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A Proton Nuclear Magnetic Resonance Study of the Site of Metal Binding in Tetracycline

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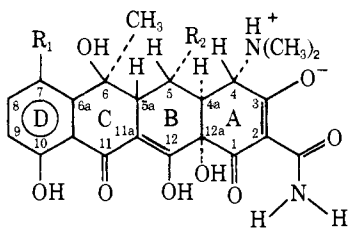
Abstract: An investigation directed toward establishing the site(s) of metal binding in the antibiotic tetracycline has been carried out in DMSO-d_6 solution using proton NMR. The paramagnetic ions Nd(III), Tb(III), V(III), Cu(II), Mn(II), and Co(II) and the diamagnetic ions La(III), Ca(II), and Mg(II) have been used. Isotropic shifts and broadening of certain tetracycline ^{1}H NMR signals are observed in the presence of paramagnetic ions. Diamagnetic ions also affect some of these ^{1}H NMR signals. Analysis of the selective effects of these ions on the ^{1}H NMR signals has led to the conclusion that in DMSO solution metal binding occurs at the tricarbonylmethane function of ring A, probably through oxygen donors.

The tetracyclines are a family of broad-spectrum antibiotics used extensively by the medical profession for more than 2 decades. The structures of 7-chlorotetracycline (Aureomycin; I, R_1 = Cl, R_2 = H) and 5-hydroxytetracycline (Terramycin; I, R_1 = H, R_2 = OH) were first deduced from chemical experiments and later confirmed through X-ray crystallographic studies. A review of the structural work and reaction chemistry of tetracyclines appeared in 1968.[2]



I

The multiplicity of potential metal binding sites present in the tetracyclines has attracted much interest. Albert[3,4] measured stability constants of metal complexes of these antibiotics in the early 1950's. The stability constants found for a number of metal ions are of the same order as those for β-diketonate or α-aminoacidate complexes of these ions,[5] and Albert recognized that tetracyclines must compete for metal ions in the human body. Concurrently it was discovered that the presence of excess metal ions neutralizes the effects of tetracyclines.[6,7] A series of experiments by Doluisio and Martin showed that therapeutically active tetracyclines form 2:1 ligand-to-metal complexes with Cu^{2+}, Ni^{2+}, and Zn^{2+}, whereas certain therapeutically inactive derivatives form only 1:1 complexes with these ions.[8] Also, using metal-free conalbumen as a model metalloenzyme drug receptor, they found binding of active tetracyclines to the receptor is greatly enhanced in the presence of Cu^{2+}, suggesting the existence of ternary drug-metal-receptor complexes.[9] Acting on the hypothesis that tetracyclines act by uncoupling oxidative phosphorylation through inhibition of metalloflavoenzymes, Colaizzi and coworkers measured the extent of inhibition of the metalloflavoenzyme NADH–cytochrome *c* oxidoreductase by a series of therapeutically active and inactive tetracyclines.[10] They presented evidence that inhibition results from chelation of iron in the enzyme by the drugs and suggested that the mode of action of tetracycline antibiotics involves inhibition of bacterial metalloflavoenzymes by chelation of enzymatically bound metal.

It is now generally agreed that the ultimate effect of tetracycline antibiotics in minimum doses is inhibition of bacterial protein synthesis as a result of binding of the drugs to bacterial ribosomes,[11–21] possibly mediated by metal ions such as magnesium.[11,13–16,18–21] It has also been proposed that metal ions serve to neutralize the charge on tetracyclines, thus enhancing transport through lipophilic bacterial cell walls.[22] There is some evidence that membrane penetration by tetracycline involves reversible association of the drug with membrane-associated cations, since chelating agents such as EDTA and ATP have marked inhibitory effects on uptake of tetracycline by membranes.[23] Although

the role of metal ions in the mode of action of tetracycline antibiotics is not settled at present, evidence accumulated over the years suggests that the chelating properties of the drugs may be important. It is not surprising that a number of investigators have attempted to establish the site or sites at which metal binding occurs. A variety of techniques has been employed, but there is general disagreement as to the site of binding. Proposed chelating groups are (a) the C_{10}–C_{11} ketophenol,[24] (b) the C_{11}–C_{12} β-diketone,[10,25,26] (c) the C_4 dimethylamine and the C_3 or C_{12a} hydroxyl,[8] (d) the C_1–C_3 tricarbonyl methane,[10,27] and (e) multidentate combination of the C_{11}–C_{12} β-diketone and the C_1–C_3 tricarbonyl methane achieved through folding the molecule along the C_{4a}–C_{12a} axis.[28]

It is clear that the binding site is not firmly established for any metal ion under a given set of conditions. Thus we are directing our efforts toward determining binding sites of a variety of metal ions in tetracycline antibiotics using a sensitive NMR technique involving paramagnetic ion probes. This method has been employed by a number of investigators in recent years to determine metal binding sites on molecules of biological interest.[29-36] In this paper are described the methods we have developed and the results obtained for tetracycline (I, $R_1 = R_2 = H$) in the presence of several paramagnetic and diamagnetic metal ions in dimethyl sulfoxide solution.

Experimental Section

Materials. Samples of tetracycline or its hydrochloride salt in the solid state are stable for at least several weeks at room temperature. Bulk samples were stored at −20°. The purity of samples used in the NMR experiments was determined by measuring the ratio of absorbances at 254 and 276 nm for solutions in 0.1 *N* H_2SO_4.[37] Tetracycline and its derivatives were dried by heating at 60° in vacuo for 8 hr. Deuteration of the labile protons in tetracycline was achieved by exchange with D_2O.

Anhydrous nitrate salts of Mg(II), Ca(II), and La(III), were prepared by published methods.[38-40] Nitrate salts of Nd(III) and Tb(III) were prepared from the corresponding oxides. Anhydrous VCl_3 obtained from Alfa Inorganics was used without further purification. Finely ground samples of $Ni(NO_3)_2 \cdot 6H_2O$, $Co(NO_3)_2 \cdot 6H_2O$ and $CuCl_2 \cdot 2H_2O$ were dehydrated by heating. Anhydrous manganous nitrate was prepared by treating a solution of $MnCl_2 \cdot 4H_2O$ in absolute ethanol with $AgNO_3$. After removing AgCl by filtration, the solvent was removed in vacuo. $Mn(NO_3)_2$ is extremely deliquescent, so it was stored in form of a DMSO-d_6 stock solution over molecular sieves.

