Binding of Mn(II) by Tetracycline. A Carbon-13 NMR Spin–Lattice Relaxation Study

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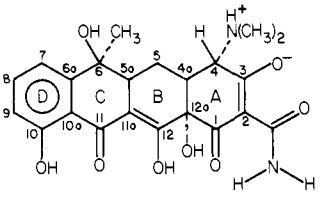
Abstract: Mn^{2+}-enhanced spin–lattice relaxation rates of ^{13}C nuclei on tetracycline in 80:20 (v/v) Me_2SO-d_6:D_2O have been measured at pH 7.0, 7.5, and 8.0, using a Mn^{2+}–tetracycline mole ratio of 3×10^{-4}. The data show that Mn^{2+} binds to two sites of the ring A tricarbonylmethane group and to a third site at $C_{12}O$ and $C_{12a}O$ under these conditions. The residence time of Mn^{2+} at a given site is short on the NMR time scale, and a number of Mn^{2+}–tetracycline complexes, differing in the mode of ligation, are in rapid equilibrium. With increasing pH, the mole fraction of bound Mn^{2+} decreases but no new binding sites are observed. Attempts to use the T_1 data to determine the position of bound Mn^{2+} accurately are complicated by the apparent occurrence of spin delocalization involving π orbitals of the tricarbonylmethane group of tetracycline.

The tetracycline antibotics have complex molecular structures which have intrigued chemists for nearly 3 decades.[1] At least two distinct molecular conformations are possible for most tetracyclines through rotation about the C_{12a}–C_{4a} bond. Several tautomeric forms can occur within the C_{11}–C_{12} β-diketone and C_1–C_{am}–C_3 tricarbonylmethane moieties. Tetracyclines are found both as neutral molecules and in the zwitterionic form, as shown in I for tetracycline. Species differing in orientation of the amide functions, via rotation about C_2–C_{amide}, are known, and epimerization at C_4 is relatively facile.



I

In the 1950's, Albert[2,3] showed that tetracyclines have strong affinities for many metal cations. This prompted numerous investigations directed toward establishing binding sites and metal–ligand stoichiometries under a variety of conditions. These efforts have been summarized in a previous publication.[4] The possibility that cation binding, particularly with Mg^{2+}, plays a role in the antibiotic mode of action of tetracyclines remains controversial.[5-8] However, in view of the magnitude of the stability constants observed for tetracyclines with cations found in living organisms, it is likely that these antibiotics exist largely in the cation–bound form in vivo. Aside from the possible biological importance of cation binding by tetracyclines, these antibiotics are of interest as unusual and complicated ligands which still present a challenge to coordination chemists since structural details of their complexes are not well established.

In a series of earlier publications from this laboratory, strong evidence was presented that binding of a variety of cations occurs at the ring A tricarbonylmethane group for tetracycline, hereafter abbreviated TC, in Me_2SO solution or in 70:30 (v/v) Me_2SO:D_2O at pH 7.[4,9-11] Similar conclusions were reached for binding of 5-hydroxytetracycline, 4-epitetracycline, and tetracyclinemethiodide to lanthanide series ion in Me_2SO.[11] These conclusions were based on ^1H and ^{13}C NMR studies in which paramagnetic cations were used as probes of the binding site. Significant paramagnetic effects (signal shifts or broadening) were observed only for nuclei on or near ring A.

In hopes of gaining a better understanding of the details of cation complexation by TC, we have measured the enhancement of ^{13}C spin–lattice relaxation rates for TC in the presence of Mn^{2+}. This method, which has been used extensively to study metalloenzymes,[12] is in principle capable of revealing the position of a bound paramagnetic cation to within a few tenths of an ångström or less.[13-15]

Experimental Section

Instrumentation. All NMR experiments were carried out on a Bruker WP-80 Fourier transform spectrometer equipped with a Bruker ASP-2000 computer and operating at 80.0 MHz for protons and 20.1 MHz for carbon-13. Quadrature phase detection was used in all cases. Sample temperatures were monitored with a Bruker B-VT-1000 temperature controller. Procedures used to calibrate the 90°/180° pulse width and to test data acquistion methods are described in detail elsewhere.[16]

Relaxation Time Measurements. Spin–lattice relaxation data for ^{13}C nuclei of tetracycline and 2-carbamoyldimedone were collected with the use of a time-saving modification of the inversion–recovery method proposed by Canet, Levy, and Peat, referred to as FIRFT.[17] The sequence is $(T\text{–}180°\text{–}\tau\text{–}90°)_n$ where $T < 5T_1$ and the first FID is not retained. Generally, 1000 FID's were accumulated and stored on a hard disk for each τ.

