Rance, M., Smith, I. C. P., & Jarrell, H. C. (1983) *Chem. Phys. Lipids 32*, 57–71.
Saito, Y., Silvius, J. R., & McElhaney, R. N. (1977) *Arch. Biochem. Biophys. 182*, 443–455.
Seelig, A., & Seelig, J. (1975) *Biochim. Biophys. Acta 406*, 1–5.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–415.
Seelig, J. (1980) *Q. Rev. Biophys. 13*, 19–61.
Seelig, J., & Waespe-Šarčevič, N. (1978) *Biochemistry 17*, 3310–3315.
Silvius, J. R., & McElhaney, R. N. (1979) *Chem. Phys. Lipids 25*, 125–134.
Silvius, J. R., Saito, Y., & McElhaney, R. N. (1977) *Arch. Biochem. Biophys. 182*, 455–464.
Silvius, J. R., Mak, N., & McElhaney, R. N. (1980) *Biochim. Biophys. Acta 597*, 199–215.
Smith, I. C. P. (1983) in *Biomembranes* (Manson, L. A., & Kates, M., Eds.) Plenum Press, New York (in press).
Smith, I. C. P., Butler, K. W., Tulloch, A. P., Davis, J. H., & Bloom, M. (1979) *FEBS Lett. 100*, 57–61.
Souzer, H. (1982) *Biochim. Biophys. Acta 691*, 161–170.
Stockton, G. W., & Smith, I. C. P. (1976) *Chem. Phys. Lipids 17*, 251–263.
Stockton, G. W., Johnson, K. G., Butler, K. W., Polnaszek, C. F., Cyr, R., & Smith, I. C. P. (1975) *Biochim. Biophys. Acta 401*, 535–539.
Taylor, M. G., Akiyama, T., & Smith, I. C. P. (1981) *Chem. Phys. Lipids 29*, 327–339.
Taylor, M. G., Akiyama, T., Saitô, H., & Smith, I. C. P. (1982) *Chem. Phys. Lipids 31*, 359–379.
Tulloch, A. P. (1979) *Chem. Phys. Lipids 24*, 391–406.
Winstein, S., Friedrich, E. C., Baker, R., & Lin, Y. (1966) *Tetrahedron, Suppl. 8*, 621–645.

Gadolinium(III) and Manganese(II) Binding by a Polyether Ionophore. Influence of Cation Charge and Solvent Polarity on the Binding Sites of Lasalocid A (X-537A)†

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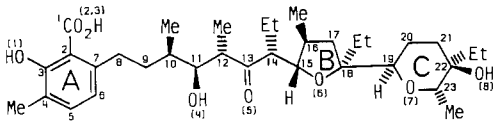
ABSTRACT: Gadolinium(III) and manganese(II) binding sites for the carboxylic polyether antibiotic lasalocid A (X-537A) were determined in a polar solvent, *N,N*-dimethylformamide, and in a relatively nonpolar solvent, chloroform-d_1, by carbon-13 NMR spin–lattice relaxation methods. The results show that binding sites used by the ionophore depend upon both the cation charge and the solvent polarity. In *N,N*-dimethylformamide, Gd(III) binds only at the anionic carboxylate moiety, whereas Mn(II) binds not only at this group but also at O_4 and O_7. In chloroform solution, both cations bind lasalocid via the carboxylate group, O_4, O_7, and O_8. There is some evidence for two modes of binding involving the carboxylate group in chloroform, and these appear to be in rapid exchange at ambient temperature. Methods are given for preparing $Gd(LAS)_3 \cdot XCHCl_3$, $La(LAS)_3 \cdot YCHCl_3$, and $Mn(LAS)_2 \cdot {}^1/_2CHCl_3$, where $X = {}^3/_2$ or ${}^5/_2$, $Y = 1$ or 2, and LAS is the anion of lasalocid A.

Lasalocid A (I),[1] an antibiotic of the polyether series, is



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well-known for its ability to transport metal cations and biogenic amines across natural and artificial membranes (Westley, 1975, 1982; Ovchinnikov & Kolosov, 1979; Poonia & Bajaj, 1979; Pressman, 1976). X-ray crystallographic studies have been carried out for Ba^{2+}, Ag^+, and Na^+ complexes of the lasalocid A anion (hereafter abbreviated LAS) (Johnson et al., 1970; Maier & Paul, 1971; Schmidt et al., 1974; Chiang & Paul, 1977; Smith et al., 1978). In all but one (Chiang & Paul, 1977) of these structures two molecules of LAS, each in a cyclic conformation stabilized by intramolecular hydrogen bonds, are found with the cation(s) sandwiched between them. Most of the oxygens are directed inward, resulting in a hydrophobic outer surface for the complex. If this structure is maintained in the solution phase, it could account for the high solubilities of the complexes in nonpolar solvents and in the interior of lipid bilayer membranes. In all of the crystal structures, the five oxygens O_4, O_5, O_6, O_7, and O_8 are involved in cation binding. In only two cases (Johnson et al., 1970; Schmidt et al., 1974) is a carboxylate oxygen bound to the cation, and in no case is O_1 involved in cation binding.

Relatively little is definitely known of the structures of LAS complexes in the biologically more relevant solution phase. Indeed, evidence is accumulating that solution-phase structures differ considerably from those found in the solid state and that they depend upon both cation charge and solvent polarity. For example, the ^{13}C NMR spectrum of the Tl^+ complex in $CDCl_3$ at low temperature shows $^{203,205}Tl$–^{13}C spin coupling indicative of Tl^+ binding at a carboxylate O, O_5, O_6, and O_8 (Lallemand & Michon, 1978). The effects of Cu^{2+} on LAS ^{13}C NMR

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[1] The numbering scheme used here is that proposed by Westley for polyether antibiotics (Westley, 1976). Oxygen numbers are shown in parentheses.

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line widths and T_1 values in $CDCl_3$ solution show Cu^{2+} binds primarily to a carboxylate O, O_1, O_4, and O_8 (Lallemand et al., 1980). In methanol solution, Pr^{3+} binding results in shifts only of ^1H and ^{13}C NMR signals assigned to the salicylate moiety of LAS (Chen & Springer, 1978), and an open-chain structure with binding only at the salicylate "head" was proposed. In a more recent study (Richardson & Gupta, 1981) of the interaction of several Ln^{3+} ions with LAS in methanol, carried out by using various electronic absorption and emission techniques, it was concluded that the strongest binding interaction involves the carboxylate group. However, a cyclic LAS structure in which other oxygens are also bound was proposed to account for the observed optical activity of the complexes.

Earlier studies showed that the stoichiometries of LAS complexes are solvent dependent (Degani & Friedman, 1974; Patel & Shen, 1976; Shen & Patel, 1976). Most evidence supports a cyclic conformation for cation-bound LAS in nonpolar solvents, but the conformation in polar media is less certain (Degani & Friedman, 1974; Patel & Shen, 1976; Shen & Patel, 1976; Alpha & Brady, 1973; Anteunis, 1976). Observed trends in LAS-cation exchange rates in methanol indicate that the conformation may depend to some extent upon cation size (Krishnan et al., 1978). Fluorescence studies of membrane surface-bound LAS complexes suggest an open-chain LAS conformation with cation binding only via the carboxyl group (Haynes & Pressman, 1974).

