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BINDING OF THE ALKALI METAL CATIONS TO TETRACYCLINE

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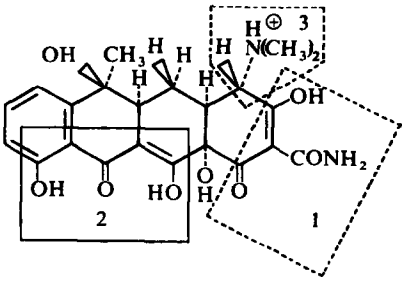
Abstract—Cation binding by tetracycline is studied in aqueous solution at pH 2.3 and 8.6, using sodium-23 nuclear magnetic resonance. It is possible to determine the binding constants not only for the sodium but also, through competition experiments, for the other alkali metal cations. While no binding occurs at pH 2.3, at pH 8.6 complexation follows the sequence: $Li^+ > Na^+$, $Cs^+ > K^+ > Rb^+$. The dissociation constants for the 1:1 complexes between tetracycline and the univalent alkali metal cations are of the order of 50 mM. Hence, the antibiotic is complexed by metal cations (Mg^{2+}, K^+ and, to a lesser extent, Na^+) when it binds to the ribosome.

Tetracyclines are antibiotics with a very large spectrum of action. They have been in common use for the last 30 yr for treatment of a variety of bacterial infections [1, 2]. Inhibition of the growth of microorganisms results from the blocking of protein synthesis at the ribosomes: the aminoacyl-*t*-RNA molecules are prevented from attaching themselves onto the ribosomes [1, 3, 4]. Even though several hundred tetracycline molecules can bind to a single ribosome [5], a single primary site (or a small number of sites) is responsible for the pharmacological activity [6, 7]. Tritton [8] recently suggested that binding of a single tetracycline molecule is sufficient to prohibit the conformational changes in the ribosome that accompany polypeptide synthesis [9].

Four proton dissociations have been observed in the tetracycline molecule, with pK_a's of 3.3, 7.7, 9.7 and10.7 [10–13]: the first ionization is from site 1, the second and fourth ionizations arise within site 2, and site 3 is responsible for the third ionization [11, 12, 14].



The second and fourth ionizations originate, the former from the enolic OH (on C-12), the latter from the phenolic OH (on C-10) [14]. Thus, tetracyclines exist in the zwitterionic form $1^-2^03^+$—using an obvious notation—in the pH range 4.3–6.7. It is also the active form, since the optimum antibacterial activity is in the pH range 5.5–6.0 [15]. The zwitterion is also the most soluble form in lipophilic phases [16] or in DMSO [17, 18] according to some authors, but others have shown that the un-ionized form $1^02^03^0$ is responsible for the solubility of tetracyclines in lipids [19] and in non-aqueous solvents [20].

In view of their molecular structure, it is not surprising that tetracyclines are cation binders and chelators [21–23]. Cation binding affects their biological activity. The attendant charge neutralization may facilitate antibiotic transport across cellular membranes [24]: chelating agents such as EDTA and ATP indeed inhibit considerably absorption of tetracyclines by membranes [25]. Cations, especially divalent cations such as Ca^{2+} and Mg^{2+}, also influence binding of tetracyclines to the ribosome [26–27]. Numerous techniques (circular dichroism, fluorescence, u.v.-visible, i.r., ^1H and ^{13}C NMR, X-ray), have indeed been applied to the measurement of binding constants, and to the determination of binding sites. Unfortunately, all these studies differ in their choice of solvent: DMSO, DMSO–water or alcohol–water mixtures: few studies are done in water solution because of the low solubility ($< 10^{-2}$ M) of tetracycline at neutral pH. Nevertheless, there appears to be consensus as to the binding sites: the chelating groups correspond to site 1 in the above scheme [12, 17, 18, 28, 29], to the bidentate enolized β-diketone of site 2 [28–35], or to both these sites [12, 31, 32, 36].