Syntheses. Tetracyclinonitrile (in which the 2-carboxamide function is converted to a nitrile) was prepared by the method of McCormick et al.[37] 2-Carbamoyldimedone was prepared by the method of Scarborough and Gould.[41]

Bis(tetracyclinato)nickel(II) and bis(tetracyclinato)cobalt(II) were made according to the procedure of Baker and Brown.[27] Tris(tetracyclinato)vanadium(III) was prepared under a nitrogen atmosphere by mixing methanol solutions of ligand and VCl_3 in a 4:1 mole ratio, respectively, then adding triethylamine as base. The solution was stirred for 2 hr, then the yellow-brown precipitate was collected and recrystallized twice from a solvent mixture of 3:1 1,2-dichloroethane:toluene under nitrogen. After drying for 8 hr at 65°, the following analysis was obtained. Anal. Calcd values for $V(C_{22}H_{24}O_8N_2)_3 \cdot 3H_2O$: C, 55.23; H, 5.26; N, 5.85. Found: C, 55.66; H, 5.08; N, 5.72.

NMR Experiments. The DMSO-d_6 (99.5 or 99.9% deuterated) used for NMR studies was obtained from Stohler Isotope Chemicals and stored over molecular sieves. Tetracyclines are known to be unstable in aqueous solution.[37] DMSO solutions of tetracyclines showed noticeable changes in color after only 3 hr,[42] thus NMR studies were carried out as rapidly as possible using freshly prepared solutions.

It is important to maintain tetracylines, metal salts, and solvents as dry as possible since the water signal interferes with some of the tetracycline resonances. Also water causes small but measurable upfield shifts in the tetracycline signals. Similar effects have been observed previously for 2′-deoxyadenosine in DMSO[43] and have been attributed to hydration[43] or to solute–DMSO interactions.[44]

Stock solutions of anhydrous tetracycline (0.2 *M*) in dry DMSO-d_6 containing tetramethylsilane (TMS) were prepared in dry 5-ml volumetric flasks. Similarly, stock solutions of the anhydrous metal salts (0.2 *M*) in DMSO-d_6 were prepared. These solutions were sealed with rubber serum caps. Aliquots were transferred to NMR tubes using calibrated 0.25-ml or 10-μl syringes. Immediately prior to each set of NMR experiments, the NMR spectrum of tetracycline free base was recorded. This provides a means of monitoring any slight chemical shift variations brought about by traces of moisture.[43,44] Then 0.01-ml increments of the metal ion stock solution were added to the known volume of tetracycline solution (usually 0.48 ml), and the NMR spectrum was recorded after each addition. Thus the mole ratios of metal ion to drug are known to within the errors involved in preparing the stock solutions and the accuracy of calibration of the syringes. During experiments with the air-sensitive ions V(III) and Co(II), all solvents were degassed, and solutions were kept under a nitrogen atmosphere.

NMR spectra were recorded on a Varian Model HA-100 spectrometer in the HR mode and calibrated by linear interpolation using the 2-KHz side band of TMS. The probe temperature was 32°. Double resonance experiments were carried out in the HA mode with the field locked on the signal of benzene added to 0.1 *M* solutions of tetracycline in DMSO-d_6. A few spectra were recorded at 60 MHz using a Varian Model A-60 spectrometer.

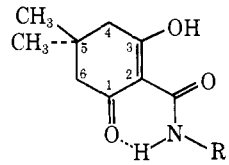
Results and Discussion

The Paramagnetic Ion Probe Method. Nuclear magnetic resonance signals are perturbed by the presence of paramagnetic ions in solution, giving rise to two basic effects: (a) signal broadening and (b) signal shifts (isotropic shifts). Useful information may be gained from either of these two experimentally observable effects. Although in some cases both these effects are observed, often one predominates. Analysis of *selective* broadening and/or shifting may reveal the immediate environment of the paramagnetic ion (and hence the binding site). The reader is referred to a recent comprehensive treatment of NMR of paramagnetic species.[45]

Although analysis and interpretation of NMR spectra of paramagnetic molecules are often difficult, a number of simple systems are well understood. Some of these systems are structurally related to various portions of tetracycline and its derivatives, and the arguments used in interpreting spectra of the simpler systems will be applied to tetracyclines bound to paramagnetic metal ions. In this investigation NMR spectra of tetracycline, hereafter abbreviated TC, in DMSO-d_6 solutions to which paramagnetic metal ions were added were examined for the occurrence of selective broadening and/or isotropic shifts over a range of metal ion:TC ratios. DMSO was chosen as the solvent in this initial study since both TC and the metal salts used are sufficiently soluble for NMR. TC has only limited solubility in water at neutral pH.

When complexes are labile on the NMR time scale the observed chemical shifts (or isotropic shifts) and line widths are weighted averages of those of free and bound ligand. Plots of $[M^{n+}]/[L]$ vs. isotropic shifts are expected to be linear only when $[M^{n+}] \ll [L]$, where $[M^{n+}]$ and [L] represent total metal ion and ligand concentrations, respectively. In the case of TC the slopes of these plots will be determined by the proportions of 1:1, 2:1, 3:1, etc., complexes in equilibrium. The situation is further complicated for TC by the possibility of metal ions coordinating to more than one site on the ligand. Thus plots of $[M^{n+}]/[TC]$ vs. isotropic shift are used here only in a qualitative sense to demonstrate the effects of added metal, and no effort is made to evaluate equilibrium constants, limiting isotropic shifts, or ligand–metal ratios from slopes of these plots.

NMR Signal Assignments. It is necessary to assign proton NMR signals of TC and its derivatives in DMSO, since no complete signal assignments in this solvent have previously been made.[46] Signal positions, particularly of the N-H and O-H protons, vary considerably from solvent to solvent. A number of TC derivatives were examined previously by NMR by Schach von Wittenau and Blackwood[47] using a variety of solvents, but DMSO was used only for the hydrochloride salt of TC. Dudek and Volpp[48] have made NMR assignments for 2-carbamoyldimedone, II (R = H), and



II

other related molecules which are believed to be good models of ring A of tetracyclines. The NMR spectrum of chelocardin, a related antibiotic, in DMSO solution has been reported more recently.[49] The following assignments, which are reasonable but not unequivocal in all cases, are made from a combination of data from the literature, observations on model systems, and spin decoupling and deuterium exchange experiments. Water-free TC and its derivatives were used in the NMR experiments to reduce the intensity of the water signal around 2.8 ppm. The observed chemical shifts of TC vary slightly depending upon the dryness of the sample and solvent (see Experimental Section). However, chemical shifts are independent of concentration in the 0.2–0.5 *M* range used in this study.

The proton NMR spectrum of TC in DMSO-d_6 solution is shown in Figure 1. Two intense signals at 1.5 and 2.4 ppm downfield of tetramethylsilane are assigned to the C_6 methyl and C_4 dimethylamino groups, respectively. The 2.4 ppm signal also contains the DMSO-d_6 resonance. The D-ring aromatic protons are responsible for the multiplets at 6.7–7.5 ppm.[47] These reduce to a pair of doublets in 7-chlorotetracycline.