Thus, it is rather apparent that the specific oxygens used by LAS for cation binding vary under different conditions in solution and that the solid-state structures are not necessarily good models of LAS complexes in solution or in lipid bilayer membranes. In hopes that a more sensitive binding site probe would provide definitive structural information in solution and reveal the effects of solvent polarity and cation charge on the structure, we have carried out ^{13}C NMR spin–lattice relaxation rate, T_1^{-1}, measurements on LAS in a polar solvent (*N*,*N*-dimethylformamide, hereafter DMF) and in a relatively nonpolar solvent (chloroform-d_1, hereafter $CDCl_3$) in the presence of the paramagnetic probe ions Gd^{3+} and Mn^{2+}. These ions are used here because of their ability to enhance dipolar relaxation of nearby nuclei without significant scalar contributions and because they are regarded as good probes of Ca^{2+} and Mg^{2+} binding, respectively (James, 1975; Dobson & Levine, 1976; Mildvan & Gupta, 1978).

The data may be used to identify LAS oxygen donors to Gd^{3+} or Mn^{2+} in each solvent, since under conditions of rapid cation exchange the paramagnetic enhancement of the relaxation rate, $T_{1P_i}^{-1}$, of a given carbon nucleus is inversely proportional to r_i^6, the C_i-cation distance. Details of the theory behind this approach are given in several reviews (James, 1975; Dobson & Levine, 1976; Mildvan & Gupta, 1978) and are summarized in previous publications from this laboratory for the experimental conditions used here (Lee et al., 1981; Lee & Everett, 1981). The pertinent relationships are shown in eq 1 and 2 where T_{1M}^{-1} is the spin–lattice relaxation rate of

$$T_{1M}^{-1} = \frac{K}{r^6}\left[\frac{3\tau_C}{1+\omega_I^2\tau_C^2} + \frac{7\tau_C}{1+\omega_S^2\tau_C^2}\right] \quad (1)$$

$$T_{1P}^{-1} = \frac{P_M}{T_{1M}+\tau_m} \quad (2)$$

a nucleus in a molecule bound to a paramagnetic ion, T_{1P}^{-1} is the difference in relaxation rates observed in the presence and absence of the paramagnetic probe ion, K represents a series of known magnetic constants, τ_c is the dipolar correlation time, ω_I and ω_S are nuclear and electron Larmor frequencies, respectively, P_M is the mole fraction of cation-bound substrate, and τ_m is the lifetime of the complex. In (1), scalar contributions to T_{1M}^{-1} are neglected, as is the usual case for Mn^{2+} and Gd^{3+}. Also, in view of the steric bulk of the bound LAS ligands which should prevent close approach of ligands not bound to the Gd^{3+} ion, outer-sphere contributions to T_{1P}^{-1} are assumed to be negligibly small. This was found to be the case in a previous study with a smaller ligand where outer-sphere effects were measured (Lee et al., 1981). Fast exchange, as defined here, requires $T_{1M} \gg \tau_m$ so that T_{1P}^{-1} values can be related directly to T_{1M}^{-1} and r.

Experimental Procedures

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 ^{13}C. Quadrature phase detection was used in all cases. Sample temperatures were monitored with a Bruker B-VT-1000 temperature controller. The 90°/180° pulse calibration was checked regularly by using the procedure described previously (Lee et al., 1981).

Relaxation Time Measurements. Experimental techniques used for collecting and reducing T_1 data are very nearly the same as those described in detail in previous papers (Lee et al., 1981; Lee & Everett, 1981). Data were collected by using a time-saving modification of the inversion–recovery method, proposed by Canet et al. and referred to as FIRFT (Canet et al., 1975). 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 with 16K data points over a 5000 Hz spectral width. Samples were contained in cylindrical or spherical inserts, approximately 8 mm in diameter, which were positioned inside 10-mm o.d. cylindrical sample tubes so as to confine the sample to the dimensions of the transmitter coil. To avoid systematic errors during data acquisition, a microprogram was used which collects a few FID's for each delay (τ) and then cycles repeatedly through the list of delays. An exponential multiplication factor, corresponding to line broadening of 5 Hz, was applied to the accumulated FID's. Temperatures were maintained constant to within ±1 °C.

Computer programs designed to compare normalized signal intensities and to determine T_1's by a three-parameter, nonlinear least-squares routine were run on a Honeywell 66/60 computer located on campus. Generally, 16–25 data points, including points on both sides of the null position, were used in the least-squares calculations.

Sample Preparation. 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 ethylenediaminetetraacetic acid (EDTA) solution prior to use.

In experiments involving Gd^{3+}, the method of sample preparation varied among the solvents. In DMF, $Gd(NO_3)_3 \cdot 5H_2O$ (Alfa, 99.9%) was added to a 0.5 M solution of NaLAS until the Gd^{3+}/LAS mole ratio was sufficiently large (3.9×10^{-5}) to cause measurable relaxation enhancement without severe NMR signal broadening. With $CDCl_3$, a solution of $Gd(NO_3)_3$ in DMF was added to a 0.5 M solution of NaLAS in $CDCl_3$ to achieve the desired Gd^{3+}/LAS ratio (2.6×10^{-4}). The resulting solution contained 2% (v/v) DMF. Due to the low solubility of $Gd(NO_3)_3$ and NaLAS in cyclohexane–chloroform mixed solvent systems, $Gd(LAS)_3$ and

Table I: Paramagnetic Contributions to Carbon-13 Spin-Lattice Relaxation Rates for Lasalocid A

	N,N-dimethylformamide			chloroform-d_1		
position	δ [a]	$T_{1P}^{-1}(Gd^{3+})$ [b,c]	$T_{1P}^{-1}(Mn^{2+})$ [b,d]	δ [a]	$T_{1P}^{-1}(Gd^{3+})$ [b,e]	$T_{1P}^{-1}(Mn^{2+})$ [b,f]
C_1	174.7	3.54 (55)	2.14 (33)	176.3	3.83 (50)	6.23 (120)
C_2	116.8	0.655 (096)	0.328 (044)	118.0	2.76 (28)	4.17 (34)
C_3	160.2	g	g	160.8	1.70 (13)	2.69 (28)
C_4	122.4	0.082 (040)	0.135 (041)	122.7	0.690 (060)	1.66 (10)
C_5	131.2	0.639 (490)	3.11 (60)	131.1	1.60 (47)	6.97 (74)
C_6	118.7	0.402 (526)	1.94 (49)	119.4	1.94 (32)	7.80 (70)
C_7	143.6	0.296 (080)	0.273 (054)	143.0	1.56 (10)	3.15 (55)
C_{11}	70.7	0.742 (563)	2.46 (56)	70.2	2.22 (38)	8.85 (84)
C_{13}	218.4	0.046 (077)	0.201 (087)	218.6	0.756 (090)	2.96 (38)
C_{15}	82.4	0.483 (763)	2.76 (1.03)	82.6	1.65 (34)	8.89 (83)
C_{18}	86.4	0.088 (069)	0.294 (071)	87.0	0.390 (037)	2.61 (15)
C_{19}	68.8	0.338 (529)	1.86 (39)	68.0	1.93 (44)	8.27 (66)
C_{22}	70.0	0.092 (068)	0.464 (083)	70.6	1.46 (09)	4.86 (42)
C_{23}	75.0	0.378 (690)	2.69 (69)	76.6	2.94 (58)	6.66 (172)

[a] Chemical shift in ppm from Me_4Si. [b] Observed relaxation rate less T_{1F}^{-1} in s^{-1}; numbers in parentheses are standard deviations in the least significant digits. [c] Gd(III)/LAS mole ratio is 3.9×10^{-5}. [d] Mn(II)/LAS mole ratio is 3.53×10^{-4}. [e] Gd(III)/LAS mole ratio is 2.6×10^{-4}; contains 2% (v/v) DMF. [f] Mn(II)/LAS mole ratio is 1.84×10^{-2}. [g] NMR signal partially obscured by solvent signal.