Binding constants have been determined for a number of divalent cations: Cu^{2+}, Ni^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, etc. and also for trivalent cations, e.g. lanthanides [28, 37]. Alkali metal cations: Li^+, Na^+, K^+, Rb^+ and Cs^+ have been neglected,

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presumably because they are expected to be much weaker binders. However, given the high extracellular Na^+ and intracellular K^+ concentrations, sodium- and potassium-binding may be important: we launched this investigation in order to determine whether tetracycline is likely to attach itself to the ribosome as the anion or as an ion pair. We were encouraged in this respect by the report of a shift from binding sites 1 to 2 of the lanthanide ions, in DMSO–water binary mixtures, when the sodium/tetracycline molar ratio becomes greater than unity [37].

MATERIALS AND METHODS

Preparation of samples

The salts are obtained as indicated: LiCl (UCB "analytical grade"), NaCl, KCl (Merck "suprapure"), RbCl, CsCl (Pierce Inorganics "ultrapure"). They are dried under vacuum at 60° for several hours. Tetracycline, as the chlorhydrate (Sigma; MW = 480.9), is kept at −25°. D_2O (Merck Uvasol; 99.8%) is used as the solvent. The various quantities of salts added, with a maximum concentration of 70 cM, produce only very weak decreases of the pH. Since tetracycline has a low solubility in water at neutral pH (less than 0.5 mg/ml at 25°), we have chosen to study cation binding at acidic pH (2.3), and mostly at a weakly basic pH (8.6). Because of the great sensitivity of tetracycline to air [2, 38], solutions are prepared a few minutes before spectra are recorded on the spectrometer: 2 ml of NaOH (4.74 cM) are introduced in a glass tube containing 19.2 mg of tetracycline in 2 ml of D_2O, plus a variable amount of salt. The resulting pH of approximately 8.6 corresponds to neutralization of two acidic functions of the antibiotic. The sample thus prepared is transferred into a 10 mm NMR tube (Bruker Spectrometer) or 8 mm NMR tube (Cameca Spectrometer). In no case, spectral accumulation and recording required more than 3 hr. During this period, the solutions of the antibiotic suffer little alteration from air contact. In a few samples, a thin layer of oxidized solution appears at the interface. We have checked that our results are unaffected by the presence of oxygen by working under an inert nitrogen atmosphere.

Sodium-23 nuclear magnetic resonance [39]

The spectra are obtained with the Bruker HFX-90 and WP-80 Fourier-transform spectrometers, with operating frequencies of 23.81 and 21.16 MHz, respectively. Field stabilization uses a deuterium lock signal from the D_2O solvent. Sample temperature is maintained at 298 ± 1 K. Variable temperature measurements, together with the preliminary observations, were performed with the Cameca 250 instrument, at a 62.86 MHz operating frequency.

In all the experiments reported here, it was checked that the lineshape is of lorentzian type. Chemical shifts vary little with respect to a 3 M aqueous solution of sodium chloride. Linewidths $\Delta\nu_{1/2}$ are measured at half-peak height for a constant concentration of tetracycline, and with variable amounts of added salts; they are known to within ±5 per cent.

Mathematical analysis

A. Sodium complexation. When the sodium cation exchanges fast between free sodium in the aqueous solution (Na^+) and tetracycline-bound sodium (NaTC), the observed linewidth $\nu_{1/2}$ is the weighted mean of the limiting linewidths $(\nu_{1/2})^{Na^+}$ and $(\nu_{1/2})^{NaTC}$; assuming a 1:1 stoichiometry:

$$\nu_{1/2} = (\nu_{1/2})^{Na^+}\,(Na^+)/(Na^+)_t + (\nu_{1/2})^{NaTC} \cdot (NaTC)/(Na^+)_t. \quad (1)$$