All ^{13}C spectra were run under conditions of proton broad-band decoupling using 16K data points over a 5000 Hz spectral width. Samples were contained in cylindrical inserts, approximately 8 mm in diameter by 10 mm in length, which were positioned inside 10 mm o.d. cylindrical sample tubes so as to confine the sample to the dimensions of the transmitter coil.

During data acquisition, short and long delays (τ) were placed in random order in most cases to avoid systematic errors. At least two fully relaxed spectra ($\tau > 5T_1$) were obtained during runs lasting more than 1 h in order to provide a check on changes in H_0 homogeneity. Further compensation for small changes in H_0 homogeneity with time was made by applying an exponential multiplication factor to the accumulated FID's, corresponding to line broadening of 5 Hz. Temperatures were maintained at 310 ± 1 K in all cases. Generally, 10–15 data points,

(1) Dürckheimer, W. *Angew. Chem., Int. Ed. Engl.* **1975**, *14*, 721–734.
(2) Albert, A. *Nature (London)* **1953**, *172*, 201.
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(4) Williamson, D. E.; Everett, G. W. *J. Am. Chem. Soc.* **1975**, *97*, 2397–2405.
(5) White, J. P.; Cantor, C. R. *J. Mol. Biol.* **1971**, *58*, 397–400.
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(15) Dobson, C. M.; Levine, B. A. In "New Techniques in Biophyics and Cell Biology", Pain, R. H.; Smith, B. J.; Eds.; John Wiley and Sons: New York, 1976; Vol. 3.
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(17) Canet, D.; Levy, G. C.; Peat, I. R. *J. Magn. Reson.* **1975**, *18*, 199–204.

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including points on both sides of the null position, were used in determining T_1.

Sample Preparation. In order to avoid problems due to adventitious paramagnetic ions, all solutions and solvents were prevented from coming into contact with metal objects such as syringe needles, vortex rods, spatulas, etc., and all glassware was soaked in an EDTA solution prior to use.

Alfa "ultrapure" $Mn(NO_3)_2 \cdot xH_2O$ was analyzed by atomic emission and found to consist of 58.7% $Mn(NO_3)_2$. This was used to prepare stock solutions of Mn^{2+} in D_2O. Carefully measured volumes of these stock solutions were added to weighed amounts of tetracycline hydrate dissolved in Me_2SO-d_6. Small amounts of NaOH in D_2O were added to adjust the apparent pH to 7.0, 7.5, or 8.0. The final solvent was 80:20 (v/v) Me_2SO-d_6:D_2O. The solutions were 0.3 M in TC and had a $[Mn^{2+}]/[TC]$ ratio of $\sim 3 \times 10^{-4}$. The $[Mn^{2+}]/[TC]$ ratio was chosen empirically so as to maximize ^{13}C T_1 relaxation enhancement without severe signal broadening.

Solutions of 2-carbamoyldimedone were prepared in a similar manner except that they also contained triethylamine at the same molar concentration as 2-carbamoyldimedone. The amine was intended to serve as a base analogous to the NMe_2 group of TC.

Error limits given for relaxation rates were obtained by using the Student's t-test distribution (95% confidence interval) as discussed previously.[16]

Theory

The effects of paramagnetic ions on NMR chemical shifts and relaxation rates are well documented.[13-15,18] Of interest here is the enhancement of spin–lattice relaxation rates, T_1^{-1}, since structural information can be extracted from these data under appropriate experimental conditions. If relaxation occurs via an electron–nuclear dipolar interaction, the enhancement in T_1^{-1} is large for nuclei near the paramagnetic ion but decreases as r^{-6}, where r is the cation–nuclear distance. The theory and applications have been discussed in considerable detail in several reviews,[13-15] and only a brief summary is given here.