$La(LAS)_3$ were prepared and mixed to give a useful Gd^{3+}/LAS ratio.

In experiments involving Mn^{2+}, the Mn^{2+} ion was introduced in the form of $Mn(LAS)_2$. This was added to solutions of NaLAS in DMF and in $CDCl_3$ until the Mn^{2+}/LAS ratios were 3.5×10^{-4} and 1.8×10^{-2}, respectively.

Preparation of LAS Complexes. The following general procedure was used in preparation of $Mn(LAS)_2$, $Gd(LAS)_3$, and $La(LAS)_3$: Aqueous solutions containing approximately 5 mmol of $La(NO_3)_3 \cdot 6H_2O$ (Alfa "Ultrapure"), $Gd(NO_3)_3 \cdot 5H_2O$ (Alfa 99.9%), or $MnCO_3$ (Fischer Reagent) in 30 mL are prepared. To a solution of 850 mg (1.4 mmol) of NaLAS (Aldrich Chemical Co.) in 25 mL of $CHCl_3$ is added a 10-mL portion of one of the above aqueous solutions. The mixture is stirred vigorously for 2–3 h, then the aqueous layer is replaced with a fresh 10-mL portion of the cation-containing solution, and the mixture is again stirred for 2–3 h. This procedure is repeated until the aqueous solution is consumed. The $CHCl_3$ layer is washed with 10 mL of H_2O, which is subsequently removed. Then the $CHCl_3$ solution is filtered, and the solvent is removed in vacuo by using a rotary evaporator cooled by liquid N_2. Elemental analyses of the resulting solids indicated the presence of $^1/_2$ to $2^1/_2$ molecules of $CHCl_3$ per formula unit, depending upon how long and at what temperature the crystalline products are evacuated. Anal. Calcd for $Gd(LAS)_3 \cdot {}^3/_2CHCl_3$: C, 59.04; H, 7.68. Found: C, 59.04; H, 7.63. Anal. Calcd for $Gd(LAS)_3 \cdot {}^5/_2CHCl_3$: C, 56.41; H, 7.32. Found: C, 56.48; H, 7.55. Anal. Calcd for $La(LAS)_3 \cdot CHCl_3$: C, 61.01; H, 7.95. Found: C, 60.86; H, 8.28. Anal. Calcd for $La(LAS)_3 \cdot 2CHCl_3$: C, 58.18; H, 7.56. Found: C, 58.70; H, 7.46. Anal. Calcd for $Mn(LAS)_2 \cdot {}^1/_2CHCl_3$: C, 63.57; H, 8.29. Found: C, 63.83; H, 8.20. The presence of $CHCl_3$ was verified by observation of a ^1H NMR signal at 7.1 ppm for these complexes in cyclohexane-d_{12} solutions.

Results and Discussion

Carbon-13 NMR signal assignments for H–LAS and several cation complexes of LAS in $CDCl_3$ and CD_2Cl_2 have been reported (Lallemand & Michon, 1978; Lallemand et al., 1980; Chen & Springer, 1978; Seto et al., 1978) and are consistent with results of off-resonance proton decoupling experiments carried out in this laboratory. A typical spectrum of NaLAS in $CDCl_3$ with signal assignments is shown in Figure 1. As a result of signal overlap at the magnetic field of our spectrometer (1.88 T), T_1 measurements were not possible for all

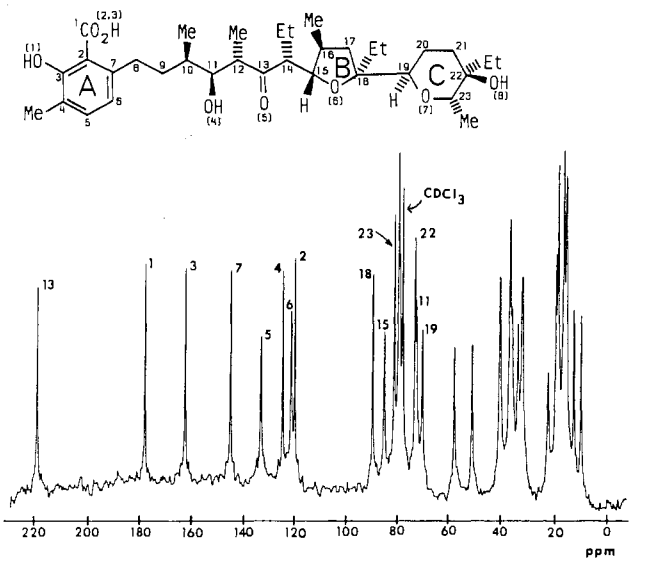


FIGURE 1: 20-MHz carbon-13 NMR spectrum of NaLAS in chloroform-d_1 solution.

carbon nuclei of LAS. However, data are available in most cases for carbons adjacent to potential cation binding sites. Table I gives paramagnetic contributions, T_{1P}^{-1}, to the ^{13}C relaxation rates. These were determined by using

$$T_{1P}^{-1} = T_{1_{obsd}}^{-1} - T_{1F}^{-1} \quad (3)$$

where $T_{1_{obsd}}^{-1}$ is the relaxation rate observed in the presence of a paramagnetic cation and T_{1F}^{-1} is the corresponding rate for a diamagnetic complex. T_{1F}^{-1} data were obtained by using NaLAS alone (for the Mn^{2+} study) or, for the Gd^{3+} study, NaLAS in the presence of La^{3+} at the same Ln^{3+}/LAS ratio used during experiments with Gd^{3+}.

Gd(III) Binding. (1) In N,N-Dimethylformamide. The most polar solvent used was DMF, which has a dielectric constant of 36.7 and a donor number of 24.0 (Gutmann, 1977). T_1 measurements were carried out for five independent samples covering a 100-fold range of Gd^{3+}/LAS mole ratios. Signals of salicylate carbons were often not visible at the higher ratios as a result of paramagnetic signal broadening. Data in Table I are from a sample where all signals are visible. The largest relaxation enhancement occurs for C_1, and with the exception of nearby carbons C_2 and C_7, all other $T_{1_{obsd}}^{-1}$ values are within one or two standard deviations of those determined for the diamagnetic complex, indicating no significant paramagnetic

relaxation enhancement. This demonstrates that Gd^{3+} binding occurs only at the carboxyl group and possibly at O_1 (data for C_3 are unavailable as a result of overlap with a solvent resonance). All other oxygens are relatively remote from the cation, and LAS may be in an open-chain configuration. This mode of coordination is quite different from those found in the solid state, but it is consistent with the findings of Chen & Springer (1978) for the Pr^{3+} complex in methanol and with the structure proposed by Haynes & Pressman (1974) for LAS complexes bound to a membrane surface.

