Conformation of Various Tetracycline Species determined with the Aid of a Nuclear Magnetic Resonance Relaxation Probe

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The aqueous solution conformation of the gadolinium(III) complexes of the protonated, neutral, and singly deprotonated tetracycline species have been determined. The complete proton resonance assignment for each species is given. Evidence is presented for the site(s) of protonation or deprotonation as well as for intramolecular hydrogen bonding.

THE conformations of the tetracycline antibiotics and the mode of interaction with metal ions have been a subject of study for many years. It has been recognised that metal ions such as magnesium and calcium may mediate the activity of the antibiotics although their precise role has not been established.[1-14] Attempts have been made to establish either the conformation of the molecules or the metal binding site but the only solution studies aimed at determining both have been through examination of c.d. spectra.[15-17] *X*-Ray studies have been reported on chlortetracycline hydrochloride,[18] oxytetracycline hydrochloride,[19,20] and its 5,12a-diacetyl derivative [21,22] and recently of the tetracycline free base.[23] Metal binding sites have been inferred from stability constant studies,[24] c.d. spectra,[15-17] and n.m.r. work in non-aqueous solvents.[25-29] However, results involving the use of lanthanides interpreted in terms of the correlation of maximum shifts with proximity of binding must be treated with caution especially when the magnetic symmetry of the complex formed is unknown.

Tetracycline exhibits a multitude of potential metal binding sites most of which have been assigned as dominant by different authors. It is the purpose of this paper to quantitatively determine the conformation of the tetracycline species bound to gadolinium(III) which may be regarded as a good probe for calcium.

RESULTS AND DISCUSSION

We have previously described the application and limitations of the use of paramagnetic relaxation probes in the assignment of proton resonances and determination of aqueous solution conformations.[30] Gd^{III} induces changes in the proton relaxation times falling off with distance from the metal ion according to a sixth-power relationship.

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No complete proton resonance assignment for tetracycline in aqueous solution has been made, nor is it readily available from the examination of coupling constants because of the complexity of the resonances arising from the A and B rings (Figure 1) and the partial

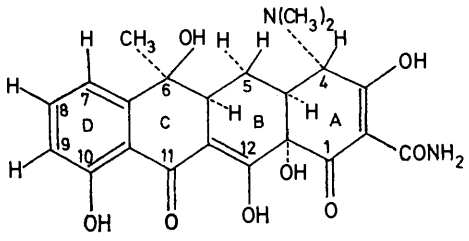

FIGURE 1

obscuring of the 4a-H and 5a-H resonances by that of the dimethylamino-group. However, a partial assignment of the A and B ring protons in [$^2H_6$]DMSO has been made [25] and our results are in agreement with this. There remains, however, the relative assignment of the 5-methylene protons and the 7-H and 9-H resonances. By titration of a [$^2H_6$]DMSO solution with ^2H_2O we are able to produce the partial assignment for the free base shown in Table 1. The assignment of the protonated and singly deprotonated species follows by pH titration. The complete assignment comes out of the search for an acceptable fit of the relaxation data.

In attempting to interpret shift or relaxation measurements in polar media a further complication arises from the observation of specific changes in chemical shifts, particularly of the D ring resonances, on dilution and changing the temperature. This indicates stacking in solution. Only by working at 333 K and at low concentration, 0.04M, is this stacking negligible.

Tetracycline Hydrochloride.—At pH 2.0 the protonated species predominates, the proton most probably being associated with the dimethylamino-group. Titration with Gd^{III} induces line broadening in all the resonances but preferentially those of the 5-methylene and 4-H protons. The least affected are those of the D ring (Table 2). Clearly the metal binds in the vicinity of the A and B rings. In attempting to fit the data we have considered the five postulated conformations of the A and B rings [17] in conjunction with the following metal binding sites: (a) 3-hydroxy-amide carbonyl, (b) 3-hydroxy-amide nitrogen, (c) 1-ketone-amide carbonyl, (d) 1-ketone-amide nitrogen, (e) 12-hydroxy-1-ketone, (f) 3-hydroxy-dimethylamino, and (g) 12a-hydroxy-dimethylamino. Two additional chelating sites, (h) 11,12-β-diketone and (i) 10,11-β-diketone are clearly not involved in this species. As we observe that one of the 5-methylene protons lies closest to the metal ion co-ordination involving the dimethylamino-group or the 3-hydroxy can be eliminated as these would give rise to preferential broadening of 4-H. Searching the available conformations and chelating sites only one provides an acceptable fit of the data. All other permutations would require at least a 100% error in $(fT_{2p})^{-1}$ for one or more protons. To achieve this fit requires the assignment of the high field (δ 2.28) 5-methylene resonance to 5_u-H — *i.e.* the proton lies on the same side of the molecule as the 6-hydroxy. With the reverse assignment no acceptable fit of the data is possible. We observe the A ring to be folded up out of the plane of the B—D rings with the

