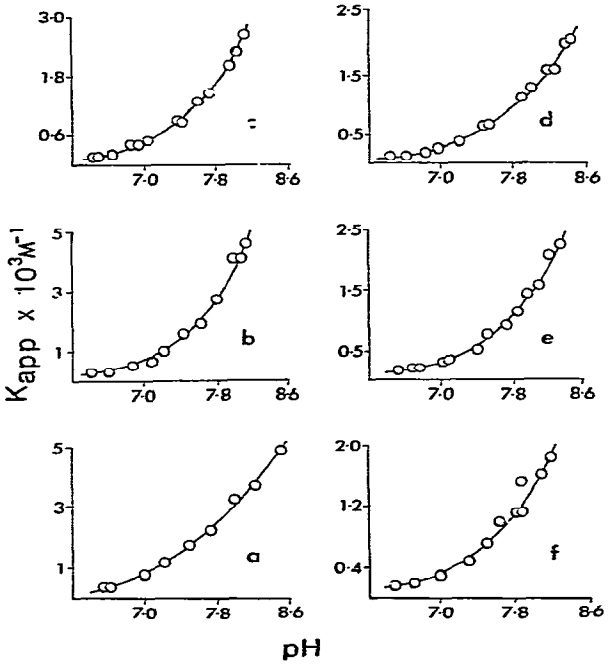

Fig. 3. Dependence of K_{app} upon pH for Mg^{2+} + tetracycline (a); Mg^{2+} + oxytetracycline (b); Mg^{2+} + chlorotetracycline (c); Ca^{2+} + tetracycline (d); Ca^{2+} + chlorotetracycline (e); Ca^{2+} + oxytetracycline (f). The solid lines show the computed best-fit to the data (see text for details).

the complexes formed with calcium are slightly weaker than those formed with magnesium [9,15] but the detailed pattern of coordination differs from that previously reported (see Discussion).

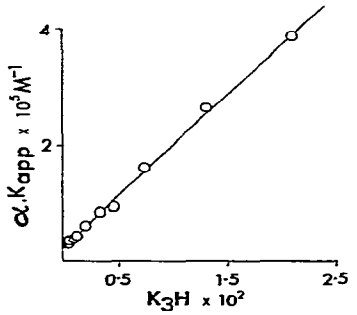

Fig. 4. Plot of αK_{app} against $K_3[H]$ according to eq. (4) for the interaction of Mg^{2+} with oxytetracycline.

Table 2
Values for the association constants of Tetracyclines with Mg^{2+} and Ca^{2+} at 23.5°C (I = 0.15 M)

Derivative	Log K_{ML}		Log K_{MHL}	
	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}
Tetracycline	4.19 (0.07)	3.96 (0.05)	3.50 (0.03)	3.04 (0.02)
Chlorotetracycline	4.08 (0.05)	3.94 (0.04)	3.16 (0.02)	2.92 (0.04)
Oxytetracycline	4.28 (0.04)	3.81 (0.07)	3.27 (0.03)	2.93 (0.02)

3.2. Relaxation studies

Measurements were made in the pH range 5.5–7.1 where two relaxation effects were observed. The faster of these (10–20 μs) could be observed in solutions containing no added metal ion, suggesting that it is associated with proton-transfer reactions of the ligand. The slower effect (1–5 ms) was dependent upon both pH and added metal ion concentration.

At the pH values used in these experiments the concentration of L^{2-} is negligible compared with that of HL^- and we proposed that the slow relaxation effect observed (see Materials and Methods) is associated with formation of MHL. This reaction must be considered as a two-step one, and it will be associated with two relaxation times

$$\text{(i)} \qquad H_2L$$
$$k_{12} \uparrow\downarrow k_{21}$$
$$\text{(ii)} \qquad H^+ + HL^- + Mg^{2+} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} MgHL^+ + H^+ . \qquad \text{(iii)}$$