Broad resonances at 13.6, 11.7, 9.0, 8.6, and 4.9 ppm are attributed to N-H and O-H since these disappear or are significantly reduced in intensity in samples which have been deuterium exchanged. The signals at 9.0 and 8.6 ppm are assigned to the A-ring amide protons, since these are absent in the spectrum of tetracyclinonitrile. Signals at very nearly these same positions for II (R = H) in DMSO solution (9.2 and 8.5 ppm) have been attributed to the carboxamide protons, and the one at lower field has been assigned to the proton involved in an intramolecular hydrogen bond.[50] The resonance at 11.7 ppm is assigned to the C_{10} phenolic proton since the phenolic proton of 2-hydroxy-5-methylacetophenone, believed to be a reasonable model of ring D of TC, resonates at 11.9 ppm in DMSO. Spectra of the hydrochloride salts of TC and 5-hydroxytetracycline show a moderately broad signal at 14.7–14.8 ppm in DMSO. This signal is absent in spectra of the free bases (zwitterions), but does occur at 14.1 ppm for tetracyclinonitrile in DMSO. It seems reasonable to assign this to the C_3 OH.[46] The analogous signal for II in DMSO occurs at 18.4 ppm;[50] however, the absence of the adjacent dimethylammonium group in II is likely responsible for this difference. Assuming the above assignments are correct, the broad signal at 4.9 ppm and the very broad signal at ~13.6 ppm must arise from the C_6, C_{12}, or C_{12a} hydroxyls or the N-H from the dimethylammonium group. The enol OH of 2,4-pentanedione in DMSO generates a very broad signal around 15.4 ppm, implying that the C_{12} OH signal of tetracycline should also be far downfield and very broad. Thus the ~13.6 ppm signal is tentatively assigned to C_{12} OH. The resonance at 4.9 ppm cannot be assigned with certainty, but there is some evidence that this arises from the C_6 OH. Thus in 7-chlorotetracycline this signal shifts downfield to 5.0 ppm accompanied by a downfield shift of similar magnitude for the C_6 methyl. The possibility of the 4.9 ppm signal arising from the C_{12a} OH cannot be excluded, however, since hydroxyl proton signals of 10% solutions of *tert*-butyl alcohol and ethanol in DMSO occur at 4.2 and 4.3 ppm, respectively. The environment of OH in these molecules should roughly approximate that of C_6 OH or C_{12a} OH in tetracyclines. A broad signal largely obscured by the amide proton resonances cannot be assigned at present.

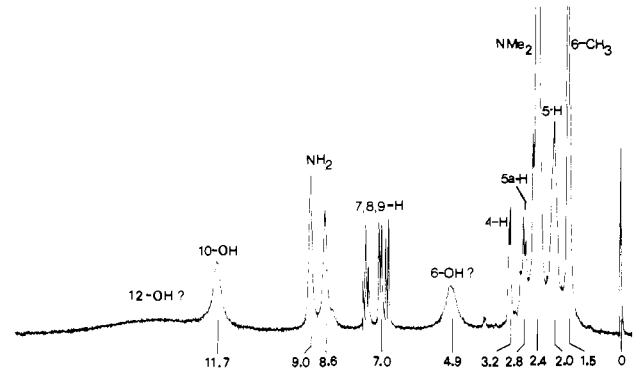


Figure 1. Proton NMR spectrum of tetracycline in DMSO-d_6 at 100 MHz. Chemical shifts are in parts per million relative to tetramethylsilane.

Signals of the remaining protons at C_4, C_{4a}, C_5, and C_{5a} are expected to be narrow, to occur at high field, and to show spin-spin splitting. A poorly resolved multiplet centered around 2.0 ppm is attributed to the C_5 methylene protons on basis of its high field position and its area, which is close to that expected for two protons. In 5-hydroxytetracycline this signal is absent but is replaced by one of half its area at 4.2 ppm. A similar downfield shift of the C_5 proton resonance with hydroxylation at C_5 was noted earlier for other solvents.[47] Signals arising from protons at C_4, C_{4a}, and C_{5a} in tetracycline are assigned from double resonance experiments, assuming the C_5 signal is correctly assigned. The resonances at 3.2 and 2.8 ppm, each of area 1.0, are shown to be a spin doublet and triplet, respectively, by comparison of spectra at 60 and 100 MHz. A doublet is expected for the C_4 proton, and a triplet might be expected for the C_{5a} proton from the simplest approach. Irradiation of the signal at 2.0 ppm, assigned to the C_5 protons, causes collapse of the triplet at 2.8 ppm to a singlet. The latter is thus assigned to the C_{5a} proton. Any effect of irradiation at this frequency on the signal at 2.6 ppm (shoulder) is not apparent due to the proximity of the dimethylamino group resonance. However, irradiation at 2.6 ppm reduces the doublet at 3.2 ppm into a singlet as expected if the doublet arises from the C_4 proton and the signal at 2.6 ppm is due to the C_{4a} proton.

The spectra of 5-hydroxytetracycline and 7-chlorotetracycline in DMSO are generally very similar to that of tetracycline, with only small changes in chemical shifts for most signals (see Table I). As noted above, a signal at 4.2 ppm for 5-hydroxytetracycline is attributed to the C_5 proton. This is not affected by deuterium exchange as is the case for the adjacent signals at 5.2 and 4.6 ppm. The 5.2 ppm signal is probably due to the C_6 or C_{12a} hydroxyl as discussed above for tetracycline, and the signal at 4.6 ppm (not present in the spectrum of tetracycline) is assigned to the C_5 hy-

Table I. Summary of NMR Signal Assignments for Tetracycline and Derivatives in DMSO[a]

	Position										
	2	3	4	4a	5	5a	6	7–9	10	12	12a
Tetracycline	8.6 9.0		2.4 (CH_3)[b] 3.2 (H)[e]	2.6[c]	2.0[f]	2.8[g]	1.5 (CH_3) 4.9 (OH)[d]	6.7–7.5	11.7	13.6 (?)	4.9 (?)[d]
Tetracycline·HCl	8.9 9.3	14.7	2.9 (CH_3) 4.3 (H)	2.8[c]	1.7–2.2	2.8[c]	1.5 (CH_3) 4.9 (OH)[d]	6.7–7.5	11.6		
5-Hydroxytetracycline	8.7 9.0		2.4 (CH_3) 3.4 (H)	2.6[c]	4.2 (H) 4.6 (OH)	2.8	1.7 (CH_3) 5.2 (OH)[d]	6.8–7.5	11.6		5.2 (?)[d]
5-Hydroxytetracycline·HCl	9.0 9.4	14.8	2.9 (CH_3) 4.7 (H)	2.8[c]	3.8 (H)	2.8[c]	1.7 (CH_3) 5.7 (OH)	6.8–7.5	11.5		
7-Chlorotetracycline	8.1 8.4		2.2 (CH_3) 3.0 (H)	2.4[c]	1.8	2.7	1.7 (CH_3) 5.0 (OH)	6.4–7.0	11.5		
7-Chlorotetracycline·HCl	8.4 8.9	14.4	2.7 (CH_3) 4.0 (H)	2.7[c]	1.7–2.1	2.7[c]	1.7 (CH_3) 5.0 (OH)	6.4–7.0	11.3		
Tetracyclinonitrile		14.1	2.6 (CH_3) 3.5 (H)	2.5[c]	1.8	2.5[c]	1.4 (CH_3) 4.3 (OH)	6.4–7.1	11.0		

[a] Chemical shifts in parts per million relative to TMS for water-free tetracyclines in dry DMSO-d_6. [b] Signal envelope includes the DMSO-d_5 resonance. [c] Signal largely obscured by the methyl resonance. [d] Tentatively assigned to C_6 OH but may be due to C_{12a} OH. [e] Spin doublet. [f] Poorly resolved multiplet. [g] Spin triplet.

droxyl. As expected, the signal at 2.8 ppm, which is a spin triplet for TC and assigned to the C_{5a} proton, becomes a doublet in 5-hydroxytetracycline.