TABLE 1

Observed chemical shifts of tetracycline resonances at 333 K [a]

	6-CH_3	5_d-H	5_u-H	4a-H	4-$N(CH_3)_2$	5a-H	4-H	8-H	7-H or 9-H
Tetracycline [$^2H_6$]DMSO	1.55	1.96		b	2.41	2.88	2.24	7.52	7.10 6.92
Tetracycline ^2H_2O pH 2.0	1.64	1.92	2.28	2.96 [c]	3.07	b	4.08	7.60	7.22 7.00
Tetracycline ^2H_2O pH 6.5	1.64	1.84	2.20	2.84 [c]	2.96	b	3.80	7.60	7.20 7.00
Tetracycline ^2H_2O pH 8.6	1.56	1.80	2.08	2.78 [c]	2.84	b	3.44	7.44	7.07 6.90

[a] Measured from sodium 3-trimethylsilylpropanesulphonate. [b] Under the dimethylamino-resonance. [c] Partly obscured by the dimethylamino-resonance.

TABLE 2

Relaxation data and comparison of experimental and calculated (best fit) internuclear distances normalised on 4-H

	pH 2.0			pH 6.5			pH 8.6		
	$(fT_{2p})^{-1}_{exp.}$ [a]	$r_{exp.}$ [b]	$r_{calc.}$	$(fT_{2p})^{-1}_{exp.}$	$r_{exp.}$	$r_{calc.}$	$(fT_{2p})^{-1}_{exp.}$	$r_{exp.}$	$r_{calc.}$
4-H	5.23×10^4	1.00	1.00	2.92×10^4	1.00	1.00	4.43×10^3	1.00	1.00
4-$N(CH_3)_2$	1.37×10^4	1.25	1.29	2.82×10^4	1.01	1.02	5.34×10^3	0.966	0.987
6-CH_3	0.636×10^4	1.42	1.37	0.285×10^4	1.48	1.37	6.37×10^3	0.938	0.950
7-H	0.312×10^4	1.60	1.53	0.178×10^4	1.60	1.53	6.56×10^3	0.933	0.962
8-H	0.224×10^4	1.69	1.72	0.132×10^4	1.68	1.72	6.25×10^3	0.941	1.00
9-H	0.250×10^4	1.66	1.64	0.136×10^4	1.68	1.64	12.1×10^3	0.843	0.812
5_u-H	9.83×10^4	0.90	0.86	4.32×10^4	0.87	0.86	17.8×10^3	0.790	0.750
5_d-H	3.68×10^4	1.06	1.10	2.22×10^4	1.05	1.10	7.15×10^3	0.920	0.887

[a] f is the ratio of paramagnetic ion concentration to ligand concentration and T_{2p} is the change in the proton relaxation time(s) induced by the paramagnetic ion. [b] r is the observed proton–metal ion internuclear distance (Å).

metal ion bound to the amide carbonyl and 1-ketone (Figure 2). It is of interest to know whether the metal

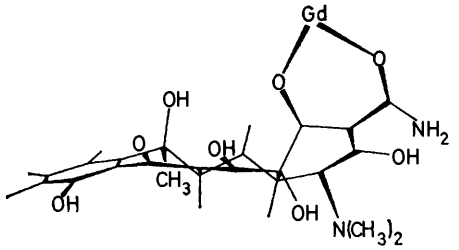

FIGURE 2 Observed conformation of the tetracycline–gadolinium(III) complex at pH 2.0

ion has induced any conformational changes upon complexation. Binding at this site imposes very little restraint upon the A and B ring conformations except in so far as rotation of the amide group is concerned. However additional evidence suggests that the observed conformation is likely to be retained by the free ligand. First the observed dihedral angle 4-H–4a-H of *ca.* 110° is in accord with the coupling constant of 1.8 Hz in the free ligand. Secondly the addition of diamagnetic $La(NO_3)_3$ does not change the observed coupling constants in the A and B rings. The dimethylamino-group is in a position such that the dihedral angles 4-H–C-4–N–CH_3 are approximately +60 and −60°. In such a position the proton on the nitrogen is almost certainly not involved in hydrogen bonding.