Since $(Na^+)_t = (Na^+) + (NaTC)$:

$$\nu_{1/2} - (\nu_{1/2})^{Na^+} = \frac{(NaTC)}{(Na^+)_t}[(\nu_{1/2})^{NaTC} - (\nu_{1/2})^{Na^+}],$$

or, equivalently:

$$\Delta\nu_{1/2} = \frac{(NaTC)}{(Na^+)_t}\cdot(\Delta\nu_{1/2})^{NaTC} \quad (2)$$

The dissociation constant for the complex is:

$$K_d = \frac{(Na^+)(TC)}{(NaTC)}, \quad (3)$$

with $(TC)_t = (TC) + (NaTC)$, so that

$$K_d = \frac{[(Na^+)_t - (NaTC)]\,[(TC)_t - (NaTC)]}{(NaTC)}. \quad (4)$$

so that the concentration of bound sodium is:

$$(NaTC) = \tfrac{1}{2}[[(Na^+)_t + (TC)_t + K_d - \{[(Na^+)_t + (TC)_t + K_d]^2 - 4(Na^+)_t(TC)_t\}^{1/2}]. \quad (5)$$

Combining equations (2) and (5):

$$\Delta\nu_{1/2} = \frac{(\Delta\nu_{1/2})^{NaTC}}{2(Na^+)_t}[[(Na^+)_t + (TC)_t + K_d - \{[(Na^+)_t + (TC)_t + K_d]^2 - 4(Na^+)_t(TC)_t\}^{1/2}] \quad (6)$$

The procedure followed for determining the two unknowns K_d and $(\Delta\nu_{1/2})^{NaTC}$ is fitting the data to equation (6): a set of arbitrary input parameters is introduced into (6), leading to a calculated value of $\Delta\nu_{1/2}$ which is then compared with the actual value, until agreement is obtained as measured by the sum of the squares of the deviations. This procedure is followed with each of the experimental points, and leads to the values for K_d and $(\Delta\nu_{1/2})^{NaTC}$. Conceivably, this limiting linewidth $(\Delta\nu_{1/2})^{NaTC}$ could have been determined experimentally in the presence of a large excess of sodium, for instance with $(Na^+)_t >$ 1.5 M. However, at such large sodium concentration, the viscosity of the solutions does not remain approximately constant as with the experimental conditions we used, and would affect markedly the observed linewidths. The unknowns K_d and $(\Delta\nu_{1/2})^{NaTC}$ are determined from multi-parameter fitting of equations (2) and (4) to the experimental points using the Simplex optimization procedure [40].

B. Competition between binding of sodium and other cations. The two competing equilibria can be written:

$$Na^+ + TC \overset{K_d}{\rightleftarrows} NaTC$$

$$M^+ + TC \overset{K}{\rightleftarrows} MTC$$

with:

$$K_d = \frac{[(Na^+)_t - (NaTC)]\,[(TC)_t - (NaTC) - (MTC)]}{(NaTC)} \quad (7)$$

and

$$K = \frac{[(M^+)_t - (MTC)][(TC)_t - (NaTC) - (MTC)]}{(MTC)} \quad (8)$$

The observed linewidth indicates the amount of bound sodium for each experimental point, using equation (2) since K_d and $(\Delta\nu_{1/2})^{NaTC}$ are already known from measurement of the linewidth as a function of sodium concentration. K is determined in the following manner: starting with an arbitrary trial value of K, one calculates the concentration (MTC), from which the concentration (NaTC) and the line broadening $\Delta\nu_{1/2}$ can be inferred.