The relaxation time for the protonation step, (i)–(ii) is fast, while that for the chelation step (ii)–(iii) is comparatively slow. In order to simplify the expression for the relaxation time τ, the hydrogen ion concentration was buffered. The total magnesium concentration, C_{Mg} was also effectively buffered by using magnesium concentrations in large excess over the total Tetracycline concentration. The relaxation expression for this mechanism is then given by [22]:

$$\tau^{-1} = \frac{k_{23}C_{Mg}}{1 + K_2[H^+]} + k_{32} , \qquad (5)$$

Table 3
Kinetic constants for the interaction of Mg^{2+} with the Tetracyclines

Derivative	k_{23} $M^{-1}\,s^{-1}$	k_{32} s^{-1}	Log K_{MHL}
Tetracycline	$3.78(0.2)\times10^5$	109.4(17)	3.54(0.1)
Chlorotetracycline	$1.95(0.2)\times10^5$	151.3(18)	3.11(0.1)
Oxytetracycline	$2.91(0.2)\times10^5$	118.2(18)	3.39(0.1)

and by measuring τ^{-1} as a function of both pH and C_{Mg} the individual rate constants were determined. The results are summarized in table 3 and plots of the data according to eq. (5) are shown in fig. 5. The agreement between the kinetically and spectrophotometrically determined values of K_{MHL} is excellent.

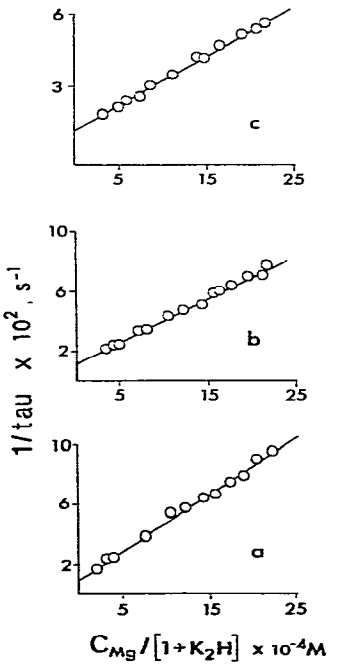

Fig. 5. Plots of τ^{-1} against $C_{Mg}/(1 + K_2[H])$ for the interaction of Mg^{2+} with tetracycline (a), oxytetracycline (b) and chlorotetracycline (c).

4. Discussion

Whilst general agreement exists regarding the values for the macroscopic pKa's of the Tetracyclines there is some disagreement regarding the appropriate assignment of the various acid groupings to the respective macroscopic dissociation constants [23–25]. Thus, whereas Stephens et al. [23] proposed that the three pK values represent succesive ionizations from carboxamide, dimethylammonium and β diketone moieties, Leeson et al. [25] proposed a reversal of the second and third assignments. Rigler et al. [24] have further characterized the protonation scheme in terms of twelve microscopic dissociation constants for the eight possible microspecies.

The long wavelength absorption band of the Tetracyclines is known to be associated with the BCD chromophore [15]. Changes in intensity and wavelength of maximum absorption for this band as a function of pH have been used here to measure pK_2 and pK_3 for the Tetracyclines. The large bathochromic shift observed in the pH range 6.5–8.5 may be taken to indicate that dissociation of the second proton is from the BCD chromophore. The additional small shift at high pH (used to measure pK_3) may indicate the dissociation of a further proton from this group and thus the existence of a microspecies of the dianion which has a doubly deprotonated BCD chromophore. However, Rigler et al. [24] have demonstrated that the acid strength of any particular group is quite dependent upon the degree of protonation of the others, so that the small, high pH shift may arise from electronic interaction between acid groups rather than from the formation of a microspecies of the dianion.

CD studies of the Tetracyclines have been undertaken in an attempt to clarify some of these points. The Tetracyclines have major CD bands at 360, 325, 290 and 265 nm (see, for example, ref. [26]) but only the latter band is associated with the A ring [26]. All of these bands show intensity changes *throughout* the pH range 6 to 11. However the A-ring band (265 nm) shows major changes at high pH whilst, for example, the 290 nm band, shows only small changes in this region. These changes are complex and will be discussed in detail elsewhere [27] but preliminary results are in agreement with the assignments of Leeson et al. [25] and with the model of Rigler et al. [24].

