The Influence of Different Solvents on the Interaction between Metal Ions and Tetracycline

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Z. Naturforsch. **34 c,** 1156 – 1161 (1979); received June 25, 1979

Tetracycline, Antibiotics, Metal Ions, Complexation, Optical Absorption

The interaction of the antibiotic tetracycline with a few metal ions, esp. Cu(II), in different solvents has been investigated by means of IR, ESR, and UV/VIS spectroscopy. It could be shown that the site of complexation depends on the solvent used. In DMSO the interaction occurs mainly via the A ring. In water (pH = 5.7) and octanol, the A chromophore as well as the BCD entity of the molecule are involved in complexation. If the tetracycline concentration exceeds the Cu(II) concentration, a 2 : 1 (antibiotic : Cu(II)) complex seems to be formed, at least in DMSO.

1. Introduction

The tetracyclines are a group of broadspectrum antibiotics which have been used for over twenty years in the treatment of both human and animal diseases. It is now generally agreed upon that the ultimate effect of the tetracyclines at concentrations found *in vivo* is the inhibition of bacterial protein biosynthesis as a result of binding of the drugs to bacterial ribosomes.

All therapeutically used tetracyclines have a very pronounced ability to form complexes with divalent cations. The stability constants for these complexes depend on the nature of the metal ion, ranging (for 1 : 1 complexes) from about 10^8 M^{-1} for Cu(II) to about 10^4 M^{-1} for Mg(II) [1 – 3]. Assuming an average serum concentration of 1.75×10^{-3} M for Mg(II) and Ca(II) [4] and a therapeutically sufficient serum concentration of tetracycline (2×10^{-6} M), more than 90 per cent of the tetracycline molecules should be found in a complex form. Several authors [5 – 7] have suggested that ternary complexes, involving the antibiotic, the bacterial ribosome, and a metal ion might play an important role in the inhibition of protein synthesis. Nevertheless the role of metal ion complexation in the mode of action of the tetracyclines is not yet well understood.

Several attempts have been made in order to elucidate type and loci of tetracycline-metal ion complex formation. Binding at the BCD chromophore as well as to the A chromophore has been proposed (s. Fig. 1).

Conover [8] proposed the oxygen atoms at C(11) and C(12) as the binding site, based on a spectrophotometric comparison of 5-hydroxytetracycline-metal ion complexes in methanolic and ethanolic solutions with complexes obtained with model compounds representing subgroups of the tetracycline molecule.

Ibsen and Urist [9] studied the complexes of 5-hydroxytetracycline with Ca(II), Mg(II), and Cu(II) in aqueous solution by means of UV/VIS spectroscopy as well as pH determination. According to their results the BCD chromophore should be the preferential binding site, too.

Mitscher *et al.* [10, 11] have studied the conformations of several tetracycline-metal ion complexes by circular dichroism in aqueous solution. They con-

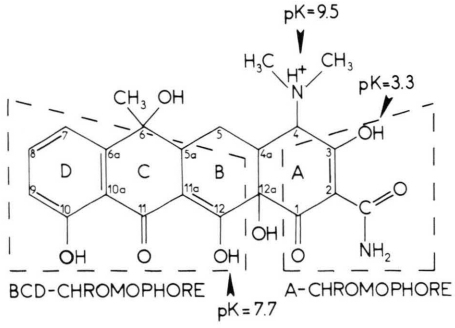

Fig. 1. The two chromophores and the pK_a-values of the tetracycline molecule.

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0341-0382/79/1200-1156 $ 01.00/0

cluded that the studied metal ions Ca(II), Mg(II), Cu(II), and Al(III) bind to the BCD chromophore. For pH values above 7.5, they suggested that an additional complex between the C(12 a) hydroxyl and the C(4) dimethylamine nitrogen and Ca(II) is formed.

Based on potentiometric titrations and calorimetric measurements Benet and Goydn [12, 13] proposed the formation of a 1 : 1 inner sphere complex at the C(10), C(11) site of five tetracycline analogs and Cu(II) in aqueous solution. A second tetracycline molecule is assumed to be attached outer sphere to the metal ion.

Baker and Brown [14] isolated Ni(II) and Co(II) complexes of different tetracyclines and concluded, by using reflectance spectroscopy and magnetic susceptibility measurements, binding via oxygen atoms in the C(1), C(2), C(3)-tricarbonyl-methane system without ruling out additional binding to the BCD moiety.