For nuclei on substrates bound to Mn^{2+}, scalar contributions to spin–lattice relaxation are negligible, and the relaxation rate is[13-15]

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right] \quad (1)$$

In the equation ω_s and ω_I are Larmor frequencies for the electron and nucleus, respectively, τ_c is the dipolar correlation time, and r is defined above. The other symbols represent magnetic constants and have their usual meanings. Values for T_{1M}^{-1} may be determined by experiment, and if τ_c is known, structural information is available through calculated values of r. An alternative procedure, which avoids the difficulties of measuring τ_c accurately, is to use T_{1M}^{-1} data to determine *ratios* of r's:

$$T_{1M_i}^{-1}/T_{1M_j}^{-1} = r_j^6/r_i^6 \quad (2)$$

Experimentally, the Mn^{2+}:substrate mole ratio must be small to avoid NMR signal loss through broadening, and eq 3 relates the observed relaxation rate, T_{1OBS}^{-1}, to T_{1M}^{-1}:

$$T_{1P}^{-1} = T_{1OBS}^{-1} - T_{1F}^{-1} = \frac{P_M}{T_{1M}+\tau_m} + T_{1OS}^{-1} \quad (3)$$

Here, T_{1p}^{-1} is the *paramagnetic* contribution to the observed relaxation rate, T_{1F}^{-1} is the relaxation rate in a diamagnetic environment, P_M is the mole fraction of cation-bound substrate, τ_m is the lifetime of the cation–substrate complex, and T_{1OS}^{-1} is a contribution to relaxation enhancement by paramagnetic ions not bound to the substrate (outer-sphere effects). If T_{1OS}^{-1} is known, and if it can be demonstrated that $\tau_m << T_{1M}$ (fast exchange), then, combining eq 2 and 3,

$$\frac{T_{1Pi}^{-1}}{T_{1Pj}^{-1}} = \frac{T_{1OBSi}^{-1} - T_{1Fi}^{-1} - T_{1OSi}^{-1}}{T_{1OBSj}^{-1} - T_{1Fj}^{-1} - T_{1OSj}^{-1}} = \frac{r_j^6}{r_i^6} \quad (4)$$

(18) LaMar, G. N.; Horrocks, W. DeW.; Holm, R. H.; Eds., "NMR of Paramagnetic Molecules", Academic Press: New York, 1973.

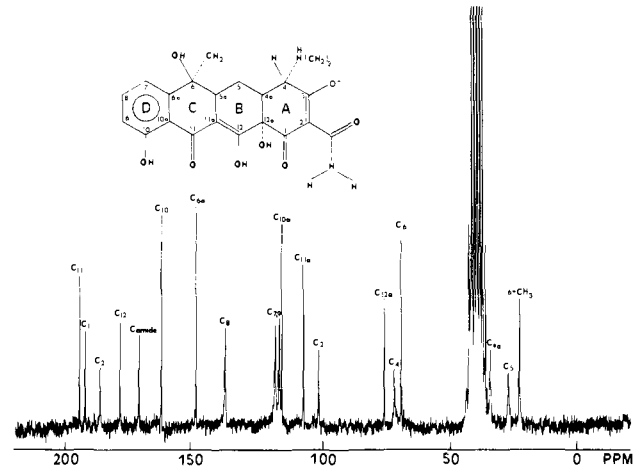

Figure 1. Carbon-13 NMR spectrum of tetracycline in 80:20 (v/v) Me_2SO-d_6:D_2O showing signal assignments.

where the symbol T_{1PI}^{-1} is used to denote the "inner sphere" paramagnetic relaxation enhancement.

Results and Discussion

Relaxation Rate Measurements. In order to use eq 4 to determine distance ratios from relaxation rate data, it must be demonstrated that fast exchange occurs, i.e., $T_{1M} >> \tau_m$. The occurrence of fast exchange may be tested conveniently by observing the temperature dependence of the paramagnetic enhancement of proton transverse relaxation rates. Thus, proton spectra of TC in 80:20 Me_2SO-d_6:D_2O were recorded at several intervals in the temperature range 290–340 °K and compared with analogous spectra taken in the presence of Mn^{2+}. Differences in line width in the presence and absence of Mn^{2+} were plotted against K^{-1}. Slopes of these plots were positive, indicating fast exchange in this temperature range.[13]

Carbon-13 spin–lattice relaxation rates for TC in the presence of $Mn(NO_3)_2$ were determined at pHs 7.0, 7.5, and 8.0 under the conditions described in the Experimental Section. Carbon-13 NMR signal assignments for TC and several of its derivatives in Me_2SO and in D_2O have been made by Asleson and Frank[19] and are used in this investigation (see Figure 1). Signals attributed to C_{5a} and the NMe_2 group are obscured by Me_2SO solvent signals. Relaxation rates for C_5, C_{4a}, and C_4 proved difficult to measure accurately due to their low amplitudes, and data for these nuclei are not used in this paper.