We then minimize the sum of the deviations in absolute magnitude, between the calculated and the observed line broadenings $\Delta\nu_{1/2}$. These calculations are performed on the basis of equation (9) and (10), which are simply derived from equations (2), (7) and (8):

$$(MTC) = \frac{A}{2} - \frac{1}{2}\sqrt{A^2 - 4(M^+)_t\left[(TC)_t + \frac{\Delta\nu_{1/2}}{(\Delta\nu_{1/2})^{NaTC}}(Na^+)_t\right]} \quad (9)$$

with

$$A = (TC)_t + (M)_t + K - \frac{\Delta\nu_{1/2}}{(\Delta\nu_{1/2})^{NaTC}}(Na^+)_t$$

and:

$$(\Delta\nu_{1/2})^{calc} = [2(Na^+)_t]^{-1}.\{B - \sqrt{B^2 - 4(Na^+)_t\,[(TC)_t - (MTC)]}\}.(\Delta\nu_{1/2})^{NaTC} \quad (10)$$

with

$$B = (TC)_t + (Na^+)_t + K_d - (MTC).$$

RESULTS

In acidic aqueous solution, at pH = 2.3, and in the presence of tetracycline (10 mM), both the chemical shift and the linewidth of the observed single ^{23}Na resonance are identical to those of the reference: in this acidic medium tetracycline does not bind the sodium cation, in contrast with the cations of heavy metals which bind unto site 1 [28].

At pH = 8.6, the linewidth of the ^{23}Na resonance depends markedly upon the concentrations of sodium and of tetracycline (Fig. 1). There is fast exchange