Williamson and Everett [15] have reported a study of metal ion binding to tetracycline by means of ^1H NMR spectroscopy in DMSO solution. Based on the isotropic shifts and the broadening of certain signals, they concluded that binding should occur with the A ring, probably via oxygen donors.

Similar conclusions were drawn by Gulbis and Everett who studied the interaction between rare earth ions and tetracyclines in DMSO [16] and in 70 : 30 (v/v) DMSO: D_2O [17].

A comparison of the results mentioned suggests that, in aqueous solution, the BCD chromophore is always involved in complexation. On the other hand, measurements in DMSO, DMSO:D_2O mixtures, and in the solid state showed a preferential or even exclusive involvement of the A chromophore.

In order to elucidate the influence of the solvent on the tetracycline-metal ion complex formation, the effect of H_2O, DMSO, and octanol on these complexes has been investigated by means of ESR- and UV/VIS- (Cu(II)) and IR-measurements (Ni(II), Co(II), Ca(II), Mg(II)).

2. Materials and Methods

Crystalline tetracycline (TC) was obtained from Sigma and stored below 0 °C. Tetracycline nitrile (2-carboxamide is replaced by nitrile) and some other tetracycline analogs are a gift of Dr. W. Dürckheimer (Hoechst, Frankfurt). The metal salts used, DSMO, and octanol were purchased from Merck, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) from Fluka.

The UV/VIS spectra have been recorded as difference spectra on a Zeiss DMR 10 spectrophotometer. Different tetracycline : metal ion concentration ratios have been investigated. The tetracycline concentration was kept constant.

Infrared spectra were recorded on a Perkin Elmer 283 grating spectrometer. A cell with KBr windows and a pathlength of 25 μm was used. Solvent bands were compensated by a variable pathlength cell in the reference beam.

ESR spectra were obtained with 5 mM of $Cu(NO_3)_2$ solutions and variable concentrations of tetracycline using a Varian E 9 spectrometer with a 100 kc field modulation. DPPH was used as a standard. The pH was measured with a Knick precision pH meter using an Ingold glass electrode.

3. Results

3.1. UV/VIS measurements

The spectrum of tetracyline (TC) in aqueous solution shows two main peaks with maxima at 273 nm and 354 nm for a pH value of 5.7. Addition of Cu(II)

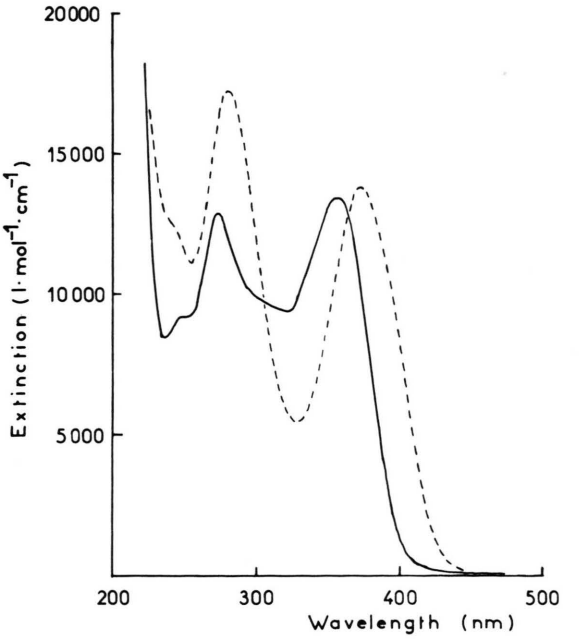

Fig. 2. UV/VIS absorption spectrum of TC in H_2O and the influence of the complexation with Cu(II), ——— TC only (pH 5.7), – – – – [TC] : [$Cu(NO_3)_2$] = 1 : 1 (pH 5.7).