Before attempting to use T_{1P}^{-1} data to determine cation-C distances, it must be demonstrated that fast exchange, $T_{1M} \gg \tau_m$ in eq 2, occurs under the experimental conditions of temperature, concentration, and P_M used to obtain the T_{1P}^{-1} data. A convenient method of testing for fast exchange is to examine the temperature dependence of T_{2P}^{-1} for NMR signals of protons near the proposed cation binding sites. Here, T_{2P}^{-1} is defined as

$$T_{2P}^{-1} = T_{2_{obsd}}^{-1} - T_{2F}^{-1} \quad (4)$$

where $T_{2_{obsd}}^{-1}$ and T_{2F}^{-1} are determined from line-width measurements on paramagnetic and diamagnetic samples, respectively. A decrease in T_{2P}^{-1} with increasing temperature implies $T_{2M} \gg \tau_M$ in an equation analogous to (2). Since $T_{1M} \geq T_{2M}$, this would demonstrate $T_{1M} \gg \tau_M$ for protons and also for carbons at comparable r values since $T_{1M}(^{13}C) > T_{1M}(^{1}H)$. Similarly, the temperature dependences of ^{13}C T_{1P}'s or T_{2P}'s could be examined, but this would require unreasonable amounts of spectrometer time for LAS. The C_4–CH_3 group of LAS provides the closest observable protons to the carboxyl binding site and is a well-isolated singlet. The T_{2P}^{-1} of this signal clearly decreases with increasing temperature over the range 303–325 K, thus indicating fast exchange.

Use of eq 1 to determine Gd^{3+}–C distances directly requires knowledge of the dipolar correlation time, τ_C, which cannot always be determined accurately. Another approach, which we have used previously (Lee et al., 1981; Lee & Everett, 1981), assumes τ_C and P_M are constant for all carbons of LAS. Then, under fast-exchange conditions, eq 1 and 2 may be combined to give eq 5 which is valid for any pair of nuclei of the same kind.

$$\frac{T_{1P_j}^{-1/6}}{T_{1P_i}^{-1/6}} = \frac{r_i}{r_j} \quad (5)$$

We have written a computer program designed to find the best-fit position of the bound cation among chemically reasonable trial structures by iterative procedures which compare experimental and calculated r_i/r_j values. T_{1P}^{-1} data for a minimum of three carbons are required by the program, which searches for a least-squares fit to three r_i/r_j ratios. The calculated ratios are obtained by using relative atomic positions for LAS carbons and oxygens reported for the "unprimed" anion in the crystal structure of the Ba^{2+} complex (Johnson et al., 1970). The program generates a three-dimensional map in two-dimensional cross sections showing sums of squares of differences ($\sum\delta^2$) between calculated and experimental distance ratios. Minima on the map are assumed to represent the best-fit positions for the cation.

In the case of LAS and Gd^{3+} in DMF, T_{1P}^{-1} data for C_1, C_2, and C_7 were used in eq 5 to determine experimental distance ratios. The computer-generated error maps show a crescent-shaped island of minimal error ranging from 3.0 to 4.0 Å from C_1 and ~1.5–2.0 Å above the salicylate plane (see Figure 2). The $\sum\delta^2$ values within this region are 10^{-5}–10^{-4}. Uncertainty in the NMR distance ratios, arising from experimental uncertainty in T_{1P}^{-1} values, may account for $\sum\delta^2$ values as small as $\sim 7 \times 10^{-3}$. Error values of this magnitude or smaller are restricted to an area occupying roughly 7 Å^3, which contains, and is centered on, the region of best fit. It is evident that Gd^{3+} cannot be located accurately from the T_1 data. However, O_1 is definitely too far from Gd^{3+} for binding. For the region of best fit ($\sum\delta^2 \sim 10^{-5}$), a reasonable Gd^{3+}–O bond distance (Sievers, 1973; Cunningham & Sievers, 1980) of 2.5 Å can be maintained to one of the carboxylate oxygens by rotation of the carboxylate group about the C_1–C_2 bond. If the group is so rotated, there can be no appreciable hydrogen-bonding interactions between either of the carboxylate oxygens and the C_3 hydroxyl hydrogen in the cation-bound substrate. The Gd–O–C_1 bond angles obtained range from 96° to 135°. If the Gd^{3+} ion is centered in the region of the deepest minimum, a Gd–O bond distance of 2.4–2.5 Å results in a Gd–O–C_1 bond angle of ~117° and requires the dihedral angle between the salicylate plane and the carboxylate group to be ~26–30°.

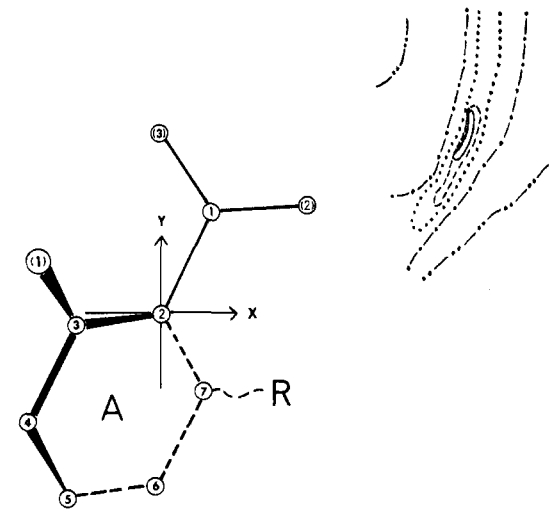


FIGURE 2: Error map illustrating the best-fit position of Gd^{3+} and Mn^{2+} cations relative to ring A of LAS in DMF solution: (—) $\sum\delta^2 \leq 10^{-5}$; (---) $\sum\delta^2 \leq 10^{-4}$; (···) $\sum\delta^2 \leq 10^{-3}$; (-·-) $\sum\delta^2 \leq 10^{-2}$; (-···-) $\sum\delta \leq 10^{-1}$; where $\sum\delta^2$'s are sums of squares of differences between calculated and experimental distance ratios (see text). The errors are shown for a section at $z = 0.45$. The carboxylate group is in the xy plane of a coordinate system with the origin at C_2. The dihedral angle between the aromatic ring and the carboxylate group is 30°.

Thus, in DMF solution, the following binding model emerges: Only the carboxylate group is significantly involved in binding to Gd^{3+}. The Gd^{3+} ion appears to be situated above (or below) the plane of the salicylate ring. The most reasonable Gd–O bond distances are found if the carboxylate group is monodentate and is not coplanar with the aromatic ring. By comparison, in the crystal structures of $Ba(LAS)_2 \cdot H_2O$ (Johnson et al., 1970) and one form of Na_2(6-Br-LAS)$_2$ (Schmidt et al., 1974) the cations are bound to one of the carboxylate oxygens and are not in the plane of the aromatic ring. In the Ba^{2+} complex, the carboxylate-aromatic ring dihedral angle is ~24°.

(2) In Chloroform. Chloroform, having a dielectric constant of 4.7, is considerably less polar than DMF, and LAS complexes are very soluble in chloroform. The T_{1P}^{-1} data obtained in this solvent are given in Table I. The results of strikingly different from those obtained in DMF. Relatively large T_{1P}^{-1} values are found for C_1, C_2, C_6, C_{11}, C_{19}, and C_{23}, indicating that Gd^{3+} binds not only to the salicylate "head" of LAS but also to O_4 and O_7 but not to O_5. It is not immediately certain whether O_6 and O_8 bind to Gd^{3+}. If binding occurs at O_6, one

expects significant and comparable T_{1P}^{-1}'s for *both* C_{15} and C_{18}, which is not the case.