Effects of Metal Ions on NMR Spectra. The metal ions most likely to play a role in the antibiotic behavior of TC and its derivatives are Mg^{2+} and Ca^{2+}, thus the sites of binding of these ions are of primary importance. However, in order to reveal the overall picture of metal binding in tetracyclines as unusual ambidentate ligands, it is also of interest to determine binding sites for other metal ions. Results obtained for the latter are discussed first.

Vanadium(III). This ion was chosen for several reasons. V(III) complexes are known to exhibit ligand NMR signals which are extremely narrow for paramagnetic species.[51-54] The observed isotropic shifts are often large and are believed to be primarily contact shifts.[51-54] Furthermore, isotropic proton shifts for V(III) complexes of salicylaldehyde[53] and β-diketones[54] have been reported and interpreted in terms of a predominant π spin delocalization mechanism. These ligands may be regarded as models of certain portions of the TC molecule; thus in TC the C_{10}–C_{11} chelating site is electronically related to salicylaldehyde, and the C_{11}–C_{12} and C_1–C_2–C_3 sites may be regarded as β-diketone analogs. If V(III) binds to these sites, the pattern of observed proton isotropic shifts should parallel those observed for the model complexes.

Addition of VCl_3 to DMSO-d_6 solutions of TC as described in the Experimental Section causes significant shifts only for C_4–H, the 8.6 ppm amide-H, and NMe_2 NMR signals as shown in Figure 2. At $[V^{3+}]/[TC]$ ratios greater than 0.2 the signals become too broad for accurate chemical shift measurement. No shifts are detected for the aromatic ring-D protons. Since appreciable isotropic shifts are observed for the aromatic protons of tris(salicylaldehydato)-vanadium(III), it must be concluded that V(III) does not bind to the C_{10}–C_{11} site of TC. Binding at the C_{11}–C_{12} β-diketone site is expected to result in alternating upfield and downfield shifts for the ring-D protons and an upfield shift for C_{5a}–H, since the latter is analogous to an α-methyl proton on a β-diketonate ligand.[54] Clearly significant binding must occur only at the ring-A sites. Several tautomers are possible for the C_1–C_2–C_3 tricarbonyl methane function, and β-diketone-like chelate rings can be formed assuming binding through oxygens associated with the amide group and either C_1 or C_3. The pattern of proton isotropic shifts expected from either of these two binding modes is identical. The amide NH's and C_4–H are expected to receive negative (low field) shifts in analogy to the methyl protons of

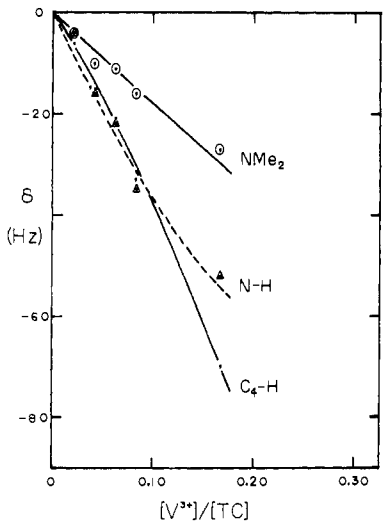


Figure 2. Plot of apparent isotropic shifts for tetracycline protons vs. $[V^{3+}]/[TC]$.

tris(acetylacetonato)vanadium(III).[51,52] Contact shifts of the NMe_2 protons involve σ spin delocalization and are expected to be negative and much smaller than those of the amide NH's or C_4–H. The direction of observed shifts is consistent with binding of V(III) through oxygens of ring A. However, the relative magnitude of observed shift for the NMe_2 signal is larger than expected. *Isotropic* shifts are probably smaller than the observed shifts, since small but significant downfield shifts are also observed for NMe_2 and C_4–H signals of TC in the presence of diamagnetic metal ions (vide infra). At present there is no reliable means of correcting the observed shifts for these effects in the presence of V(III). Direct metal binding to NMe_2 is deemed unlikely in view of the poor binding properties of tertiary amines relative to β-diketones.[5] Binding through the amide nitrogen, which can become part of a π delocalized chelate ring through tautomerization, should produce qualitatively the same pattern of isotropic shifts as binding through either combination of oxygens.[55] However, an extremely large isotropic shift would then be expected for N–H due to its proximity to V(III).

It is concluded from the NMR data presented here that binding of V(III) to TC likely involves oxygen atoms of the tricarbonyl methane function of ring A. At present a distinction between the two binding modes, amide oxygen plus

either C_1 or C_3 oxygens, cannot be made. The small isotropic shifts observed in this investigation for V(III) and other paramagnetic ions relative to those normally found in discrete bis or tris complexes is attributed to competition by DMSO for coordination sites of the metal. Thus an appreciable portion of the added metal ion may be solvated rather than bound to TC. The NMR spectrum of $V(TC)_3$ was recorded; however, signals proved too broad to allow definite assignments.

Cobalt(II). Dipolar shifts can make significant contributions to observed isotropic shifts for Co(II) complexes.[45] The dipolar contribution can be calculated if the orientation of a given nucleus relative to the principal magnetic axes is known, and values for the principal magnetic susceptibilities and the metal-nuclear distance are available.[56] Conversely, if dipolar shifts are known for several nuclei it is possible to obtain a rough picture of the geometry of the complex (and hence the binding site). Assumptions have to be made regarding the direction of the principal magnetic axes. Unfortunately there is no reliable method of separating contact and dipolar shifts for the system consisting of Co(II), DMSO, and TC in equilibrium.

Figure 3 shows that significant NMR shifts are found only for C_{4a}-H, C_4-H, NMe_2, and the 8.6 ppm protons of TC in the presence of Co(II). All signals become broadened at high $[Co^{2+}]$. Dipolar interactions operate only over a short range, and, unless long-range contact shifts arise from an unusual amount of σ spin delocalization, the data indicate Co(II) is binding to groups on ring A. As for V(III) the observed shifts likely contain contributions other than those arising from the paramagnetism of Co(II), and these contributions cannot accurately be assessed. Although no firm conclusions can be drawn from these data, it is likely that the binding sites for Co(II) and V(III) are the same.