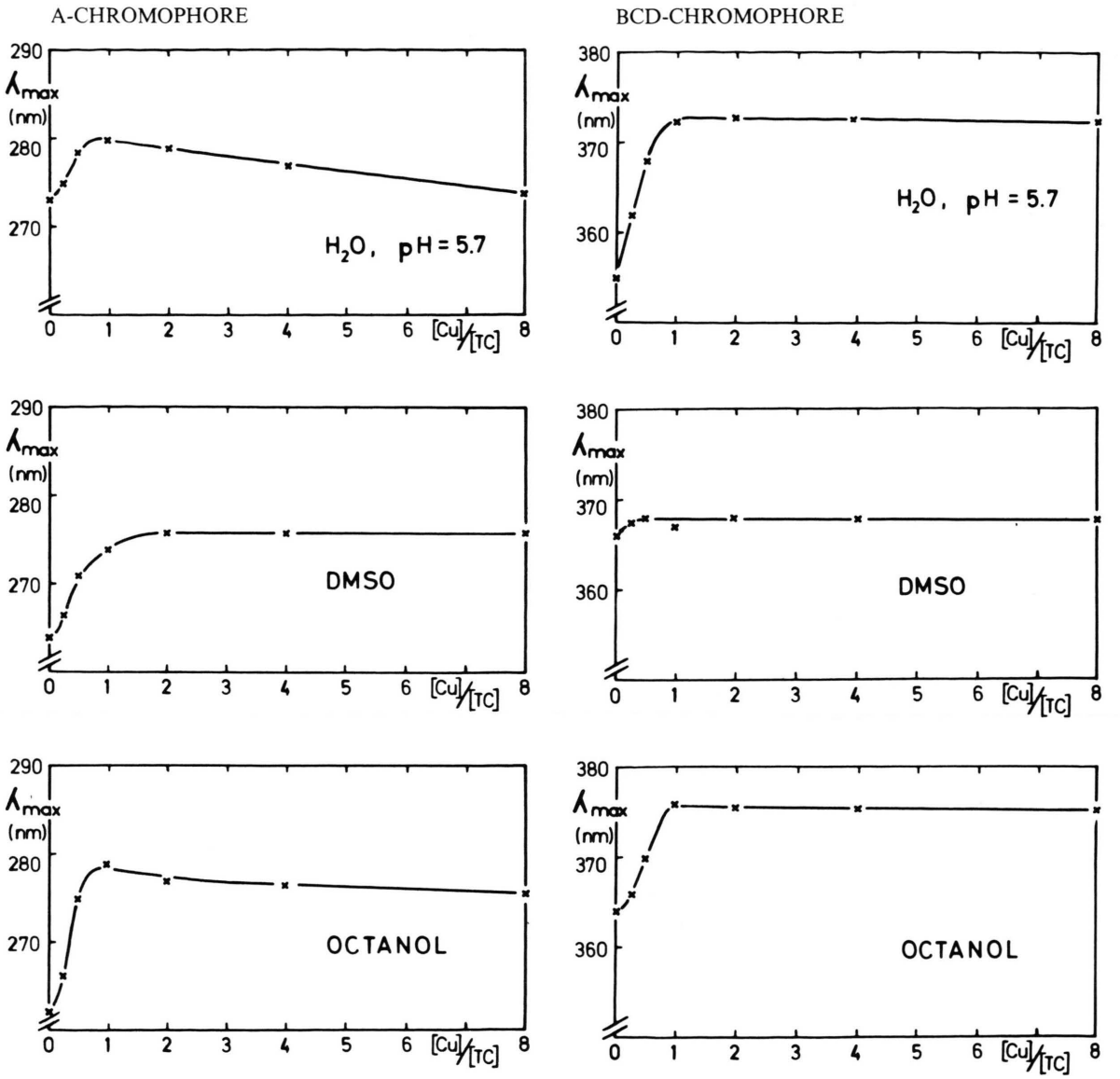

Fig. 3. The position of the maxima of the absorption bands of TC at about 270 nm and 360 nm as a function of the $[Cu(NO_3)_2]$: [TC] ratio in H_2O, DMSO, and octanol.

or other metal ions shifts both of them to greater wavelengths and increases the intensity of the 273 nm band (s. Fig. 2). A decrease of the pH value to 2.0 shifts the 273 nm peak to 266 nm, whereas the other maximum remains at 354 nm. An increase of the pH value to 9.0 shifts the 354 nm peak to 368 nm, whereas the other maximum remains unchanged. The shift of the 273 nm peak can be assigned to the protonation of the A chromophore at the C(3) hydroxil group ($pK_a = 3.3$) [12], the shift of the 354 nm peak to the deprotonation of the BCD chromophore occuring probably at the C(12)-OH site [18] ($pK_a = 7.7$) [12]. It could be shown that the 354 nm band is exclusively due to a BCD chromophore absorption [8] whereas the 273 nm band is a superposition of absorptions of the A and BCD chromophores [19]. A comparison of the UV spectra of model compounds used for the two chromophores

shows that the A chromophore contributes more to the absorption at 273 nm than the BCD chromophore [8]. The variations of the UV/VIS spectrum by changing the pH-values indicate that the two chromophores are essentially independent and that shifts of the 273 nm band are due to interactions with the A chromophore. Shifts of the 354 nm peak arise from interactions with the BCD chromophore.