A pronounced decrease in T_{2P}^{-1} with increasing temperature was found over the range 280–325 K for NMR signals of H_5, H_6, and H_{11}, which indicates fast exchange at the temperature (310 K) used to obtain the T_1 data. Further support for the condition $T_{1M} \gg \tau_M$ is evident from the *variation* of T_{1P}^{-1}'s in Table I. If the denominator of eq 2 were dominated by τ_m, all T_{1P}^{-1}'s would be identical within error. Fast-exchange conditions were found also during a ^{13}C NMR study of binding of Cu^{2+} by LAS in chloroform solution (Lallemand et al., 1980).

Because of the few multiple bonds present in LAS, the molecule is extremely flexible. A recent high-field proton NMR study of $La(LAS)_3$ in chloroform solution (Everett et al., 1983) revealed the presence of a dynamic, intramolecular process in which O_4 and O_7 from different ligands appear to take turns binding to La^{3+}. Also two environments for the salicylate group of $La(LAS)_3$ are in rapid exchange at ambient temperature but can be "frozen out" below 275 K. Such processes very likely occur also for the Gd^{3+} complex in $CDCl_3$ solution, and it cannot be assumed that each oxygen donor spends the same amount of time bound to Gd^{3+}. Thus, the effective P_M's for carbon atoms near oxygen donors may differ. In a solvent of low polarity such as chloroform, each Gd^{3+} ion is probably bound to three LAS anions via their carboxylate groups in order to prevent separation of charge. The remaining Gd^{3+} coordination sites could be occupied by additional oxygens from one or more of these same LAS anions in a cyclic conformation, but all three LAS anions are not necessarily bound identically in an instantaneous structure.

For these reasons, it is unrealistic to attempt to find a single LAS conformation and Gd^{3+} position which would simultaneously fit T_{1P}^{-1} data for all carbons. Thus, we have chosen to regard the LAS anion as comprised of several independent, relatively rigid, ligating moieties and to consider Gd^{3+} binding to these separately.

The search program described above was used with the T_{1P}^{-1} data in $CHCl_3$ solution for C_1, C_2, and C_7 and, independently, with C_{19}, C_{22}, and C_{23}. In these calculations, ring C was assumed to have the chair conformation found in the X-ray structure of the Ba^{2+} complex (Johnson et al., 1970) and predicted by energy minimization calculations (Painter et al., 1982); i.e., the C_{18}–C_{19} bond is equatorial, and O_8 and C_{23}–CH_3 are trans diaxial.

When the data for C_{19}, C_{22}, and C_{23} are used, the best fits ($\sum\delta^2 \sim 10^{-5}$) between calculated and experimental distance ratios occur when Gd^{3+} occupies a region of space ~0.5 Å long situated in a favorable position for binding to both O_7 and O_8. The center of this region is within 0.5 Å of the position found for Ba^{2+} in the crystal structure of $Ba(LAS)_2 \cdot H_2O$. Experimental uncertainties in T_{1P}'s can give rise to $\sum\delta^2$ values as small as 9×10^{-3}. Gd–O distances are 2.4–2.8 Å for O_7 and 2.6–3.2 Å for O_8. At any given point in the region of minimum fit, the Gd–O_7 distance is ~0.3 Å shorter than the Gd–O_8 distance. If rings B and C have the same relative orientation as in crystalline $Ba(LAS)_2 \cdot H_2O$, the Gd–O_6 distance is 2.9–3.1 Å. The position of the cation relative to rings B and C is shown in Figure 3. As was the case in DMF solution, experimental errors are too large to locate Gd^{3+} more accurately. However, strong binding to O_7 and weaker binding to O_8 are clearly indicated. The reason for the large difference in T_{1P}^{-1} values for C_{15} and C_{18} is not clear. If O_6 is bound to Gd^{3+}, steric strain apparently forces C_{18} to be ~20% further from the Gd^{3+} ion than C_{15}. Alternatively, it is possible that

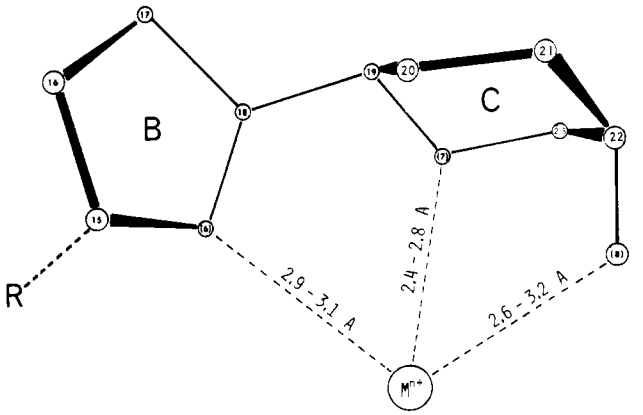


FIGURE 3: Diagram showing the best-fit position of Gd^{3+} and Mn^{2+} cations relative to rings B and C of LAS in $CDCl_3$ solution. The conformations shown for rings B and C are those found by crystallography for the Ba^{2+} complex (Johnson et al., 1970) and by energy minimization calculations (Painter et al., 1982).

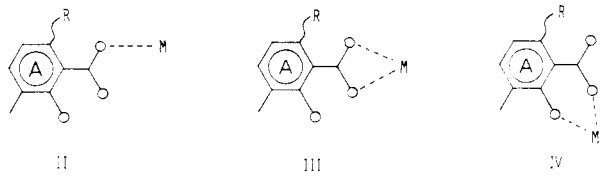
O_6 does not bind Gd^{3+}, and the T_{1P}^{-1} value found for C_{15} simply reflects a close approach of this carbon to the Gd^{3+} ion in the complex. It is perhaps of significance to point out that a relatively long Cu–O_6 distance was found in a ^{13}C T_1 study of Cu^{2+} binding by LAS in chloroform solution (Lallemand et al., 1980). Also the $^{203,205}Tl$–^{13}C coupling constant is larger for C_{15} than for C_{18} by a factor of 4 in the Tl^+ complex (Lallemand & Michon, 1978). In view of the uncertainty in the Gd^{3+} position, no attempt is made here to determine the relative orientation of rings B and C from NMR data.

The T_{1P}^{-1} data for C_{11} and C_{13} indicate significant binding to O_4 but not to O_5. Again, these results are consistent with those found in the above-mentioned Cu–LAS study. Since it cannot be assumed that P_M for O_4 is the same as that of ring C donors, data for C_{11} and ring C carbons cannot be combined to determine a reliable C_{11}–Gd distance.

At the salicylate end of LAS, the large relaxation enhancement at C_1 indicates binding through one or both of the carboxyl oxygens. However, C_2 experiences a disproportionately large enhancement with respect to C(1), and the magnitudes of T_{1P}^{-1} values for C_3, C_5, C_6, and C_7 are inconsistent with binding solely through the C_1 oxygens. An attempt was made to locate the cation by using T_{1P}^{-1} values for C_1, C_2, and C_7. A crescent-shaped region of minimum error ~4.5 Å in length was found above and roughly normal to the plane of the salicylate ring. Most of this region is beyond the range of normal Gd–O bond distances for both the carboxyl oxygens and O_1. Gd^{3+} positions which give reasonable bond distances require extraordinary Gd–O–C bond angles and close approach of Gd to C_1 and C_2. Thus, the Gd^{3+} ion cannot be located satisfactorily by using T_{1P}^{-1} data for C_1, C_2, and C_7 obtained in chloroform solution.