Baker and Brown previously prepared $CoL_2 \cdot 2H_2O$ and $NiL_2 \cdot 2H_2O$ where L = TC, anhydrotetracycline, and dedimethylaminotetracycline.[27] They presented convincing evidence from ligand field spectra that the binding site is the same for all three ligands and concluded that binding occurs through oxygens of the tricarbonyl methane system of ring A. NMR spectra of $Co(TC)_2 \cdot 2H_2O$ prepared in the present study proved to be insufficiently resolved for signal assignments.

Copper(II). Cu(II) has often been used as a paramagnetic ion probe of binding sites in molecules of biological interest.[31-32,35-36] It functions primarily as a selective broadening agent with little or no accompanying isotropic shifts. The very short-range dipolar broadening mechanism has been assumed to dominate.[57] Addition of small amounts of $Cu(NO_3)_2$ to DMSO solutions of TC results in selective broadening of C_4-H and both amide NMR signals. There is also some evidence for selective broadening of the C_5-H's, but this is not nearly so apparent as for C_4-H and NH_2. At higher $[Cu^{2+}]/[TC]$ values, all signals become broad. Again, the NMR evidence favors binding at the ring A sites. Although Cu(II) generally prefers nitrogen donors, an X-ray structural investigation of the Cu(II) complex of *N*-phenyl-2-carbamoyldimedone (II, R = phenyl) shows that binding occurs through the oxygens.[58] Results of an ir, NMR, and EPR study on N-substituted and unsubstituted 2-carbamoyldimedone complexes of Cu(II), Ni(II), Co(II), and Zn(II) are also consistent with binding through oxygens.[50] Since no selective effects on other PMR signals of TC in the presence of Cu(II) are evident, it is concluded that binding occurs through two oxygens of the ring A tricarbonyl methane group as found in the 2-carbamoyldimedones. Space-filling models show one of the C_5 protons to be in surprisingly close proximity to the proposed site of binding for one of the possible conformations of TC. This could account for the apparent selective broadening of the C_5-H_2 signal.

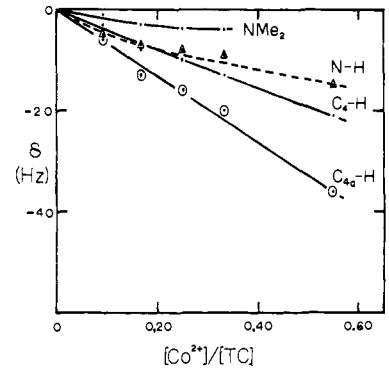


Figure 3. Plot of apparent isotropic shifts for tetracycline protons vs. $[Co^{2+}]/[TC]$.

Manganese(II). This ion has been extensively used by Cohn and coworkers as a nuclear relaxing agent to probe the active sites of enzymes which are activated by divalent ions.[59,60] The binding properties of Mn(II) and Mg(II) are sufficiently similar that Mg(II) may often be replaced by Mn(II) in biological molecules.[59] The latter is a far better magnetic and spectroscopic probe of its environment. Thus Mn(II) was chosen for the present investigation with the assumption that the binding site found for Mn(II) would be the same as for Mg(II).

The presence of small amounts of $Mn(NO_3)_2$ causes no significant NMR shifts of TC protons; however, there is definite selective broadening of the amide proton signal at 8.6 ppm. At higher $[Mn^{2+}]/[TC]$ ratios all signals are broadened. Selective broadening of the 8.6 ppm amide signal is also found with Cu(II), and significant shifts of this signal occur in the presence of V(III) and Co(II). The implication is that Mn(II) interacts with the carboxamide group, and the mode of binding is likely the same for all these ions. The fact that Mg(II) also shows a selective effect on the 8.6 ppm amide signal (vide infra) is evidence that Mn(II) and Mg(II) bind at the same site in TC.

Rare Earth Ions. Isotropic shifts for TC protons in the presence of V(III) and Co(II) were obtained by subtracting chemical shifts of free TC from the observed chemical shifts in the presence of these ions. These isotropic shifts only approximate the effects of paramagnetic ions on the spectra, since it is known that interaction of *diamagnetic* cations such as Zn^{2+} and H^+ with TC and 2-carbamoyldimedone causes nonnegligible ^1H NMR shifts for certain protons.[47,50,61]

Ideally one would like to correct for these diamagnetic shifts in all studies involving paramagnetic ion probes by taking the difference between shifts observed in the presence of diamagnetic and paramagnetic ions. However, such a procedure is justified only if (1) the binding sites for paramagnetic and diamagnetic ions are identical, and (2) the polarizing power and nature of binding (ionic, covalent, π-back-bonding, etc.) are similar for the two ions. It is reasonable to assume these requirements are satisfied for rare earth cations, using La(III) as the diamagnetic probe. Thus spectra of TC in the presence of La(III), Nd(III), and Tb(III) were examined in hopes of establishing a binding site not only for these ions, but more importantly for Ca(II) which often may be isomorphically replaced by rare earth ions in biologically important molecules.

In the presence of $La(NO_3)_3$ downfield shifts for the TC C_4-H, C_{4a}-H, and NMe_2 protons and an upfield shift for the 8.6 ppm amide proton are observed. In addition, the 8.6 ppm amide NMR signal shows a pronounced and selective

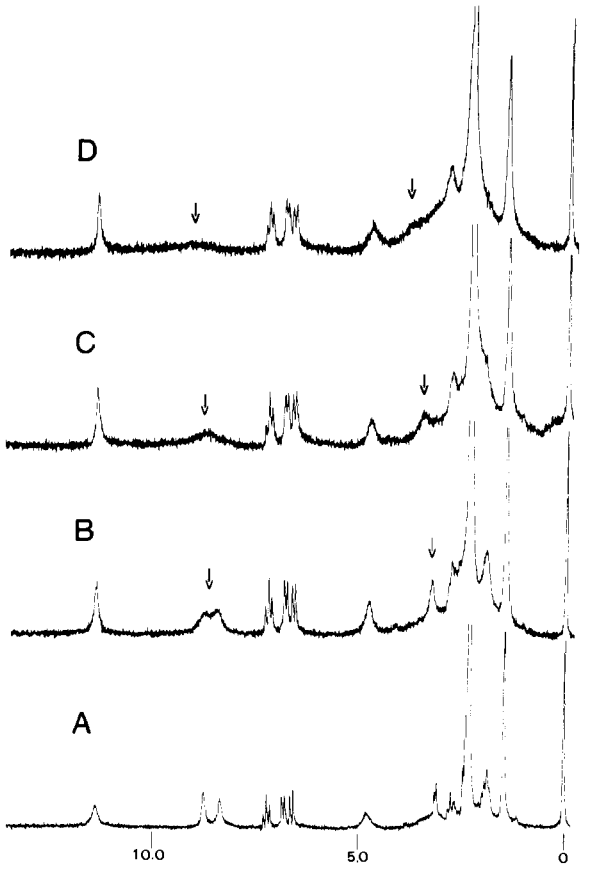


Figure 4. Proton NMR spectra of tetracycline in DMSO-d_6 at various $[Nd^{3+}]$:[TC] values: A, no Nd^{3+}; B, 1:12; C, 2:12; D, 3:12. Arrows point out signals selectively broadened.

broadening with increasing $[La^{3+}]$ and is nearly lost in the baseline at $[La^{3+}]/[TC] \geq 0.3$.