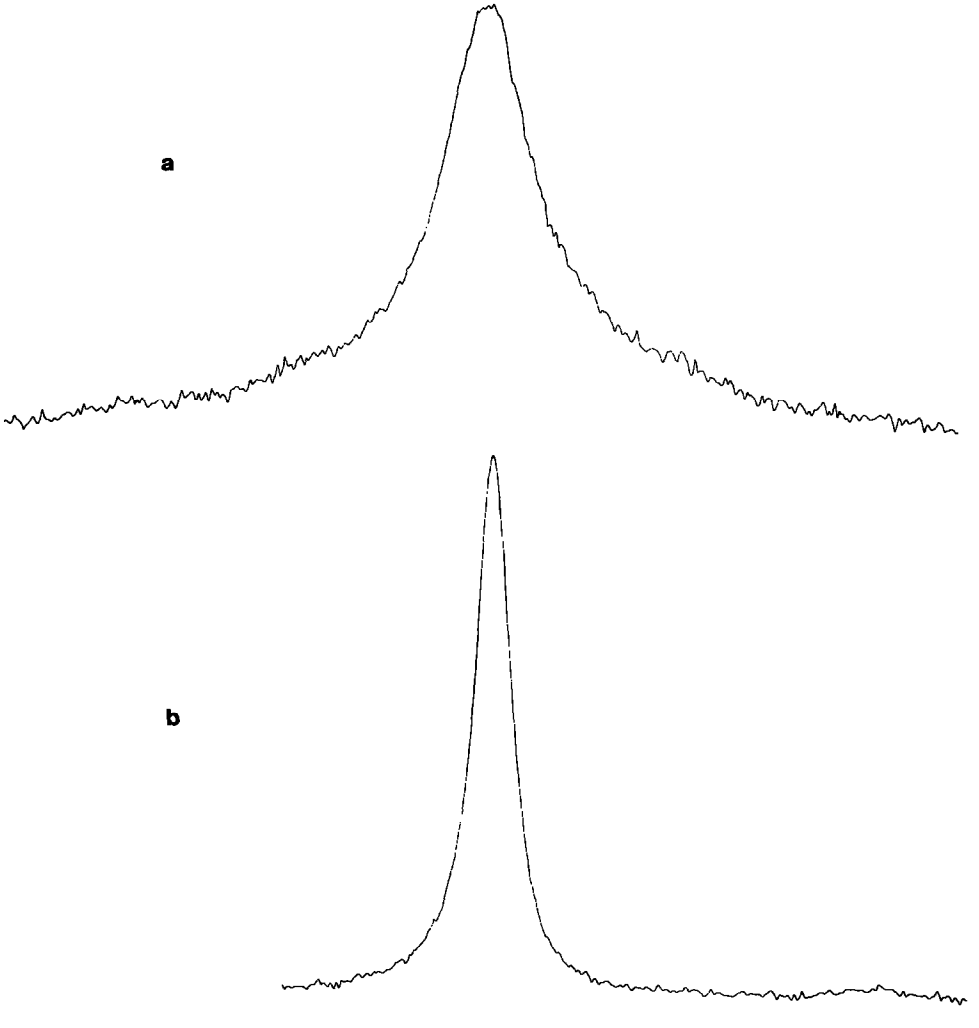

Fig. 1. ^{23}Na resonances for aqueous NaCl in the presence (a) and in the absence (b) of tetracycline (20 mM), at 28°.

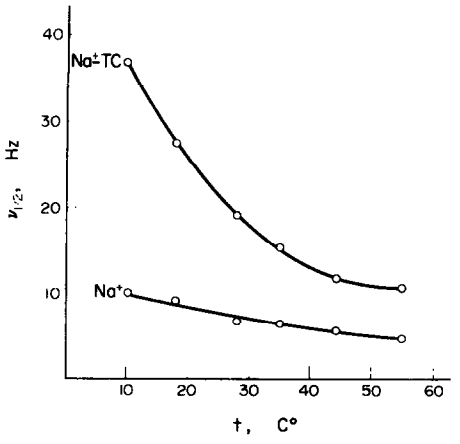

Fig. 2. Influence of temperature on the sodium-23 linewidths of aqueous NaCl in the presence and in the absence of tetracycline (20 mM).

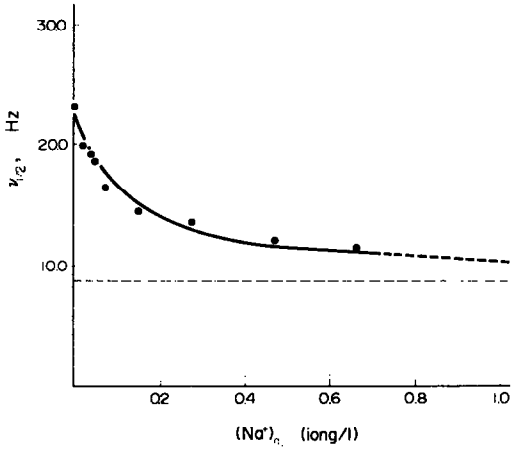

Fig. 3. Variation of the ^{23}Na linewidth with the addition of NaCl to an aqueous solution of tetracycline (20 mM).

The solid line is calculated with the final parameters indicated in the text. The horizontal dashed line corresponds to the linewidth characteristic of free sodium in water.

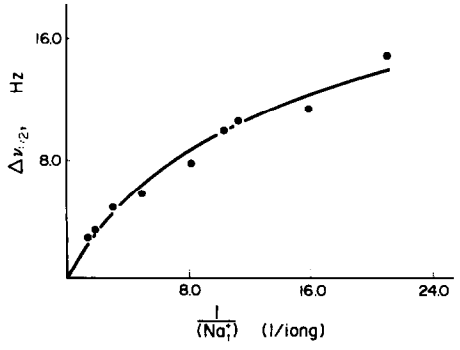

Fig. 4. Plot of the line broadening of the sodium-23 resonance in presence of tetracycline (20 mM) as a function of the reciprocal total sodium concentration.

The solid line is calculated using equations (2) and (4), with the final parameters indicated in the text. The origin corresponds to the linewidth of 3 M NaCl in D_2O, used as reference.

between the free and bound sodium states, since the observed linewidth decreases as the temperature is increased [41] (Fig. 2).

The decrease of the ^{23}Na linewidth with the addition of NaCl agrees very nicely with equation (6) predicated on the basis of a 1:1 stoichiometry (Fig. 3). However, we cannot exclude entirely a 1:n stoichiometry- -n being the number of bound Na^+ cations.