Relaxation rates for ^{13}C nuclei of TC in the absence of Mn^{2+} were also measured.[20] Differences in relaxation rates in the presence and absence of Mn^{2+} (T_{1OBS}^{-1} and T_{1F}^{-1}, respectively) represent the paramagnetic enhancement of relaxation and contain contributions both from TC-bound Mn^{2+} and from solvated Mn^{2+} ions (outer-sphere relaxation). Significant enhancement is observed only for resonances attributed to C_1, C_2, C_3, C_{amide}, C_{12}, and C_{12a}. This implies that there is no significant binding of Mn^{2+} to the oxygens bound to C_6, C_{10}, and C_{11}. The possibility that the NMe_2 group is involved in binding is not ruled out by these data. However, earlier studies involving tetracyclinonitrile[4] and tetracyclinemethiodide[11] showed that binding at this group is insignificant.

In order to use the data in a more quantitative fashion, corrections for outer-sphere relaxation, T_{1OS}^{-1}, must be made. In principle, T_{1OS}^{-1} can be determined by a graphical method which involves T_1 data measured at various Mn^{2+}:TC mole ratios.[21]

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(20) Ideally, T_{1F}^{-1} data should be obtained from TC in the presence of a diamagnetic ion having binding properties very similar to those of Mn^{2+}. Although Mg^{2+} would appear to be the ion of choice here, earlier ^1H and ^{13}C NMR work indicated that fast exchange conditions may not be upheld for the Mg–TC complex in Me_2SO/D_2O.[4,9]

(21) Levy, G. C.; Dechter, J. J.; Kowalewski, J. *J. Am. Chem. Soc.* **1978**, *100*, 2308–2314.

Table I. Paramagnetic Contributions to Spin-Lattice Relaxation Rates of Tetracycline Nuclei

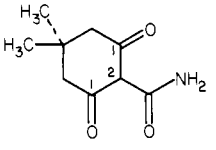
nucleus	δ^c	T_{1PI}^{-1},[a,b] s^{-1} pH 7.0	pH 7.5	pH 8.0
C_{11}	193.8	0.27 (7)	0.22 (9)	0.10 (11)
C_1	191.7	1.40 (10)	0.71 (14)	0.68 (16)
C_3	185.9	1.83 (11)	0.84 (12)	0.67 (21)
C_{12}	178.3	0.87 (7)	0.55 (12)	0.62 (18)
C_{amide}	171.0	1.72 (15)	1.09 (13)	1.01 (14)
C_{10}	162.2	0	0	0
C_{6a}	148.7	0.12 (7)	0.10 (9)	0.04 (13)
C_8	137.5	1.50 (181)	2.86 (349)	2.58 (276)
C_7	118.0	0.79 (157)	2.76 (248)	2.79 (216)
C_9	116.4	1.08 (90)	2.05 (174)	1.48 (134)
C_{10a}	115.6	0.04 (6)	0.09 (9)	0.10 (11)
C_{11a}	107.0	0.41 (8)	0.25 (12)	0.19 (14)
C_2	101.2	1.34 (8)	0.91 (13)	0.71 (17)
C_{12a}	75.7	1.01 (11)	0.58 (14)	0.43 (13)
C_6	69.2	0.37 (8)	0.31 (14)	0.29 (15)

[a] $T_{1PI}^{-1} = T_{1OBS}^{-1} - T_{1F}^{-1} - T_{1OS}^{-1}$, where T_{1OS}^{-1} is assumed to be $T_{1OBS}^{-1} - T_{1F}^{-1}$ for C_{10} at each pH (see text). [b] Errors in the least significant digits are given in parentheses (see Experimental Section). [c] Chemical shift in ppm from tetramethylsilane at pH 7.0 in 80:20 (v/v) Me_2SO-d_6:D_2O.

However, we found considerable scatter in such plots. We chose instead to use the paramagnetic enhancement found for C_{10} as an estimate of T_{1OS}^{-1} for all carbons. C_{10} has the smallest paramagnetic relaxation enhancement and thus is not close to any Mn^{2+} binding site. It is assumed that the relaxation enhancement for C_{10} is due entirely to outer-sphere interactions. Accordingly, the relaxation enhancement data for all other carbons were reduced by 0.27, 0.39, and 0.24 at pH 7.0, 7.5, and 8.0, respectively.