In the presence of Ca^{2+} and Mg^{2+}, the batho-

chromic shifts observed for the uncomplexed Tetracyclines are observed at much lower pH. This increased acidity of the BCD chromophore supports the suggestion [11] that oxygen atoms of this chromophore provide the principal site for metal ion chelation. Further evidence for this assignment comes from the observation [28,29] that isochlorotetracycline, which contains only the dimethylamino group and the $C_1-C_2-C_3$ system as potential binding sites, does not chelate Ca^{2+} (though it does chelate Ni^{2+} and Zn^{2+}). Thus, the chelation site for Ca^{2+} seems to be fairly well established. This is not the case with Mg^{2+}. Although chelation at the dimethylamino group appears unlikely from the work of Baker et al. [16] it is difficult to distinguish between chelation to the BCD chromophore or to the $C_1-C_2-C_3$ system of oxygen ligands. This is because chelation, and subsequent deprotonation, at the latter site might, through electronic interaction between the sites [16], be causing the shift in the 370 nm absorption band.

Values of K_{app} at a single pH have been reported for Mg^{2+}/tetracycline at pH 7.8 ($I = 0.1$, $K_{app} = 2500$) [5], for Mg^{2+}/chlorotetracycline at pH 7.4 ($I \approx 0$, $K_{app} = 3740$) [9] and for Ca^{2+}/chlorotetracycline at pH 7.4 ($I \approx 0$, $K_{app} = 2270$) [9]. The value for Mg^{2+}/tetracycline agrees well with the values reported here but the values for chlorotetracycline are considerably higher. This appears to be an affect of ionic strength differences and to check this the relevant values were measured in the absence of added KNO_3, obtaining 2900 (120) for magnesium (cf. ≈1000 at $I = 0.15$) and 1850 (80) for calcium (cf. ≈600 at $I = 0.15$).

Da Silva et al. [8] have reported formation of *both* MHL *and* ML only for the interaction of Mg^{2+} with tetracycline or chlorotetracycline and for Ca^{2+} with chlorotetracycline. The affinities they report are considerably higher than those obtained here. The affinities they report for the interaction of magnesium with dimethyl-chlorotetracycline and metacycline are also considerably higher than those reported by Benbough et al. [30] for these derivatives. Whilst the origin of these large differences is not clear, our results are obtained at low pH, to avoid Tetracycline degradation, and in the presence of excess metal ion which minimizes the possibility of interference from formation of ML_2 [4,19]. In attempts to measure K_{app} at pH values greater than 9.0 we found non-linear Scatchard plots, which would be consistent with interference from formation of the bis-Tetracycline complex at the lower concentrations of metal.

Inspection of table 2 shows that, for both metal ions investigated, the difference between the association constant for formation of MHL and that for formation of ML is surprisingly small. This is consistent with the suggestion that dissociation of the third proton is from the dimethylammonium group and that this group is not directly involved in chelation of the metal ion.

The pattern of magnesium complex formation is generally discussed in terms of the mechanism proposed by Eigen and Wilkins [31]. This mechanism gives a second order rate constant which is equal to the product of an outer-sphere association constant (K_{os}) and the water substitution rate, k_{ex}

$$k_f = K_{os} k_{ex} \cdot \qquad (6)$$

The value of k_{ex} is approximately 1×10^5 for magnesium [32] so that the values of K_{os} predicted by eq. (6) lie in the range 2—4 for the Tetracyclines. This is the range predicted by theory for a reaction of this type [33]. Fig. 6 compares the logarithm of the second order formation rate constant, k_{23}, with the charge product for the magnesium complex reactions reported (taken from refs. [33] and [34]). The slope indicates an approximate doubling of the rate constant per unit decrease in charge (cf. ref. [22]). The values reported for Mg^{2+}/Tetracycline complexation fit into this sequence rather well so that the normal dissociative mechanism appears to be valid.