A useful representation is that of the line broadening as a function of the reciprocal of the total sodium concentration (Fig. 4). The observed curvature indicates that the value of K_d is commensurate with the weakest sodium concentrations used in these experiments.

These results are analyzed as indicated above, on the basis of equation (6) to yield:

$$(\Delta\nu_{1/2})^{NaTC} = 90 \pm 10 \text{ Hz}$$

$$K_d = 70 \pm 20 \text{ mM}$$

We shall not discuss further the limiting linewidth in the complex: it is normal for a species of that size in water solution.

Competition of the sodium cation with the cations of other alkali metals translates into a reduction of the observed ^{23}Na NMR linewidth [42], at constant sodium and tetracycline concentration, when another alkali metal chloride is added (Fig. 5).

The competition experiments, taken together with the above determination of K_d yield the following set of apparent dissociation constants for the complexes between tetracycline and alkali metal cations:

Li^+	40 ± 20 mM
Na^+	70 ± 20 mM
K^+	90 ± 20 mM
Rb^+	110 ± 20 mM
Cs^+	60 ± 30 mM

DISCUSSION

The interaction of the sodium cation with tetracycline is weaker than with divalent cations. For instance, the corresponding dissociation constants are: Mg^{2+} ($\simeq Ca^{2+}$) = 4 × 10^{-4} M [26]; Mn^{2+} = 6.3 × 10^{-5} M; Cu^{2+} = 1.6 × 10^{-8} M; Zn^{2+} = 1.6 × 10^{-5} M [21].

It is of the same order of magnitude as the reported dissociation constants for other sodium complexes, e.g. (mM) 100 for ATP, 200 for citrate, 200 for sulfate, and 20 for EDTA [43].

Comparing the values for sodium and for the other alkali metal cations, they fall within a relatively narrow range. Because of the experimental uncertainties, it is impossible to state without ambiguity which of the Eisenman selectivity sequences [44] is followed here. One will recall that among the 120 possible permutations (5!) for the five alkali metal cations, only 14 selectivity sequences are observed in practice for inorganic systems (cation-selective glass electrodes, ion exchangers, etc.) as for organic molecules (cryptands, crown ethers, etc.) [44, 45]. Most of the literature results on organic compounds obey Eisenman's sequence XI [44], i.e. $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$ [46], dictated by the inverses of the ionic radii.