This uncertainty in the Gd^{3+} position strongly suggests that the T_{1P}^{-1} data are complicated by contributions from more than one mode of ligation. Indeed, as pointed out earlier, there is evidence that the salicylate group in $La(LAS)_3$ has two environments in rapid exchange at ambient temperature. At low temperatures, two signals having relative area ratios of approximately 1/3 can be seen for C_1 and for protons at C_4–CH_3, C_5–H, and C_6–H. The same phenomenon is expected for the Gd^{3+} complex and would account for the observed pattern of T_{1P}'s. The salicylate carbons must be sufficiently close to Gd^{3+} in each environment to experience relaxation enhancement, and it is reasonable to assume the anionic carboxylate group is bound to the cation in each environment in chloroform solution. Possible modes of salicylate binding include mono-

dentate carboxylate (II), bidentate carboxylate (III), and a



II III IV

bidentate chelate formed by O_1 and carboxylate binding (IV). The latter is supported by the magnitude of T_{1P}^{-1} for C_3 and has been shown to be consistent with T_{1P}^{-1} data obtained for LAS in chloroform solution in the presence of Cu^{2+} ion (Lallemand et al., 1980). Furthermore, II and IV have been found crystallographically for cation complexes of salicylic acid (Downie & Speakman, 1954; Klug et al., 1958; Hanic & Michalov, 1960; Kushi et al., 1970). The occurrence of a binuclear complex with a *bridging* carboxylate such as recently reported in the crystal structure of the synthetic carboxylic ionophore McN-4308 (Van Roey et al., 1982) is unlikely because of the very small Gd^{3+}/LAS mole ratio used here. In view of the errors in T_{1P}^{-1} data, we make no attempt to define in more detail the mode of Gd^{3+}–salicylate binding. A ^{13}C NMR spectrum of $Gd(LAS)_3 \cdot {}^3/_2CHCl_3$ in $CHCl_3$ showed, as expected, only a few extremely broad resonances.

Thus, we conclude that in chloroform solution, LAS binds Gd^{3+} via the carboxylate group, O_4, O_7, O_8, possibly O_6 and O_1 (in a fraction of the bound LAS ligands), but not O_5. Very likely in an instantaneous structure a Gd^{3+} ion is bound by carboxylate anions from three LAS ligands, possibly one salicylate hydroxyl, and also O_4, O_7, and O_8 from one or two of these same ligands or from different LAS ligands. Although lanthanide(III) complexes of small ligands commonly have a coordination number of 9 (Cotton & Wilkinson, 1980), steric strain may prevent this in LAS complexes. Thus, one, two, or all three carboxylate-bound LAS ligands may have "dangling" ends, depending upon whether any can fold sufficiently to bind also via O_4 and/or O_7 and O_8. Finally, there is rapid exchange among all Gd-bound ligands with those bound by Na^+ in bulk solution. This model is consistent with the fact that C_1 has the largest T_{1P}^{-1}, presumably because it has the largest P_M. Also, the model is supported by results of a proton NMR study of $La(LAS)_3$ in chloroform solution (Everett et al., 1983).

(3) Other Solvents. Attempts were made to use cyclohexane as a solvent in this work because it has a simple ^{13}C NMR spectrum which interferes minimally with LAS signals, and it might be expected to model the environment of the hydrophobic interior of a lipid bilayer membrane. However, both NaLAS and $La(LAS)_3 \cdot CHCl_3$ proved to be insufficiently soluble in cyclohexane for practical ^{13}C T_1 measurements. T_1 experiments were feasible in a solvent mixture of C_6H_{12} and $CDCl_3$ (35% v/v $CDCl_3$). However, T_1's for $La(LAS)_3$, measured in the presence of $Gd(LAS)_3$, were within the limits of error of those measured in the absence of $Gd(LAS)_3$. This indicates either no binding by Gd(III) or, more likely, slow exchange in this solvent.

Qualitative ^{13}C NMR signal broadening experiments in several $CDCl_3$/DMF mixed-solvent systems were carried out in which increasing amounts of $Gd(LAS)_3$ were added to solutions of NaLAS. Significant effects were observed only for signals from C_1, C_2, C_3, and C_7 with solvents having 20% (v/v) or more DMF. This indicates binding by the carboxyl group predominates even with a minor proportion of the polar solvent.

(4) Correlation Times. Conclusions reached thus far regarding Gd^{3+} positions and Gd–C distances have all been made by computer fitting of distance ratios (eq 5) in which numerical values for τ_C and P_M (eq 1 and 2) are not required. It is of interest to compare these Gd–C distances with "absolute" Gd–C distances, determined by using estimated values of τ_C and P_M in eq 1. An estimate of the effective correlation time can be obtained with $La(LAS)_3$ in chloroform solution. Carbon-13 nuclear Overhauser enhancement factors measured by gated decoupling techniques for C_5, C_6, C_{14}, and C_{15}, which have well-isolated signals, are within error of 2.0. This indicates that relaxation is dominated by dipolar interactions with protons, and eq 6 can be used to estimate τ_C (Lyerla & Levy, 1974). By use of $n_H = 1$, $r_{CH} = 1.05$ Å, and an average T_1 of 0.092 ± 0.004 s for the four carbons, an average $\tau_C = 4.0 \times 10^{-10}$ s is obtained.

$$T_1^{-1} = \frac{n_H \gamma_H^2 \gamma_C^2 \hbar^2 \tau_C}{r_{CH}^6} \quad (6)$$

For a given carbon atom, the effective P_M is the mole fraction of that carbon which is sufficiently close to Gd^{3+} to experience measurable paramagnetic relaxation enhancement. For small ligands P_M is normally the mole fraction of bound ligand; however, for LAS this is not necessarily the case since all ligands may not be bound identically. For simplicity we assume that in $CHCl_3$ solution only one LAS binds a given Gd^{3+} ion via O_7 and O_8, so that the effective P_M for ring A carbons is the experimental Gd/LAS mole ratio, 2.59×10^{-4}. When this P_M is used with the above τ_C and experimental T_{1P}^{-1} values for C_{19}, C_{22}, and C_{23}, Gd–C distances of 4.2, 4.4, and 3.9 Å, respectively, are obtained. Corresponding distances from the best-fit Gd^{3+} position are 3.4–3.8, 3.5–4.0, and 3.2–3.6 Å. The agreement is considered to be reasonable in view of the assumptions made in determining the absolute distances: (1) P_M is the Gd/LAS mole ratio; (2) τ_C measured for $La(LAS)_3$ (actually τ_r) is valid for the Gd^{3+} complex; i.e., τ_S does not dominate τ_C in the paramagnetic complex; (3) Gd^{3+} in the presence of excess NaLAS has the same structure as $La(LAS)_3$ in $CHCl_3$ solution.

Manganese(II) Binding. Experiments using Mn^{2+} as a binding site probe were carried out similarly to those involving Gd^{3+} except that $Mn(LAS)_2 \cdot {}^1/_2CHCl_3$ was used as a soluble source of Mn^{2+} which was added to DMF or $CDCl_3$ solutions of NaLAS. T_{1P}^{-1} data are given in Table I.

In DMF solution, significant relaxation enhancement is found for C_1, C_5, C_6, C_{11}, C_{15}, C_{19}, and C_{23}, indicating Mn(II) binding at the carboxylate group, O_4, O_7, and possibly O_6. Since these results are quite different from those obtained with Gd^{3+} in DMF, where only the carboxylate group binds significantly, the T_1 experiment with Mn^{2+} was repeated with an independent sample.[2] All T_{1P}^{-1} values agree within the limits of error for the two samples, and data shown in Table I are averages.