Additional evidence for this assignment is given by the fact that the 273 nm band of tetracyline nitride is not shifted at all between pH 2 and pH 6. This is due to the very low pK_a value of the C(3)-OH group in this molecule caused by the strong acidifying influence of the nitrile group [20]. In contrary, by increasing the pH value to 9.0 the shift of the 354 nm band is similar to the one obtained for TC.

Based on this assignment it is possible to compare the influence of metal ion complexation on the UV/VIS spectrum of tetracycline in different solvents and to determine the site of binding (s. Fig. 3). The shifts of the two bands in aqueous solution indicate that in water (pH 5.7) the two chromophores are involved in complexation. The shift of the 273 nm band is reversed for [Cu] : [TC] > 1 (s. Fig. 3). Two additional shoulders appear at about 400 and 440 nm of the absorption spectrum, a phenomena which is not yet well understood. An involvement of the two chromophores can be also observed in the case of octanol.

In DMSO, a shift of the A chromophore band can be observed only, indicating, that only this chromophore takes part in complexation. Addition of small amounts of KOH to the TC solution results in a shift of the 364 nm band of the BCD chromophore to 395 nm proving that the BCD chromophore of tetracycline in DMSO is protonated.

3.2. Infrared measurements

Because of the low solubility of tetracycline in water and octanol infrared measurements were performed in DMSO only. The tetracycline molecule shows a very characteristic IR spectrum between 1500 and 1700 cm^{-1} consisting of 4 bands at:

$$1655\ cm^{-1},\ 1601\ cm^{-1},\ 1578\ cm^{-1},\ \text{and}\ 1522\ cm^{-1}$$

(s. Fig. 4). The spectrum of tetracycline nitrile in this region consists only of the two bands at 1601 cm^{-1} and 1578 cm^{-1} suggesting that the two bands at 1655 cm^{-1} and 1522 cm^{-1} arise from the C(2) amide

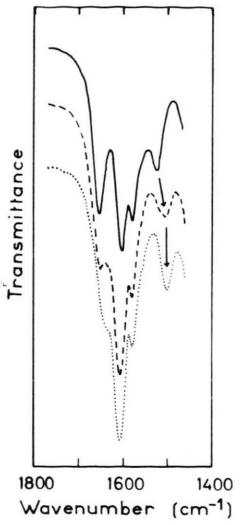

Fig. 4. IR spectra of TC (75 mM) in DMSO in the absence and the presence of different concentrations of Mg(II). ——— TC only, – – – – [TC] : [$Mg(NO_3)_2$] = 4 : 1, ····· [TC] : [$Mg(NO_3)_2$] = 2 : 1.

group. The band at 1655 cm^{-1} can be assigned essentially to the stretching vibration of the C = O moiety of the amide group (amide I), the band at 1522 cm^{-1} to a superposition of an NH_2 bending and the C-N stretching vibration (amide II).

The interaction with metal ions diminishes considerably the absorption at 1655 cm^{-1} and shifts the band at 1522 cm^{-1} to smaller wavenumbers (s. Fig. 4). This shift increases for the different metal ions used in the following order:

$$Cu(II) > Ni(II) > Co(II) > Mg(II) > Ca(II)$$

and can be correlated with the log K values reported in other publications [2, 5] (s. Fig. 5). The K_1 value for Ca(II) has not been reported by these authors.

Fig. 5 indicates that, at least in DMSO, the complexation of the different metal ions should occur at the same site. Moreover, the observed shifts of the amide bands demonstrate the involvement of the A chromophore, in particular of the C(2) amide group in complexation. This result is in good agreement with the UV/VIS measurements and the results obtained by Williamson and Everett [15].

3.3. ESR measurements

Due to complexation with ligands possessing a higher complex forming ability than the solvent mol-

ecules, the ESR signal of the paramagnetic Cu(II) ion is normally shifted to a higher magnetic field. This shift reflects essentially the interaction of the unpaired d-electron of Cu(II) with the electric fields of the new ligands.

Fig. 6 shows the high complex forming ability of the tetracycline molecule using DMSO as a solvent. At an equimolar concentration the original signal of the Cu(II)-DMSO-complex is already strongly reduced. The new signal which appears at a higher magnetic field should be due to a 1 : 1 [TC : Cu(II)] complex. If the TC concentration exceeds the Cu(II) concentration the signal is shifted to a higher magnetic field. This signal should arise from a 2 : 1 [TC : Cu(II)] complex and is the first direct spectral evidence for the existence of such a complex which has been postulated by Albert in 1953 [1] based on a potentiometric titration. Because of the low solubility in water and octanol such concentration dependent measurements could not be performed in these solvents.