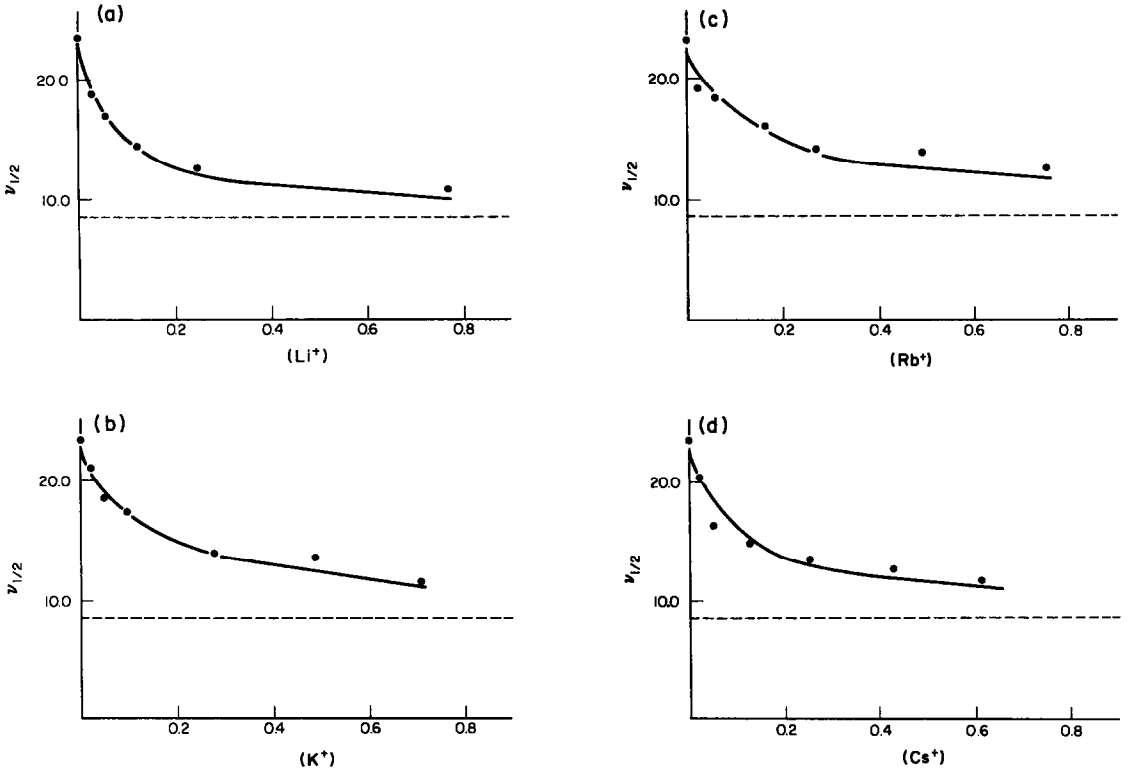

Fig. 5. Variation of the sodium-23 linewidth, with $(Na^+) = 47.4$ mM, as a function of added alkali metal chlorides, in the presence of 20 mM tetracycline.

The solid lines are calculated using equations (9) and (10), together with the final parameters in the text. a: LiCl; b: KCl; c: RbCl; d: CsCl. The horizontal dashed line corresponds to the linewidth characteristic of free sodium in water.

Furthermore, β-diketonic systems—such as that present within site 2 of tetracycline—bind lithium better than the other alkali metals, the binding constants being very similar for sodium, potassium, rubidium, and cesium [47, 48].

At the pH = 8.6 of our measurements, tetracycline exists predominantly as the anion $= 1^-2^-3^+$, so that our observations are consistent with cation binding at site 1, at site 2, or at both. Unfortunately, we could not determine the dissociation constants for the alkali metal cations–tetracycline complexes at pH 7.4 with our technique because the antibiotic is not completely soluble and the solutions are not completely homogeneous at this physiological pH.

Finally, with the binding constants obtained here, it is possible to determine the nature of the tetracycline species which attaches itself to the ribosome. Without considering binding of the metallic cations with various biomolecules (nucleic acids, proteins, etc.) and taking the intracellular concentration of tetracycline equal to the plasmatic concentration leading to inhibition of the growth of *Escherichia coli*, i.e. 2×10^{-6} M (1 μg/ml) [2, 49], the magnesium cation is in large excess (10^{-3} M) with respect to tetracycline; if it were the only cation present, given its binding constant to tetracycline, 71 per cent of the tetracycline molecules would exist as magnesium-bound ion pairs. However, the sodium (10^{-3} M) cations are also abundant in the cytoplasm: using the equilibrium constants obtained above and assuming that they would not be significantly different at pH 7.4 from their values at pH ≃ 8.6, we calculate corresponding percentages of 14 per cent sodium-bound and 53 per cent potassium-bound tetracycline. Likewise, in the extracellular fluid, several cations compete for quasi-saturation of the tetracycline binding sites $= Na^+$ (10^{-1} M) to the extent of 67 per cent; Mg^{2+} and Ca^{2+} (10^{-3} M), approximately 75 per cent each; and Zn^{2+} (5×10^{-5} M), also 75 per cent. This serves to show that the effect of the univalent cations (Na^+, K^+) is far from being negligible when compared with the divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}), even though tetracycline binds the latter more strongly by several orders of magnitude.

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