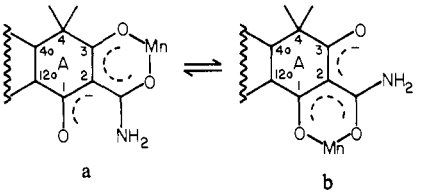
The data are shown in Table I. Error limits given are cumulative errors in T_{1OBS}^{-1}, T_{1F}^{-1} and T_{1OS}^{-1}, which originate from errors in signal intensities (see Experimental Section). For the aromatic carbons, C_7, C_8, and C_9, both T_{1OBS}^{-1} and T_{1F}^{-1} are large, and errors approach 100% when taking their differences. Thus the apparently large enhancements shown for these carbons are not significant. Errors are relatively small for those remaining carbons which show significant relaxation enhancement, viz., C_1, C_2, C_3, C_{amide}, C_{12}, and C_{12a}.

Binding at the Tricarbonylmethane Sites. It is clear that oxygens bound at both C_1 and C_3 are involved in binding to Mn^{2+}, and it is likely that the amide oxygen rather than the nitrogen is involved. Previous IR, NMR, and EPR studies of Co(II), Ni(II), and Cu(II) complexes of 2-carbamoyldimedone, II (hereafter abbreviated CDD), an analogue of ring A of TC, indicated that binding involves the amide oxygen.[22] This was substantiated later by an X-ray crystallographic determination of the Cu(II) complex of the *N*-phenyl derivative of II.[23]



II

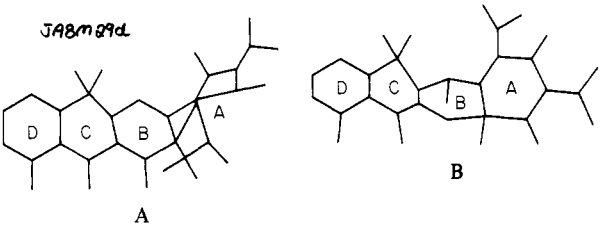
The data in Table I indicate that the ring A tricarbonylmethane moiety binds as a bidentate ligand in the two rotomeric configurations shown in III. Both of these configurations have been observed for TC derivatives in the solid state by crystallography[24-33]



III

and both are found in the same crystal in two instances.[29] The chelate rings are similar to those in β-diketonate complexes, and indeed the reported stability constants for Mn(II) complexes of TC[3] and 2,4-pentanedione[34] are nearly identical. At the very low Mn^{2+}/TC mole ratio used in these experiments, it is likely that at least two TC molecules are bound simultaneously to a given Mn^{2+} ion, and each molecule can use either of the above binding sites. Dreiding models show that no unusual steric constraints prevent binding through either configuration. At pH 7 the larger relaxation enhancement observed for C_3 compared to C_1 indicates that a larger fraction of TC molecules are bound as IIIa. The observed binding at the tricarbonylmethane group is consistent with results of pK_a measurements of TC in 50:50 Me_2SO:H_2O which show this group to be fully deprotonated in the pH range 7–8.[35]

Molecular Conformation of Tetracycline. Dreiding models readily demonstrate that TC can assume two major conformations through rotation about the C_{12a}–C_{4a} bond. These are illustrated schematically in IV. Mitscher and co-workers[36-37] presented CD



IV

evidence that most therapeutically active tetracyclines have the same conformation in aqueous solution and proposed that this conformation is the same as that found by crystallography[24] for the HCl salt of 7-chlorotetracycline, namely A in IV. Extensive crystallographic studies, carried out recently by Stezowski and co-workers and by Palenik and co-workers, have shown that conformation A occurs in the solid state for most tetracycline derivatives.[26-33] Conformation B is found for crystalline, *anhydrous* 5-hydroxytetracycline (OTC), and the molecule is not zwitterionic.[26,28]. Crystals of *hydrated* TC and OTC on the other hand, contain zwitterionic molecules in conformation A.[26]

Stezowski[26,28] has proposed that in nonaqueous media, neutral tetracyclines exist in the nonzwitterionic form and adopt conformation B, but they convert to the zwitterionic state and conformation A in the presence of water. A recent CD study of OTC, using ethanol–water solvent mixtures, presents strong evidence of a conformation change as the proportion of water in the solvent increases.[38] Very similar effects on the CD spectrum of TC in