Measurements with $CuCl_2$ instead of $Cu(NO_3)_2$ showed that the Cl^- ions can successfully compete in DMSO for the binding to the Cu(II) ions which is not observed for the NO_3^- ions. Therefore, in all experiments reported $Me(NO_3)_2$ was used as metal salt.

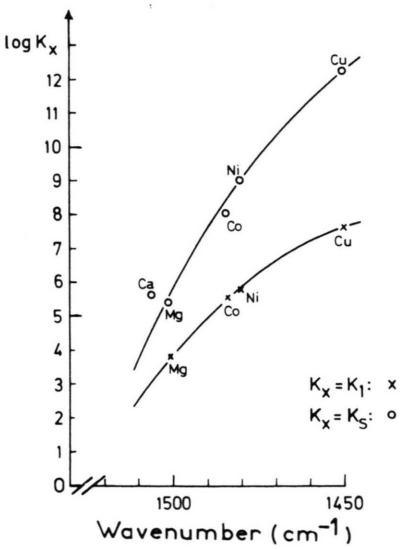

Fig. 5. The correlation between the position of the amide II band in the IR spectra of TC in DMSO and the equilibrium constants for the complex formation of TC with different divalent cations, with K_1 for: Me(II) + TC $\rightleftharpoons$ Me(II) TC, K_2 for: Me(II) TC + TC $\rightleftharpoons$ Me(II) TC_2, and $K_S = K_1 \cdot K_2$. The metal ion concentration was kept constant (75 mM).

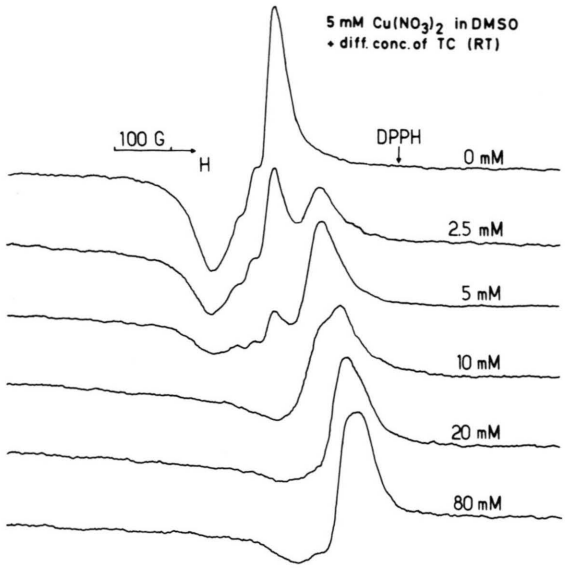

Fig. 6. ESR spectra of a 5 mM $Cu(NO_3)_2$ solution in DMSO with different concentrations of TC and measured at RT. Modulation amplitude 2 G, power 5 mW.

4. Discussion

Following the results obtained in particular by UV/VIS measurements it can be concluded that in water (pH 5.7) and octanol the BCD and the A chromophore are involved in complexation, whereas in DMSO the A chromophore only seems to take part in complexation. If the second pK_a value (7.7) can be assigned to the hydrogen of the C(12) hdroxyl group as has been proposed by Asleson [18] based on his NMR results, the direct ligands of the BCD chromophore to metal ions should be the C(11) and C(12) oxygen atoms. The IR results reported indicate clearly the involvement of the C(2) amide group when complexation occurs at the A chromophore. In this case the amide oxygen should act as an atomic ligand since a binding of the amide nitrogen to the metal ion would implicate, at least in part, a loss of resonance stabilization of the amide group [21] which amounts to 21 kcal/mol [22]. Another oxygen atom in the neighbourhood of the amide group should act as a second ligand. Depending on the tautomeric form this can be either the oxygen at C(3) or C(1).

The differences in the complexation behavior of tetracycline in the three solvents might be due to the fact that water and octanol can act as donor as well

as acceptor for hydrogen bonds, whereas DMSO can act as acceptor only. A solvent with donor and acceptor properties should be more suitable to break the internal hydrogen bonds of the tetracycline molecule (C(10)-OH . . . O = C(11) and C(11) = O . . . HO − C(12)) [23] at the C(10), C(11), C(12) sites and to make these sites more susceptible for the attachment of a metal ion.

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