Addition of $Nd(NO_3)_3$ to TC solutions results in significant downfield shifts for C_4-H and the 8.6 ppm amide proton signal. Much smaller downfield shifts are observed for the NMe_2 and C_5-H_2 NMR signals. Signals arising from C_4-H, both amide protons, and possibly the C_5-H_2 protons are selectively broadened as shown in Figure 4. In order to evaluate the paramagnetic contribution to the observed signal shifts, chemical shifts found in the presence of La(III) at various $[M^{3+}]/[TC]$ values were subtracted from those observed in the presence of the same concentrations of Nd(III). A plot of these isotropic shifts is shown in Figure 5.

Isotropic shifts caused by paramagnetic rare earth ions are believed to be predominately the short-range dipolar type.[62] Since the only signals shifted significantly here are those of protons associated with ring A of TC and since those signals selectively broadened arise from protons on or near ring A, it is concluded that binding involves donor atoms associated with ring A. As mentioned earlier, one of the C_5 protons may be quite close to these donor atoms, thus accounting for the selective broadening and shifting of the C_5-H_2 signal. Binding at the C_{10}-C_{11} site may be ruled out since the C_{10}-OH and C_7-C_9 aromatic ^1H NMR signals are not perturbed in any selective way in the presence of Nd(III). Binding at the C_{11}-C_{12} site is expected to result in more pronounced effects on NMR signals of the C_{10}-OH, C_6-OH, C_{5a}-H, and C_5-H's than on those associated with ring A. Although binding at the C_{11}-C_{12} site cannot completely be ruled out, the data favor binding at ring A. Binding of NMe_2 is unlikely in view of the relatively poor binding properties of tertiary amines and the absence of se-

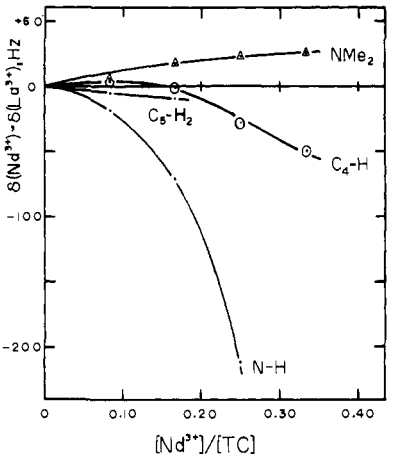
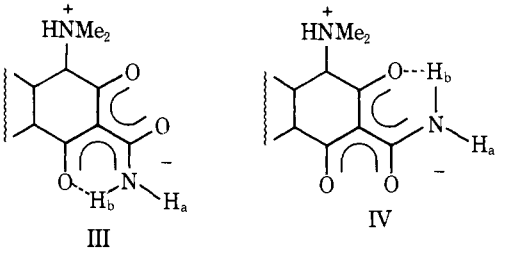


Figure 5. Plot of isotropic shifts for tetracycline protons vs. $[Nd^{3+}]/[TC]$ as determined using shifts observed at the same $[La^{3+}]/[TC]$.

lective broadening of the methyl proton signals. This leaves the tricarbonyl methane function, and binding probably occurs through oxygens of this group rather than the amide nitrogen. The rate of broadening of the amide ^1H NMR signals with increasing $[Nd^{3+}]$ is not significantly greater than that of the C_4-H signal as would be expected for N bonding; also very large isotropic shifts for the amide protons would be expected for N bonding.

No selective broadening of TC ^1H NMR signals in the presence of $Tb(NO_3)_3$ is apparent. At higher $[Tb^{3+}]/[TC]$ values general broadening of all signals occurs. Downfield shifts are observed for C_4-H, NMe_2, and 8.6 ppm amide signals. Isotropic shifts were determined using the La(III) data as for Nd(III). As is the case for Nd(III), the largest shifts occur for the amide signal, and all other shifted signals arise from protons associated with ring A. Again it is concluded that binding involves oxygen donors of the tricarbonyl methane function.

It remains to account for the selective broadening of the 8.6 ppm amide NMR signal in the presence of La(III). A number of valence bond resonance structures and tautomers may be written for the tricarbonyl methane function of ring A.[48] In solution it is perhaps best described as a completely delocalized zwitterion such as III or possibly IV where



III IV

there is a strong intramolecular hydrogen bond for one amide proton. This is consistent with bond lengths found by X-ray studies of TC derivatives.[63,64] Hydrogen bonded protons generally resonate at lower-than-normal magnetic fields,[65] and the amide NMR signal at lower field (9.0 ppm) for TC is probably H_b in III or IV. This is in agreement with assignments made earlier for the 2-carbamoyldimedone amide NMR signals.[48,50] If this assignment is correct, H_a in III and IV is selectively broadened and shifted upfield in the presence of diamagnetic La(III) (and Ca(II), vide infra). The broadening is most likely the result of a chemical exchange process induced by La(III). Any change in the nitrogen quadrupole relaxation time in the presence

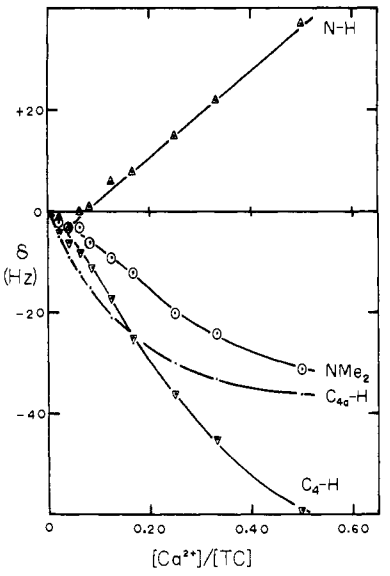


Figure 6. Plot of NMR shifts for tetracycline protons vs. $[Ca^{2+}]/[TC]$.