Similarly, in chloroform solution, T_{1P}^{-1} values from two independent samples were in good agreement, and averaged values are given in Table I. The data indicate Mn^{2+} binding at the carboxylate group, O_4, O_7, and possibly O_6 and O_8. The situation here is similar to that found for Gd^{3+} in $CDCl_3$ solution.

In both solvents attempts to demonstrate the occurrence of fast exchange of Mn^{2+} were inconclusive. No significant changes could be seen in proton T_{2P}^{-1} values over the tem-

[2] Also, T_1 experiments with Gd^{3+} in DMF were repeated with $Gd(LAS)_3 \cdot {}^3/_2CHCl_3$, rather than $Gd(NO_3)$, as the source of Gd^{3+} in order to test for effects of nitrate ion in the earlier experiments. Again, a large T_{1P}^{-1} was found for C_1, and no significant relaxation enhancement was found for C_5, C_6, C_{11}, C_{15}, C_{19}, or C_{23}.

perature range 290–320 °C. Carbon-13 T_{2P}^{-1} values at 310 °C are slightly larger than the corresponding T_{1P}^{-1} values for most oxygen-bearing carbons (average ratio = 1.10). However, this does not unambiguously distinguish between slow exchange where $T_{1P}^{-1} = T_{2P}^{-1} = P_m/\tau_m$ and fast exchange where $T_{2P}^{-1}/T_{1P}^{-1} = 7/6$ if there are no contact contributions. The best evidence for fast exchange in the Mn–LAS system lies in the *variation* of observed T_{1P}^{-1} values for LAS carbons in both solvents. Identical T_{1P}^{-1} values are expected for all carbons in the event of slow exchange.

(*1*) *In N,N-Dimethylformamide.* Mn–C distance ratios are related to the sixth root of the corresponding T_{1P}^{-1} ratios as shown by eq 5. In DMF solution, the sixth root of T_{1P}^{-1} ratios for C_2/C_1, C_7/C_1, and C_2/C_7 are within the limits of error of corresponding values obtained with Gd^{3+}. Thus, *the carboxylate moiety binds Mn^{2+} and Gd^{3+} in a similar manner in DMF*, and the binding model described earlier for Gd^{3+} applies also for carboxylate binding by Mn^{2+}. The ~0.2-Å difference in ionic radii of the two ions is much smaller than the uncertainty in the best-fit cation position (see Figure 1). Unexpectedly large T_{1P}^{-1} values are found for C_5 and C_6. We have no simple explanation for this except to point out that in an earlier binding site study (Lee & Everett, 1981) of tetracycline, with Mn^{2+} as a paramagnetic probe, large T_{1P}^{-1} values are consistently found for protonated aromatic carbons which are distant from any potential cation binding site.

Unlike Gd^{3+}, Mn^{2+} causes significant relaxation enhancement for C_{11}, C_{15}, C_{19}, and C_{23} in DMF solution. By use of T_{1P}^{-1} data for C_{19}, C_{22}, and C_{23}, the search program reveals an arc of Mn^{2+} positions having $\sum\delta^2 \leq 10^{-5}$. The arc is 1.8 Å in length and is centered about the position of minimum error ($\sum\delta^2 = 10^{-7}$) which is 2.8 Å from O_7 and 4.9 Å from O_8, and under the assumption that rings B and C have the same relative orientation as in crystalline $Ba(LAS)_2$, the Mn^{2+}–O_6 distance is 4.0 Å. Although the T_{1P}^{-1} data indicate a close approach of C_{15} to Mn^{2+}, T_{1P}^{-1} for C_{18} is considerably smaller. Similar results are found in chloroform solution with both Gd^{3+} and Mn^{2+}, and as discussed earlier, it is not clear whether O_6 actually binds these ions. Since the relative orientation of rings B and C here is not necessarily the same as in the Ba^{2+} complex in the solid state, T_{1P}^{-1} data for C_{15} could not be used in the search program with data from ring-C carbons. Normal Mn(II)–O distances fall in the range of 2.1–2.4 Å (Macgillavry & Rieck, 1968). Within the arc of Mn^{2+} positions described above, the closest approach of Mn^{2+} to O_7 is 2.4 Å and to O_8 is 4.2 Å.

In summary, there is definite evidence for Mn^{2+} binding to the carboxylate group, O_4, and O_7 in DMF solution. The carboxylate group appears to bind via one oxygen, as is the case for Gd^{3+}.

(*2*) *In Chloroform.* In chloroform solution, the sixth roots of T_{1P}^{-1} ratios for C_2/C_1, C_7/C_1, and C_2/C_7 for LAS in the presence of Mn^{2+} are within the limits of error of the values obtained in chloroform with Gd^{3+}. Similarly, sixth roots of T_{1P}^{-1} ratios for C_{19}/C_{23}, C_{22}/C_{23}, and C_{22}/C_{19} obtained in the presence of Mn^{2+} agree within error with those obtained in the presence of Gd^{3+}. Thus, in chloroform solution the two ions bind LAS similarly, and the binding model discussed earlier for Gd^{3+} applies also to Mn^{2+} (see Figure 3).

For both ions, T_{1P}^{-1} data for salicylate ring carbons are believed to have contributions from at least two types of cation-bound salicylate in rapid exchange at ambient temperature. In support of this is the observation that at temperatures below 270 K the proton NMR spectra of $Ca(LAS)_2$, believed to have a structure similar to that of $Mn(LAS)_2$, show two signals for C_6–H in chloroform solution. One of these signals has several times the intensity of the other. The occurrence of multiple salicylate NMR signals for $La(LAS)_3$ at low temperatures was discussed earlier. Also the C_1–C_2–C_7 $T_{1P}^{-1/6}$ ratios in chloroform are quite different from those obtained in DMF for both ions. In the latter solvent, the best-fit calculations indicate monodentate binding by the carboxylate group.

Significantly, $T_{1P}^{-1/6}$ ratios for C_{19}–C_{22}–C_{23} obtained with either Mn^{2+} or Gd^{3+} in chloroform are different from those obtained with Mn^{2+} in DMF (except for the C_{19}/C_{23} ratio where the agreement is within error). The difference arises from the larger relative T_{1P}^{-1} values for C_{22} in chloroform. The best-fit calculations indicate Mn^{2+} and Gd^{3+} binding to O_8 in chloroform but not in DMF.

Summary and Conclusions

In DMF solution Gd^{3+} binds solely to the carboxylate group of LAS. Mn^{2+} also binds the carboxylate group but in addition binds O_4 and O_7. In $CDCl_3$ solution both ions bind LAS in the same manner. Here the salicylate moiety appears to have at least two different modes of ligation which exchange rapidly at ambient temperature. Additional binding in $CDCl_3$ occurs via O_4, O_7, and O_8 groups from either the salicylate-bound ligands or other LAS anions in solution.

The different binding modes observed for Gd^{3+} and Mn^{2+} in DMF may result from a difference in their solvation enthalpies in this polar solvent. The trivalent ion is expected to have a higher solvation enthalpy, and apparently only the anionic carboxylate group of LAS is capable of displacing the solvation shell. With the divalent ion, on the other hand, O_4 and O_7 is addition to the carboxylate group are able to displace at least some of the coordinated DMF molecules. In $CDCl_3$ solution, where solvation enthalpies are small, the solvation shells of both ions are readily displaced by the carboxylate and other oxygen donors.