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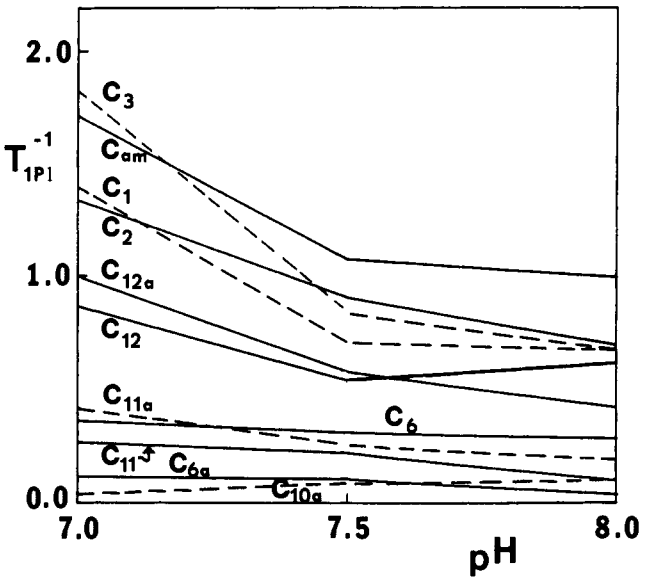

Figure 2. pH dependence of Mn(II)-enhanced spin–lattice relaxation rates for tetracycline carbons.

100% Me_2SO were found by Gulbis upon addition of water.[39,40] In the present investigation, where an 80:20 (v/v) Me_2SO–water solvent mixture is used, TC most likely occurs as a zwitterion in conformation A both in the presence and absence of Mn^{2+}.

Binding at $C_{12}O$ and $C_{12a}O$. Relaxation enhancement for C_{12} and C_{12a} is larger than expected from Mn^{2+} bound as in III, and it appears that oxygens at these carbons are also involved in Mn^{2+} binding. Since the enhancement is the same within the limits of error for both these carbons, it is likely that Mn^{2+} is chelated by the oxygen donors. These oxygens are sufficiently close for chelation only if TC adopts conformation A.

Thus we propose that chelation occurs at $C_{12}O---C_{12a}O$ and that binding at this site is independent of binding at the tricarbonylmethane sites. The smaller relaxation enhancements observed for C_{12} and C_{12a} compared to those of C_1, C_2, C_3, and C_{amide} indicate that a smaller mole fraction of TC molecules is bound at the $C_{12}O---C_{12a}$ site (assuming similar Mn–C distances in each case).

From a qualitative point of view then, one can envision a Mn^{2+} ion surrounded both by solvent and by TC molecules which are zwitterionic and in conformation A and which present one of three possible bidentate moleties for ligation. The lifetime of a given Mn^{2+}–TC complex is short on the NMR time scale, and a number of species differing in the number of bound TC's and in the mode of ligation are in rapid equilibrium.

The modes of ligation of Mn^{2+} proposed here may be compared with those found by crystallography for Hg^{2+} and K^+ complexes of OTC.[27] In the Hg^{2+} complex, zwitterionic OTC molecules in conformation A and configuration IIIb bind the Hg^{2+} ion in a bidentate fashion through C_1O and $C_{amide}O$. The K^+ complex, which was prepared under strongly basic conditions, has a more complicated structure in which two symmetry-related OTC^{2-} ions bind four K^+ ions through C_1O, $C_{amide}O$, $C_{12}O$, $C_{12a}O$, and $C_{11}O$. Two of the K^+ ions also bind to C_3O's of additional TC molecules. The divalent OTC^{2-} ions are found in a slight modified version of conformation A.

Effects of Change in pH. Figure 2 shows changes in T_{1P1}^{-1} data over the pH range investigated. The general trend[41] toward smaller T_{1P1}^{-1} values with increasing pH may result from competition of OH^- as a ligand[42] for Mn^{2+}, which would reduce the mole fraction of bound TC. Apparent exceptions to this trend occur for C_{12} and C_{10a} where the data indicate an increase in Mn^{2+} binding near these atoms at higher pH. This is readily explained for C_{12} by increased deprotonation[35] of $C_{12}OH$; however, in both cases the observed changes in T_{1P1}^{-1} are within the limits of error. Similarly, other changes in *relative* T_{1P1}^{-1}'s among different carbons with increasing pH are probably not significant except for C_1 and C_3. Here the data indicate essentially identical mole fractions of Mn^{2+} bound as in IIIa and IIIb at pH 7.5 and 8.0, but not at 7.0.

Attempts to Determine the Position of Bound Mn^{2+}. In an earlier application[16] of paramagnetic relaxation enhancement methods, using a simple substrate and Gd(III), we were able to determine the position of bound Gd(III) relative to the substrate to within a few tenths of an ångstrom by using relationship 4. In the case of TC, the situation is complicated by the occurrence of three binding sites, and the time-averaged relaxation enhancement of a given carbon (Table I) has potential contributions from Mn^{2+} at each site. The contribution from each site depends upon the mole fraction of TC molecules using that site and upon the carbon–Mn^{2+} distance, r.