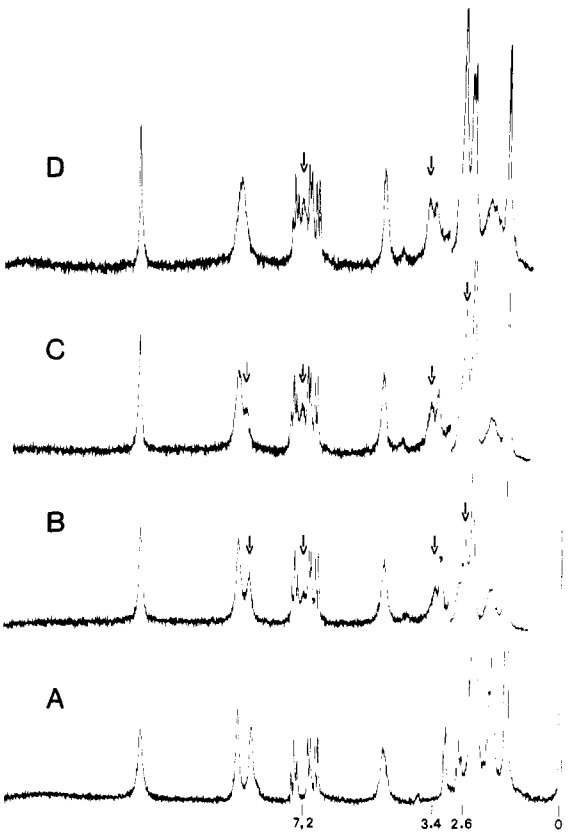


Figure 7. Proton NMR spectra of tetracycline in DMSO-d_6 at various $[Mg^{2+}]$:[TC] values: A, no Mg^{2+}; B, 2:16; C, 3:16; D, 4:16.

of La(III) would affect both amide NMR signals. The same is true for any La(III) induced structural equilibrium such as III $\rightleftarrows$ IV or rotation about the amide C–N bond. The NMR results indicate the hydrogen bond is not broken, but the environment of H_a is perturbed.

The amide NMR signal of 2-carbamoyldimedone (II, R = H) at 9.2 ppm shows only slight solvent dependence, whereas the other amide NMR signal (H_a) occurs some 2.6–3.6 ppm to higher field in $CHCl_3$ than in more basic solvents such as pyridine and DMSO.[48,50] It appears that this proton forms a hydrogen bond to pyridine and DMSO. A possible explanation for the broadening and upfield shift of the NMR signal due to H_a of TC in the presence of La(III), consistent with binding at ring-A oxygens, is that the electronic and steric effects of the nearby cation perturb the H_a–DMSO hydrogen bond. Assuming the La–TC complex is labile on the ^1H NMR time scale, the observed NMR signal is an average of those of DMSO bound TC (low field) and La–TC complex (high field). The signal position reflects the proportion of the two species and moves toward higher field as $[La^{3+}]/[TC]$ increases. The signal broadening may be explained by assuming the rate of site exchange is intermediate on the ^1H NMR time scale.

Calcium(II). The effect of Ca(II) on the ^1H NMR spectrum of TC is very similar to that of La(III). Downfield shifts of NMe_2, C_{4a}–H, and C_4–H occur, whereas the 8.6 ppm amide NMR signal is shifted upfield and broadened. The observed shifts as a function of $[Ca^{2+}]/[TC]$ are plotted in Figure 6. The broadening of N–H is not as pronounced as in the presence of La(III), perhaps as a result of the lower charge on Ca(II). The results indicate Ca^{2+} binds at the same site as La(III) and the paramagnetic rare earth ions, namely, the tricarbonyl methane function. Presumably the origin of selective effects on the 8.6 ppm amide signal is the same as in the presence of La(III).

Magnesium(II). The effects of Mg(II) on the ^1H NMR spectrum of TC are different from those of any other metal ion examined. Small downfield shifts are found for resonances of C_4–H, C_{4a}–H, and NMe_2 and are similar to those observed in the presence of Ca(II) and La(III). However, in contrast to its behavior in the presence of Ca(II) and La(III), the 8.6 ppm amide NMR signal remains fairly sharp and shifts downfield. This signal and those arising from C_4–H and NMe_2 diminish in strength as $[Mg^{2+}]$ increases. Simultaneously three new signals appear at 7.2, 3.4, and 2.6 ppm and increase in strength with increasing $[Mg^{2+}]$ (see Figure 7). These new signals are tentatively assigned to N–H (7.2 ppm), C_4–H (3.4 ppm), and NMe_2 (2.6 ppm) protons in a modified form of the TC molecule, and at high Mg(II) concentrations they predominate over the original signals.

Sheberstova et al. have recently shown that for equilibrium mixtures of TC hydrochloride and its C_4-epimer (ETC) in pyridine-d_5 solution two distinct NMR signals may be seen for the C_4–H proton, the NMe_2 protons, and the C_6–CH_3 protons.[66] If Mg(II) induces epimerization of TC in DMSO, the data indicate that Mg(II) prefers to bind the ETC, and ligand exchange is slow on the ^1H NMR time scale. However, Sheberstova et al. make no mention of effects on N–H resonances, and additionally the apparent absence of any change in the C_6–CH_3 resonance in the present work leaves some doubt as to whether the new signals arise from ETC.

X-Ray structural studies on TC derivatives have proven the existence of both limiting conformations of the TC framework obtainable by twisting about the C_{12a}–C_{4a} bond.[63] Also there is some NMR evidence for existence of different conformations among TC derivatives in solution from the magnitude of the C_4–H–C_{4a}–H coupling constant.[47] A conformational change in TC might be expected to result in new chemical shifts for protons such as C_{4a}–H, C_4–H, NMe_2, and C_5–H_2 whose environment would be significantly changed. However, the environment of NH_a is not expected to change much with a conformational change. A possible explanation of the spectral results assumes that Mg(II) prefers to bind to TC in a conformation different from that of free TC in DMSO and different from that in the presence of the other metal ions investigated.[67] This conformation change must be slow on the NMR time scale,

at least in the presence of Mg(II). The new NMR signals at 3.4 and 2.6 ppm are thus assigned to C_4-H and NMe_2, respectively, in the Mg-bound TC.[68] The new signal at 7.2 ppm is attributed to $N-H_a$ of the Mg-TC complex, but in this case the new chemical shift results directly from the presence of Mg(II) rather than from the conformational change. Note that the new $N-H_a$ signal is upfield of the original, consistent with a disruption of DMSO-TC hydrogen bonding as discussed above for TC in the presence of Ca(II) and La(III). If ligand exchange is slow in the NMR time scale for Mg(II) here, two separate signals for $N-H_a$ are expected. Further work will be necessary to verify the above interpretation and to determine which conformation is preferred by Mg(II). The interpretation given here is consistent with binding at the ring A tricarbonylmethane group.

Tests of the Binding Model. The experiments described in this paper strongly indicate binding at the ring A tricarbonylmethane function of TC for all metal ions investigated. A logical test of this binding model is to examine derivatives of TC in which this function is altered. Tetracyclinonitrile, in which the 2-carboxamide function is converted to a nitrile, was prepared from TC as described previously.[37] The ^1H NMR spectrum of tetracyclinonitrile in DMSO-d_6 in the presence of increasing amounts of Nd(III) was examined. Nd(III) was chosen for this study because it causes both shifting and selective broadening of ^1H NMR signals for TC. However, Nd(III) causes no observable signal shifts for tetracyclinonitrile, even for the C_4-H signal which had a relatively large shift under similar conditions for TC. No selecting broadening effects are apparent except for the 4.3 ppm signal which is believed due to the C_6-OH. The behavior of this signal and that arising from traces of water in the sample indicate that Nd(III) induces exchange between these protons. Clearly the mode of Nd(III) binding has been severely altered by destruction of the 2-carboxamide group.