Tetracycline Free Base.—The results at pH 6.5 on the free base are very similar to those of the hydrochloride with only one major difference observed in the effect upon the dimethylamino-protons. The position of metal binding and ring conformations are essentially unaltered upon the loss of one proton. However to accommodate the data it is necessary to rotate around 4-N by *ca.* −120°. This brings one of the methyl groups very much closer to the metal ion. We suggest that the reason for this change lies in the presence of the hydrogen bond N–H • • • 3-O (the same zwitterionic form has been observed in the *X*-ray study [23]). The conformation we observe certainly brings the proton and oxygen closer than the sum of their van der Waals radii. In the hydrochloride species 3-O would have to be protonated and any such hydrogen bond formed would be much weaker. The position of the amide group is obviously fixed by chelation with the metal ion and the conformation observed is opposite to that found in all the published *X*-ray structures of tetracyclines. Whether or not it retains this conformation in the absence of the metal ion is unknown, however, it is interesting to note that this same conformation has been recently found in a crystal structure of the free base [23] suggesting that it is perhaps not reversed by the requirements of co-ordination.

Tetracycline at pH 8.6.—At this pH tetracycline is predominantly the singly deprotonated species. The relaxation data (Table 2) are markedly different and show qualitatively that the metal ion lies close to the centre of the molecule, at or near the BC ring junction. The most likely co-ordination sites are thus (e), (h), and (i). From the data for the D ring protons and for 6-CH_3 the site is clearly defined as being (h), the 11,12β-diketone. Presumably, therefore, the proton that has been lost from the free base is from 12-hydroxy. To arrive at this conclusion for the binding site requires that the resonance centred at δ 6.90 (and δ 7.00 at pH 2.0) arises from 9-H. The reverse assignment of 9-H and 7-H would render the data meaningless.

The overall conformation is shown in Figure 3, ring A being twisted from that observed in the free base. The dihedral angle 4-H–4a-H is now *ca.* 70° again in accord with the observed coupling of 1.7 Hz in the free ligand. The dimethylamino-group is in a similar position to that of the free base and may still be constrained by hydrogen bonding.

It has been observed that, at a 1 : 1 mole ratio of Na^+ to tetracycline in a mixed DMSO–aqueous solution, Na^+ competes for binding and shifts the site of lanthanide co-ordination.[28] However the concentration of Na^+ present in this experiment is very much lower than that of tetracycline arising from titration of the free base to

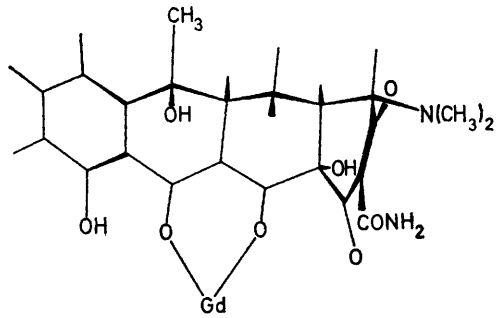

FIGURE 3 Observed conformation of the tetracycline–gadolinium(III) complex at pH 8.6

pH 8.6. Coupled with the low concentrations of lanthanide employed it seems unlikely that any shift in co-ordination site has been brought about by the presence of Na^+.

Conclusion.—The conformation of tetracycline undergoes a subtle rather than dramatic change upon deprotonation of the free base. That proton lost is probably from 12-hydroxy. Upon loss of this proton the Gd^{III} binding site shifts from 1-O amide to the 11,12β-diketone. In all three experiments more protons have been observed than are required to define the conformation of the complex. The additional data shows that there must be a predominance of a unique complex in solution and that there can be no significant contribution arising from co-ordination at a second site.

EXPERIMENTAL

Tetracycline was obtained from the Sigma Chemical Co. and gadolinium nitrate was prepared from the oxide, 99.99%, from Rare Earth Products Ltd. Tetracycline (0.04M at pH 2 and 8.6, 0.02M at pH 6.5) was dissolved in ^2H_2O and adjusted to the required pH with NaO^2H or ^2HCl. Gadolinium nitrate, 0.01M, was used at pH 2.0 and

an 0.01M solution of the EDTA complex was used at pH 6.5 and 8.6.

Spectra were recorded on a Bruker WH90-DS spectrometer. Care was taken to avoid the effect of spin decoupling by the metal ion by decoupling the resonances as far as possible. Line shapes of remaining multiplets were fitted with the aid of a computer programme. The transverse relaxation times, T_2, were estimated from the line width ($\Delta\nu$) at half peak height ($T_2^{-1} = \pi\Delta\nu$). For methyl groups the calculated internuclear distance is derived as previously.[30] 3-Trimethylsilylpropanesulphonic acid was used as a reference and to monitor any changes in bulk susceptibility. All experiments were carried out under conditions of fast chemical exchange.[30]

We acknowledge financial assistance from the University of Cape Town and the C.S.I.R.

[6/1976 *Received*, 25*th October*, 1976]