Dreiding models indicate that Mn^{2+} bound at the $C_{12}O$–$C_{12a}O$ site may be sufficiently distant from the tricarbonylmethane group that, as a result of the r^{-6} dependence, it would make a negligible contribution to the relaxation enhancement of C_2, C_3, and C_{am}. To test this, Mn^{2+}-induced relaxation enhancements for ^{13}C nuclei of CDD, II, were measured (see Experimental Section). In CDD the C_2 and C_{amide} signals appear at 102.3 and 172.9 ppm, respectively, and are analogous to those of TC (see Table I). The ratio $T_{1P}^{-1}(C_2)/T_{1P}^{-1}(C_{amide})$ for CDD is 0.77 (6) which is very close to that found for TC, 0.81 (8), or 0.78 (11) if outer-sphere corrections are made. It must be concluded that the above hypothesis is valid and that CDD and the tricarbonylmethane group of TC bind Mn^{2+} in a very similar manner. Binding at the tricarbonylmethane group involves a three-site exchange, where the three sites are IIIa, IIIb, and free TC. If there is no *direct* IIIa $\rightleftharpoons$ IIIb exchange, the following relationship holds:[43,44]

$$T_{1Pi}^{-1} = f_a T_{1Mi(a)}^{-1} + f_b T_{1Mi(b)}^{-1} \quad (5)$$

Here T_{1Pi}^{-1} is the measured relaxation enhancement for carbon i, f_a and f_b are mole fractions of TC bound as in IIIa and IIIb, respectively, and T_{1M}^{-1} s are defined for each binding site by eq 1.

A reasonable assumption is that Mn^{2+} binds in a similar fashion at sites a and b so that Mn–C_i distances are the same in each case for C_2 and C_{am}. Thus $T_{1M_{C_2(a)}}^{-1} = T_{1M_{C_2(b)}}^{-1}$ (and similarly for C_{am}) and by combining eq 5 and 2,

$$\frac{T_{1P1_{C_2}}^{-1}}{T_{1P1_{C_{amide}}}^{-1}} = \frac{T_{1M_{C_2}}^{-1}}{T_{1M_{C_{amide}}}^{-1}} = \frac{r_{C_{amide}}^{6}}{r_{C_2}^{6}} \quad (6)$$

The above relationship can, in principle, be used as in a previous example[16] to determine the position of the bound Mn^{2+} ion. In order to facilitate the calculation, crystallographically determined atomic coordinates[31] for TC in conformation A and configuration IIIb were converted to a Cartesian system calibrated in ångstroms with the origin at C_2. Mn^{2+} was assumed to be bound equally to C_1O and $C_{am}O$ with a Mn–O distance[45] of 2.15 Å. A computer

(38) Hughes, L. J.; Stezowski, J. J.; Hughes, R. E. *J. Am. Chem. Soc.* **1979**, *101*, 7655–7657.
(39) Gulbis, J. Ph.D. Thesis, The University of Kansas, 1977.
(40) Similar CD changes were also observed upon addition of Nd^{3+}, Mg^{2+}, or H^+ ions to TC in 100% Me_2SO. It was demonstrated that these changes are not due to water introduced via the metal salts.[39]
(41) Data collected on an independent sample at pH 7 are in good agreement with those reported in Table I.
(42) The K_{sp} for $Mn(OH)_2$ is reported to be 4×10^{-14} $mol^3 \cdot l^{-3}$ in water ("Handbook of Chemistry and Physics"; CRC Press: Cleveland, 1972; 53rd ed.). If the K_{sp} were smaller by $\sim 10^2$–10^3 in 80:20 (v/v) $Me_2SO:D_2O$, the solubility limit would be approached at pH 8 for the Mn^{2+} concentrations used in these experiments.
(43) Led, J. J.; Grant, D. M. *J. Am. Chem. Soc.* **1977**, *99*, 5845–5858.
(44) This equation also assumes the lifetimes of both Mn–TC complexes IIIa and IIIb are short relative to ^{13}C T_{1M}'s.
(45) Structural data for Mn(II) β-diketonate complexes appear to be unavailable. Mn(III)–O bonds average 1.99 Å in $Mn(acac)_3$.[46] Co–O bonds average 2.06 Å and 1.90 Å respectively in $Co(acac)_2$ and $Co(acac)_3$.[47] Assuming a similar difference for manganese, the estimated Mn(II)–O distance in β-diketonate complexes is ~2.15 Å.
(46) Stults, B. R.; Marianelli, R. S.; Day, V. W. *Inorg. Chem.* **1979**, *18*, 1853–1858.

program was written to vary the position of Mn^{2+} in small increments within these constraints and to calculate relative distances from Mn^{2+} to C_2 and C_{am} at each position, using the crystallographic data. These distance ratios were compared with the $r(C_{am})/r(C_2)$ ratio of 0.959 determined from the NMR data at pH 7 (Table I).