Another test of the binding model involves molecules having only the tricarbonylmethane function available for metal binding. This requirement is satisfied by 2-carbamoyldimedone (II, R = H). The 4,6-methylene and 5-methyl protons of II are analogous to the C_4-H and C_5-H_2 protons, respectively, of TC. If the proposed binding model for TC is correct, the 4,6-methylene, 5-methyl, and amide ^1H NMR signals of II should show similar behavior to their analogs in TC in the presence of a given metal ion. Quantitative addition of $Nd(NO_3)_3$ to a 1:1 molar mixture of TC and triethylamine (added to function as a base analogous to the NMe_2 of TC) results in selective broadening of both amide ^1H NMR signals as observed for the amide signals of TC in the presence of the same concentrations of Nd(III). Some broadening of the methylene signal is evident, but this effect is not so pronounced as for C_4-H in TC, perhaps due to the fact that broadening is averaged over four methylene protons undergoing rapid site exchange. Small but significant downfield shifts are observed for the methyl and methylene ^1H NMR signals. A quantitative study of these shifts, using La(III) as a diamagnetic probe, was not attempted due to interference of the ^1H NMR signals by those arising from triethylamine. However, these results are sufficiently similar to those found for TC to lend strong support to a scheme involving Nd(III) binding at the ring-A tricarbonylmethane group of TC.

Studies of structure-activity relationships in the tetracycline antibiotics have shown that a number of factors are involved in determining drug activity.[69] Although the role of metal binding in the activity of tetracyclines is not yet well defined, it is perhaps of significance with regard to the metal binding site proposed in this paper that the amide carbonyl (but not the amide nitrogen) appears to be essential for drug activity.[69] Another question that remains to be answered is whether the binding site found using DMSO as a solvent is the same as would be used by TC under physiological conditions. Further studies of metal binding in derivatives of TC are in progress.

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(46) NOTE ADDED IN PROOF. After submission of this paper we became aware of a recent article (G. L. Asleson, L. J. Stoel, E. C. Newman, and C. W. Frank, *J. Pharm. Sci.*, **63**, 1144 (1974)) in which NMR assignments for tetracycline hydrochloride in DMSO were made. Their assignments are in good agreement with those presented here for tetracycline hydrochloride except for the signal around 14.7 ppm which Asleson et al. assigned to $C_{12}OH$. However, this disagreement has no bearing on the metal binding site arguments presented in this paper for TC free base.
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Fenton's Reagent. VI. Rearrangements During Glycol Oxidations[1]

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Abstract: The acid-catalyzed rearrangement RĊOHCHOHR → RCOĊHR proposed by Norman[2] on the basis of ESR measurements has been confirmed during the oxidation of ethylene glycol and 2,3-butanediol both by studies of reaction stoichiometry and product isolation. Results are consistent with a rapid collapse of the radical to a radical cation which competes with its oxidation by Fe^{3+}. Similar processes are confirmed with glycol derivatives, ethylene chlorohydrin and glycol monophosphate, and the scope and significance of the reaction are discussed.

In 1966, two groups[2,3] reported that, in acid solution, hydroxyl radical attack on ethylene glycol yields a product with an ESR spectrum consistent with structure **2**, rather than the expected **1**.

$$\underset{\mathbf{1}}{HO\dot{C}HCH_2OH} \qquad \underset{\mathbf{2}}{O{=}CH\dot{C}H_2}$$

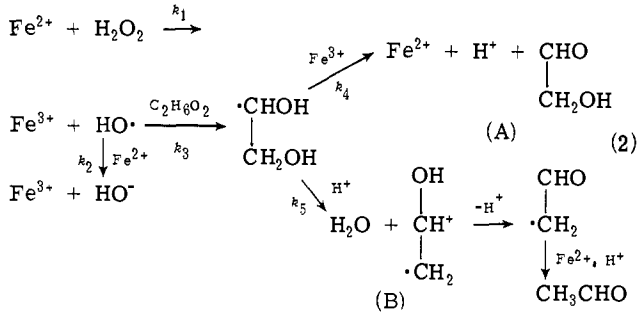
Norman's group examined the reaction further and proposed that the result is the consequence of an acid-catalyzed loss of -OH from **1**, to yield a radical cation which on proton loss yields **2** (the two steps perhaps being concerted).

$$HO\dot{C}HCH_2OH \xrightarrow{H^+} HOCH(^+)-\dot{C}H_2 \longrightarrow O{=}CH\dot{C}H_2 \quad (1)$$

They showed that the process was quite general for glycols, and that similar loss of Cl^- occurred from chlorohydrins. Subsequently, they demonstrated that the same sort of reaction occurs when similar radicals are produced by HO· addition to enol derivatives.[4] Since their conclusions were based solely on ESR spectra of transient intermediates, we have reexamined these processes by studies of the stoichiometry of Fenton's reagent reaction in such systems and by product isolation. In general, our results confirm and extend their model.

Stoichiometry of Fenton's Reagent Oxidation of Glycols

Combining Norman's proposed reactions with our own data on hydroxyl radical reactions with a variety of substrates,[5] we would anticipate reaction scheme 2 (using ethylene glycol as a model). If H_2O_2 is added to a Fe^{2+}–substrate system, to the extent that reaction follows path B, the stoichiometry (moles of H_2O_2 consumed/mole of Fe^{2+} oxidized) would be the same as if no glycol were present; i.e., the ratio $2\Delta H_2O_2/\Delta Fe^{2+}$ will be 1.[6] However, path A regenerates Fe^{2+}, and this path predicts that the ratio will increase linearly with the ratio [substrate]/[Fe^{2+}]. Actual ratios should depend upon the concentrations of acid and Fe^{3+} and the resulting relative importance of the two paths.



Results with ethylene glycol are shown in Figure 1 and nicely confirm the model. In 0.05 *M* $HClO_4$ and only the small amount of Fe^{3+} formed in the oxidation, chain lengths are only slightly larger than unity. Addition of 0.16 *M* Fe^{3+}, however, gives long chains at high [substrate]/[Fe^{2+}] ratios, but these again are drastically reduced in 0.5 *M* acid. Results with 2,3-butanediol, Figure 2, are similar but less pronounced. (The small drop in chain length at high substrate/Fe^{3+} ratios is probably an experimental artifact due to poor titration end points at high glycol concentrations.) Plainly the collapse to radical cation has an enormous effect on the oxidation chain length; the dashed lines in Figures 1 and 2 show the predicted stoichiometry if only