Acknowledgments

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Registry No. I, 25999-31-9; I·Na, 25999-20-6; *N,N*-dimethylformamide, 68-12-2; chloroform, 67-66-3.

References

Alpha, S. R., & Brady, A. H. (1973) *J. Am. Chem. Soc. 95*, 7043–7049.

Anteunis, M. J. O. (1976) *Bioorg. Chem. 5*, 327–336.

Canet, D., Levy, G. C., & Peat, I. R. (1975) *J. Magn. Reson. 18*, 199–204.

Chen, S.-T., & Springer, C. S., Jr. (1978) *Bioinorg. Chem. 9*, 101–122.

Chiang, C. C., & Paul, I. C. (1977) *Science (Washington, D.C.) 196*, 1441–1443.

Cotton, F. A., & Wilkinson, G. (1980) *Advanced Inorganic Chemistry*, 4th ed., Chapter 23, Wiley, New York.

Cunningham, J. A., & Sievers, R. E. (1980) *Inorg. Chem. 19*, 595–604.

Degani, H., & Friedman, H. L. (1974) *Biochemistry 13*, 5022–5032.

Dobson, C. M., & Levine, B. A. (1976) in *New Techniques in Biophysics and Cell Biology* (Pain, R. H., & Smith, B. J., Eds.) Vol. 3, Wiley, New York.

Downie, T. C., & Speakman, J. C. (1954) *J. Chem. Soc.*, 787–793.

Everett, G. W., Parker, S. B., & Williams, R. J. P. (1983) *Biochemistry* (in press).
Gutmann, V. (1977) *Chimia 31*, 1–7.
Hanic, F., & Michalov, J. (1960) *Acta Crystallogr. 13*, 299–302.
Haynes, D. H., & Pressman, B. C. (1974) *J. Membr. Biol. 16*, 195–205.
James, T. L. (1975) *Nuclear Magnetic Resonance in Biochemistry*, Academic Press, New York.
Johnson, S. M., Herrin, J., Liu, S. J., & Paul, I. C. (1970) *J. Am. Chem. Soc. 92*, 4428–4435.
Klug, H. P., Alexander, L. E., & Sumner, G. G. (1958) *Acta Crystallogr. 11*, 41–46.
Krishnan, C. V., Friedman, H. L., & Springer, C. S. (1978) *Biophys. Chem. 9*, 23–25.
Kushi, Y., Hosoo, T., & Kuroya, H. (1970) *J. Chem. Soc., Chem. Commun.*, 397.
Lallemand, J.-Y., & Michon, V. (1978) *J. Chem. Res., Miniprint*, 2081–2090.
Lallemand, J.-Y., Rao, R., & Prangé, T. (1980) *Nouv. J. Chim. 4*, 315–319.
Lee, J. Y., & Everett, G. W. (1981) *J. Am. Chem. Soc. 103*, 5221–5225.
Lee, J. Y., Hanna, D. A., & Everett, G. W. (1981) *Inorg. Chem. 20*, 2004–2010.
Lyerla, J. R., & Levy, G. C. (1974) in *Topics in Carbon-13 NMR Spectroscopy* (Levy, G. C., Ed.) Vol. I, Chapter 3, Wiley-Interscience, New York.
Macgillavry, C. H., & Rieck, G. D., Eds. (1968) *International Tables for X-ray Crystallography*, Vol. III, p 268, Kynoch Press, Birmingham, England.
Maier, C. A., & Paul, I. C. (1971) *J. Chem. Soc., Chem. Commun.*, 181–182.
Mildvan, A. S., & Gupta, R. K. (1978) *Methods Enzymol. 49G*, 332–359.
Ovchinnikov, Y. A. (1979) in *Frontiers in Bioorganic Chemistry and Molecular Biology* (Ovchinnikov, Y. A., & Kolosov, M. N., Eds.) Chapter 8, Elsevier, Amsterdam.
Painter, G. R., Pollack, R., & Pressman, B. C. (1982) *Biochemistry 21*, 5613–5620.
Patel, D. J., & Shen, C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1786–1790.
Poonia, N. S., & Bajaj, A. V. (1979) *Chem. Rev. 79*, 389–445.
Pressman, B. C. (1976) *Annu. Rev. Biochem. 45*, 501–530.
Richardson, F. S., & Gupta, A. D. (1981) *J. Am. Chem. Soc. 103*, 5716–5725.
Schmidt, P. G., Wang, A. H.-J., & Paul, I. C. (1974) *J. Am. Chem. Soc. 96*, 6189–6191.
Seto, H., Westley, J. W., & Pitcher, R. G. (1978) *J. Antibiot. 31*, 289–293.
Shen, C., & Patel, D. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4277–4281.
Sievers, R. E. (1973) *Nuclear Magnetic Resonance Shift Reagents*, Academic Press, New York.
Smith, G. D., Duax, W. L., & Fortier, S. (1978) *J. Am. Chem. Soc. 100*, 6725–6727.
Van Roey, P., Smith, G. D., Daux, W. L., Umen, M. J., & Maryanoff, B. E. (1982) *J. Am. Chem. Soc. 104*, 5661–5666.
Westley, J. W. (1975) *Annu. Rep. Med. Chem. 10*, 246–256.
Westley, J. W. (1976) *J. Antibiot. 29*, 584–586.
Westley, J. W. (1982) *Polyether Antibiotics: Naturally Occurring Acid Ionophores*, Vol. I and II, Marcel Dekker, Basel.

Shortest Nucleosomal Repeat Lengths during Sea Urchin Development Are Found in Two-Cell Embryos†

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ABSTRACT: Prior to fertilization, sperm possess one of the longest nucleosome repeat lengths yet determined [~250 base pairs (bp) for the sea urchin *Strongylocentrotus purpuratus*]. We show here that the two-cell embryo has an average repeat size of 189 ± 2 bp as probed by micrococcal nuclease; this is the shortest average nucleosomal subunit reported for *S. purpuratus*. By the eight-cell stage, the average nucleosome repeat increases to 201 ± 2 bp, and it subsequently increases further during development. These results indicate that a dramatic rearrangement of chromatin occurs upon fertilization and that this chromatin remodeling continues through early development. When two-cell embryos are labeled for 30 min with [^{3}H]thymidine and digested briefly, they exhibit nuclease-hypersensitive fragments averaging 308 bp in size, which are consistent with the size of protected DNA units in replication intermediate complexes at blastula stage (as described by Levy and Jacob [Levy, A., & Jacob, K. M. (1978) *Cell (Cambridge, Mass.) 14*, 259]). Our results are consistent with two general propositions: (1) long repeat lengths are found in highly differentiated cells, and (2) short repeat lengths are characteristic of cells more active in cell division. Our data would also imply that a rapid increase in the DNA complement, e.g., in the transition from haploid to diploid state following fertilization, is accompanied by a shortening of the average size of DNA in a nucleosome after replication.

The average repeat length of DNA in the nucleosome, once assumed to be nearly constant at 200 base pairs (Kornberg, 1974), has been found to vary widely from 160 bp (base pairs) in lower eukaryotes like yeast (Lohr et al., 1977) to about 250 bp in sea urchin sperm (Arceci & Gross, 1980a; Spadafora et al., 1976). Even within one species the basic subunit repeat has been observed to differ dramatically depending on the tissue origin and state of development of the cell, indicating that two tissues from the same animal can have the genome

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