The best agreement occurs when the Mn^{2+} ion is 1.5 Å out of the (approximate) plane of and over the center of the C_1O–C_1–C_2–C_{am}–$C_{am}O$ chelate ring. The location of Mn^{2+} determined in this manner varies only slightly by using (1) data from Table I at pH 7.5 and 8.0, (2) data excluding the T_{1OS}^{-1} correction, and (3) data for CDD. This position for Mn^{2+} is unrealistic from the standpoint of classical bidentate oxygen coordination and is reminiscent of certain π-bonded dienyl complexes.[48] It is likely, however, that the Mn^{2+} position determined in this manner is inaccurate as a result of spin delocalization from Mn^{2+} to ligand MO's.[55] This would cause certain ligand atoms to behave as local centers of dipolar relaxation, resulting in erroneously large T_1^{-1} enhancements and unrealistically short Mn–nuclear distances for these atoms.[49-51] The Mn^{2+} position found for TC indicates relaxation enhancement for C_2 is probably too large.

Spin delocalization via the ligand π system can occur as the result of either metal-to-ligand or ligand-to-metal charge transfer.[52] Spin density will then reside in the ligand LUMO or HFMO, respectively, and will concentrate at atoms having large coefficients for these MO's. The tricarbonylmethane moiety of TC is related electronically to the trivinylmethyl radical for which Hückel MO coefficients are available.[53] It is perhaps significant that a large coefficient is found in the HFMO of trivinylmethyl for the atom corresponding to C_2 of TC. This MO has zero coefficients for the atoms corresponding to C_{amide}, C_1, and C_3. Thus a L → M charge transfer could account for the unexpected Mn^{2+} position, using T_1 data for TC (and CDD). Because of the r^{-6} dependence, a very small amount of spin density on a carbon atom would have a measurable effect on its relaxation rate.

Spin delocalization through the π system of the tricarbonylmethane group should have little effect on T_{1PI}^{-1} values for C_{12} and C_{12a}. Also, σ spin delocalization resulting from Mn^{2+} binding at the $C_{12}O$–$C_{12a}O$ site is not a priori expected to be significant. However, the Mn–C_{12}/Mn–C_{12}a distance ratio, calculated from T_1^{-1} data, cannot be used to locate Mn^{2+} *uniquely*, assuming symmetrical bidentate chelation via O's. In principle, Mn^{2+} could be located at this site if τ_c were known accurately. Although estimates of τ_c for Mn^{2+}-free TC can be made from T_1 measurements if τ_c is dominated by molecular rotation-diffusion,[54] the effective τ_c of the Mn–TC *complex* is expected to be considerably different. Thus no attempts are made to locate Mn^{2+} within the $C_{12}O$–$C_{12a}O$ site.

Summary

Spin–lattice relaxation enhancement data in Table I clearly indicate that Mn^{2+} binds only at the tricarbonylmethane and $C_{12}O$–$C_{12a}O$ sites in 80:20 Me_2SO-d_6:D_2O solution in the pH range 7–8. These results are consistent with those of previous experiments[4,9-11] in which selective enhancement of ^1H and ^{13}C NMR line widths of TC was observed in the presence of a variety of paramagnetic cations. Crystallography has shown that OTC also uses these binding sites for K^+ and Hg^{2+} cations.[27]

The Mn^{2+} ion has a short residence time (on the NMR time scale) at a given binding site, and it is likely that Mn^{2+} is bound to more than one TC molecule at any instant. A number of distinct, instantaneous complexes, in which each bound TC may use any of three binding sites, probably exist in rapid equilibrium. It is believed that both free and Mn^{2+}-bound TC adopt conformation A under the conditions of these experiments.

Quantitative treatment of the T_1 data for TC and CDD indicates a Mn^{2+} position above the β-diketone-like rings of the tricarbonylmethane group. This position is believed to be inaccurate as a result of effects on the data of spin delocalization from Mn^{2+} to the π orbitals of TC.

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