In considering a possible role for chelation in the

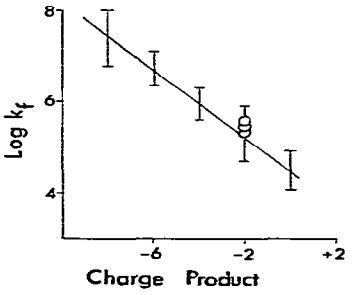

Fig. 6. Comparison of forward rate constant with charge product for magnesium 1:1 complexes. Data for the tetracyclines (○) is compared with that for a number of other ligands (|). The latter values were taken from refs. [33] and [34].

mechanism of Tetracycline action it is important to note that the concentration of drug effective both in vivo and in vitro is generally at least two orders of magnitude *less* than the total magnesium concentration. A simple mechanism involving non-specific reduction of the Mg^{2+} concentration (and resulting destabilization of the ribosome) can thus be ruled out.

The observation [36] that Tetracyclines will not bind to DNA in the absence of magnesium suggests that the metal ion may mediate binding by forming a bridging link between the drug and the macromolecule. Evidence for such a role in the binding of Tetracyclines to ribosomes has been presented [35]. The large excess of total magnesium over drug, in view of the moderate association constants reported here, probably ensures complete complexation of the antibiotic by magnesium. This suggests that it is probably the metal-complexed form of the drug that interacts with the ribosome and that although the metal in such a complex may form an electrostatic link to a negative site on the ribosome this is a consequence of the metal-drug equilibrium rather than the direct reason for inhibition [6].

The observation [6] that a single Tetracycline molecule is sufficient to inhibit a single ribosome argues for a single, high-affinity, drug sensitive binding site. Tritton [6] has argued that occupancy of this site interferes with ribosome function by decreasing the flexibility of the ribosome rather than by affecting some highly specific magnesium interaction. This suggestion does not, of course, exclude the possibility that magnesium mediates the binding at this site. It is interesting to note the kinetic constants reported [6] for the interaction of tetracycline with 70 S ribosomes at 25.2°C:

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

$$k_1 = 2.85 \times 10^5\ \mathrm{M^{-1}\,s^{-1}} , \qquad k_2 = 315\ \mathrm{s^{-1}} ,$$

$$k_{-1} = 15\ \mathrm{s^{-1}} , \qquad k_{-2} = 236\ \mathrm{s^{-1}} .$$

Although the unusually low value of k_1 (approximately three orders of magnitude less than that expected for a diffusion controlled reaction) has been explained [6] by postulating a restricted orientation of the drug binding site, the value is remarkably close to that reported here for the interaction of tetracycline with aqueous magnesium (3.8×10^5). Forward rate constants of this magnitude have also been reported for the interaction of streptomycin [37] and erythromycin [38] with ribosomes. This suggests the possibility that the rate of binding at this site may well be determined by the rate of water loss from a magnesium ion and thus supports the suggestion of magnesium mediated binding. The K_{app} value reported [6] for tetracycline binding to ribosomes (44 000 M^{-1} at pH 7.5) is an order of magnitude higher than K_{app} for Mg^{2+}/tetracycline interaction at this pH, thus enabling the site to compete with "free" Mg^{2+} for the available tetracycline. At high Mg^{2+} concentrations where protein synthesis is largely inhibited even in the absence of the drug, tetracycline has no further inhibitory action. This has been explained [6] in terms of the high magnesium concentration "overstabilizing" the ribosome structure such that tetracycline has no further effect. An alternative explanation is that the higher Mg^{2+} concentration is sufficiently high to compete effectively with the strongly interacting site. Such an argument is difficult to quantify, especially as the affinity of phosphate bound Mg^{2+} may be considerably less than that of free aqueous Mg^{2+}, but the kinetic similarities discussed above strongly support the idea of magnesium mediated binding.

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